PRODUCTION OF FRUCTO-OLIGOSACCHARIDES FROM THE ENZYMATIC CONVERSION OF COCONUT SUGAR CATALYZED BY FRUCTOSYLTRANSFERASE IN A BATCH REACTOR

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for the award of the degree of
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CENTRE OF GRADUATE STUDIES

We certify that the thesis entitled “The Production of Fructo-oligosaccharides from Natural Coconut” is written by Noormazlinah Binti Ahmad. We have examined the final copy of this thesis and in our opinion; it is fully adequate in terms of scope and quality for the award of the degree of “Master of Engineering in Bioprocess”. We herewith recommend that it be accepted in fulfillment of the requirements for the degree of “Master of Engineering specializing in Bioprocess”.

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I hereby declare that the work in the thesis is my own research except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature

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Date: 29 November 2010

To my beloved husband, Mohd Fadilah Bin Omar and my twin daughters Nuriffah Najihah binti Mohd Fadilah and Nuriffah Nasuha binti Mohd Fadilah

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ABSTRACT

This thesis discusses the maximization of fructooligosaccharides (FOS) production from a novel substrate coconut sugar as a new source of sucrose. The enzymatic reaction has been performed in batch reactor with enzyme which is fructosyltransferase (FTase). To elucidate the study, two phases of framework has been carried out which are characterization of materials and experimental work. The materials that have been characterized are coconut sugar, FTase and FOS for qualitative and quantitative analysis purposes. The spectral analysis of the materials has been studied by using Fourier Transform Infra Red (FTIR) to determine the functional group of each material and obtained that coconut sugar and FOS subsist of carbohydrate family while FTase contains complex of protein compounds. Coconut sugar contains of 71% sucrose for its total sugar. It is leads from other coconut parts (coconut water and coconut milk) that approved as the most suitable substrate of the study. The molecular weight of FTase is 142 kDa with 401 U/mL of enzyme activity while it is more stable between 50 to 60 ^{o}C and pH 5.5. The experimental work initially has been carried out with six reaction parameters (coconut sugar concentration, enzyme concentration, temperature, pH level, reaction time and agitation speed) was performed to facilitate the appropriate range prior to conducting a fractional factorial design approach. The initial screening performed in batch reactor using two-level fractional factorial design, indicated that reaction temperature and coconut sugar concentration were significant factors in FOS production. All these significant factors were then optimized using RSM in order to maximise the FOS concentration and conversion yield. The FOS concentration and conversion yield after optimization were 243.23 g/L and 32.52%, respectively as compared to the initial FOS concentration and conversion yield (197.31 g/L and 28.14%), indicating an improvement of 45.92 g/L and 18.83%. The optimum conditions of reaction temperature and coconut sugar concentration were found to be at 54.34 ºC and 750.73 g/L. Finally, the self-fabricated 10L batch reactor has been utilized for scale up purposes consecutively for industry application which resulted 30.03% for the yield of FOS. It is proved that the equation is applicable for industrial scale with difference of 2% with lab scale.

ABSTRAK

Tesis ini membincangkan tentang memaksimumkan penghasilan Fruktooligosakarida (FOS) daripada gula Melaka sebagai sumber baru sukrosa. Tindakbalas enzim telah dijalankan di dalam reaktor berkelompok dengan menggunakan enzim Fruktosil-transferase (FTase). Bagi melaksanakan kajian ini, dua fasa kerja telah dijalankan iaitu pencirian bahan-bahan dan kerja-kerja eksperimen. Bahan-bahan yang dicirikan ialah gula Melaka, FTase dan FOS piawai untuk tujuan analisis kualitatif dan kuantitatif. Analisis spektra setiap bahan telah dikaji dengan menggunakan Spektrofotometer Inframerah Tranformasi Fourier (FTIR) untuk menentukan kumpulan berfungsi setiap bahan dan didapati bahawa gula Melaka dan FOS terdiri daripada keluarga karbohidrat ataupun gula manakala FTase mengandungi komponen protin kompleks. Gula Melaka terdiri daripada 71% sukrosa daripada jumlah gulanya. Gula melaka mengandungi sukrosa yang tinggi berbanding dengan bahagian kelapa yang lain (air kelapa dan santan kelapa) dan ini telah membuktikan bahawa gula Melaka adalah paling sesuai digunakan sebagai bahan mentah dalam penyelidikan ini. Berat molekul bagi FTase ialah 142 kDa dengan 401 U/mL aktiviti enzim manakala enzim ini paling stabil pada suhu diantara 50 hingga 60 ^{o}C dan pH 5.5. Kerja-kerja eksperimen telah dimulakan dengan enam parameter (kepekatan gula Melaka, kepekatan enzim, suhu, pH, masa bertindakbalas dan kelajuan pengaduk) dijalankan bagi menentukan had yang sesuai sebelum melaksanakan pendekatan rekabentuk faktorial separa (FFD). Penyaringan awal telah dijalankan didalam reaktor berkelompok menggunakan dua aras rekabentuk faktorial separa, menunjukkan bahawa suhu dan kepekatan gula Melaka merupakan faktor yang paling signifikan kepada penghasilan FOS. Faktor-faktor yang signifikan ini kemudiannya talah dioptimumkan menggunakan RSM untuk memaksimumkan kepekatan dan hasil penukaran FOS. Kepekatan FOS dan hasil penukaran setelah dioptimumkan ialah 243.23 g/L dan 32.52%, dibandingkan dengan permulaanya (197.31 g/L dan 28.14%) dan telah menunujukkan peningkatan sebanyak 45.92 g/L dan 18.83%. Keadaan optimum bagi suhu tindakbalas dan kepekatan gula Melaka didapati pada 54.34 ^{o}C dan 750.73 g/L. Akhir sekali, dengan menggunakan 10 L reaktor berkelompok yang telah diubahsuai untuk tujuan proses skala besar bagi aplikasi industri menunujukkan memberikan penghasilan 30.03%. Ini membuktikan bahawa persamaan yang dihasilkan boleh digunakan untuk skala industri kerana perbezaan skala industri dan skala makmal hanya 2.37%.

TABLE OF CONTENTS

		Page
SUPERVISOR'S DECLARATION		ii
STUDENT'S DECLARATION		iii
ACKNOWLEDGEMENTS		iv
ABSTRACT		vi
TRANSLATION OF ABSTRACT		vii
TABLE OF CONTENTS		viii
LIST OF TABLES		xii
LIST OF FIGURES		xiv
CHAPTER 1	**INTRODUCTION**	
1.1	Background of the problem	1
1.2	Problem statements	2
1.3	Objectives of the study	3
1.4	Scope of the study	3
1.5	Rationale and Significance	4
CHAPTER 2	**LITERATURE REVIEW**	
2.1	Introduction	6
2.2	Sucrose as Crucial Substrate for FOS production	7
	2.2.1 Advantages of Coconut Sugar as Substrate	8
2.3	The Novelty of FTase for FOS Production	10
	2.3.1 Bacterial FTase	11
	2.3.2 Fungal FTase	13
	2.3.3 Commercial FTase	14
	2.3.4 Cell Recycling for the Production of FTase	15
2.4.	Type of Process in FOS Production	
	2.4.1 Chemical Synthesis in production of FOS	15
	2.4.2 Enzymatic Reaction in production of FOS	16
	2.4.2.1 Microbial Production of FOS	16

2.4.2.2Fermentative methods of Microbial production 18
2.4.3 Continuous Production of FOS 18
2.4.3 Yield of FOS 19
2.5 Parameters of Enzymatic Reaction on Production of FOS 20
2.6 Characterization and Purification of Raw Materials. 25
2.7 Response Surface Methodology (RSM) 25
2.8 Application of FOS 29
2.8.1 Role of FOS in Control of Diabetes 30
2.8.2 FOS as Prebiotic 30
2.8.3 FOS as Dietary Fiber 31
2.8.4 FOS and Mineral Absorption 33
2.8.5 Role of FOS in Defense Functions 34
2.8.6 Anticancerous Effect of FOS 35
2.8.7 Additional Health Effect of FOS 35
2.8.8 Applications of FOS in Food Formulations 36
37

CHAPTER 3 MATERIALS AND METHODOLGY

3.1 Operational Framework
3.1.1 Phase 1: Characterization of Materials 39
3.1.2 Phase 2: Reaction Process in Batch System 39
3.2 Preparation of the Materials for Enzymatic Reaction of FOS 39
Production 41
3.2.1 Coconut Sugar 41
3.2.2 FTase and FOS 42
3.2.3 Buffer 43
3.3 Characterization of Materials 43
3.3.1 Coconut Sugar 43
3.3.1.1 The Total Sugar Contents in Coconut's Part 43
3.3.1.2 Total Sugar Contents in Coconut Sugar 44
3.3.1.3 Moisture Content 44
3.3.1.4 Spectral Analysis 45
3.3.1.5 Morphology Study 45
3.3.1.6 Viscosity of Coconut Sugar 46
3.3.2 Characterization of FTase 47
3.3.2.1 Molecular Weight Determination 47
3.3.2.2 Enzyme Activity 48
3.3.2.3 Stability of FTase 49
3.3.2.3.1 Thermal Stability 49
3.3.2.3.2 pH Stability 49
3.3.2.4 Spectral Analysis of FTase (FTIR) 50
3.3.3 Characterization of FOS 50

3.3.3.1 Spectral analysis of FOS 50
3.4 Standard of FOS Production 51
3.5 FOS Analysis by HPLC 51
3.6 One Factor at One Time (OFAT) 51
3.7 Determination of Kinetic Parameters in Batch System 52
3.8 Experimental Design 53
3.8.1 Screening Process using Two Level Fractional Factorial Design (FFD) 54
3.8.2 Optimization Process using Central Composite Design (CCD) 55
3.8.3 Validation of Model Empirical Equation 55
3.9 Scale up 56

CHAPTER 4 RESULTS AND FINDINGS

4.1 Characterization of Materials 58
4.1.1 Characterization of Coconut Sugar 59
4.1.1.1 Total Sugar Contents in Coconut's Part 59
4.1.1.2 Total Sugar Contents in Coconut Sugar 60
4.1.1.3 Moisture Contents 62
4.1.1.4 Spectral Analysis 62
4.1.1.5 Morphology Study 63
4.1.1.6 Viscosity Study 65
4.1.2 Characterization of FTase 68
4.1.2.1 Molecular Weight by using SDS PAGE 68
4.1.2.2 Enzyme Activity Assay 68
4.1.2.3 Stability of FTase 69
4.1.2.3.1 Thermal Stability 69
4.1.2.3.2 pH Stability 71
4.1.2.4 Spectral Analysis of FTase 72
4.1.3 Characterization of FOS 74
4.1.3.1 Spectral Analysis (FTIR) 74
4.2 Kinetic Parameter in Batch System 75
4.3 One Factor at One Time 78
4.3.1 Time Course Study on the FOS Production and other Reducing Sugar 79
4.3.2 Effect of Reaction Time 80
4.3.3 Effect of Substrate Concentration 81
4.3.4 Effect of Enzyme Concentration 82
4.3.5 Effect of Temperature 83
4.3.6 Effect of pH 84

4.3.7 Effect of Agitation Speed 85
4.3.8 Concluding Remarks 86

4.4 Screening Process by Fractional Factorial Design (FFD) 87

4.4.1 Analysis and Findings 87
4.4.2 Concluding Remarks of FFD Study 95

4.5 Optimization by Response Surface Methodology (RSM) 96

4.5.1 Concluding Remarks of CCD study 103

4.6 Validation of Empirical Model Adequacy 103

4.7 Scale Up 105

CHAPTER5 GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK 106

5.1 General Conclusion 108

5.2 Recommendations for Future Works 109

REFERENCES

APPENDICES

A Operational Framework, Correlation Chart, Standard Methods and Standard Curves 123

B Data Generations for OFAT, FFD and RSM study 136

C List of Publications 142

TABLE OF CONTENTS

		Page
SUPERVISOR'S DECLARATION		ii
STUDENT'S DECLARATION		iii
ACKNOWLEDGEMENTS		iv
ABSTRACT		vi
TRANSLATION OF ABSTRACT		vii
TABLE OF CONTENTS		viii
LIST OF TABLES		xii
LIST OF FIGURES		xiv
CHAPTER 1	**INTRODUCTION**	
1.6	Background of the problem	1
1.7	Problem statements	2
1.8	Objectives of the study	3
1.9	Scope of the study	3
1.10	Rationale and Significance	4
CHAPTER 2	**LITERATURE REVIEW**	
2.3	Introduction	6
2.4	Sucrose as Crucial Substrate for FOS production	7
	2.2.1 Advantages of Coconut Sugar as Substrate	8
2.3	The Novelty of FTase for FOS Production	10
	2.3.1 Bacterial FTase	11
	2.3.2 Fungal FTase	13
	2.3.3 Commercial FTase	14
	2.3.4 Cell Recycling for the Production of FTase	15
2.4.	Type of Process in FOS Production	
	2.4.1 Chemical Synthesis in production of FOS	15
	2.4.2 Enzymatic Reaction in production of FOS	16
	2.4.2.1 Microbial Production of FOS	16

2.4.2.2Fermentative methods of Microbial production 18
2.4.3 Continuous Production of FOS 18
2.4.3 Yield of FOS 19
2.5 Parameters of Enzymatic Reaction on Production of FOS 20
2.6 Characterization and Purification of Raw Materials. 25
2.7 Response Surface Methodology (RSM) 25
2.8 Application of FOS 29
2.8.1 Role of FOS in Control of Diabetes 30
2.8.2 FOS as Prebiotic 30
2.8.3 FOS as Dietary Fiber 31
2.8.4 FOS and Mineral Absorption 33
2.8.5 Role of FOS in Defense Functions 34
2.8.6 Anticancerous Effect of FOS 35
2.8.7 Additional Health Effect of FOS 35
2.8.8 Applications of FOS in Food Formulations 36
37

CHAPTER 3 MATERIALS AND METHODOLGY

3.4 Operational Framework
3.4.1 Phase 1: Characterization of Materials 39
3.4.2 Phase 2: Reaction Process in Batch System
39
3.5 Preparation of the Materials for Enzymatic Reaction of FOS 39
Production 41
3.5.1 Coconut Sugar 41
3.5.2 FTase and FOS 42
3.5.3 Buffer 43
3.6 Characterization of Materials 43
3.6.1 Coconut Sugar 43
3.6.1.1 The Total Sugar Contents in Coconut's Part 43
3.6.1.2 Total Sugar Contents in Coconut Sugar 44
3.6.1.3 Moisture Content 44
3.6.1.4 Spectral Analysis 45
3.6.1.5 Morphology Study 45
3.6.1.6 Viscosity of Coconut Sugar 46
3.6.2 Characterization of FTase 47
3.3.2.4 Molecular Weight Determination 47
3.3.2.5 Enzyme Activity 48
3.3.2.6 Stability of FTase 49
3.3.2.3.3 Thermal Stability 49
3.3.2.3.4 pH Stability 49
3.6.2.4 Spectral Analysis of FTase (FTIR) 50
3.6.3 Characterization of FOS 50

3.3.3.1 Spectral analysis of FOS 50
3.7 Standard of FOS Production 51
3.8 FOS Analysis by HPLC 51
3.9 One Factor at One Time (OFAT) 51
3.7 Determination of Kinetic Parameters in Batch System 52
3.8 Experimental Design 53
3.8.1 Screening Process using Two Level Fractional Factorial Design (FFD) 54
3.8.4 Optimization Process using Central Composite Design (CCD) 55
3.8.5 Validation of Model Empirical Equation 55
3.9 Scale up 56

CHAPTER 4 RESULTS AND FINDINGS

4.8 Characterization of Materials 58
4.8.1 Characterization of Coconut Sugar 59
4.8.1.1 Total Sugar Contents in Coconut's Part 59
4.8.1.2 Total Sugar Contents in Coconut Sugar 60
4.8.1.3 Moisture Contents 62
4.8.1.4 Spectral Analysis 62
4.8.1.5 Morphology Study 63
4.8.1.6 Viscosity Study 65
4.8.2 Characterization of FTase 68
4.8.2.1 Molecular Weight by using SDS PAGE 68
4.8.2.2 Enzyme Activity Assay 68
4.8.2.3 Stability of FTase 69
4.8.2.3.1 Thermal Stability 69
4.8.2.3.2 pH Stability 71
4.8.2.4 Spectral Analysis of FTase 72
4.8.3 Characterization of FOS 74
4.8.3.1 Spectral Analysis (FTIR) 74
4.9 Kinetic Parameter in Batch System 75
4.10 One Factor at One Time 78
4.10.1 Time Course Study on the FOS Production and other Reducing Sugar 79
4.10.2 Effect of Reaction Time 80
4.10.3 Effect of Substrate Concentration 81
4.10.4 Effect of Enzyme Concentration 82
4.10.5 Effect of Temperature 83
4.10.6 Effect of pH 84

4.10.7 Effect of Agitation Speed 85
4.10.8 Concluding Remarks 86

4.11 Screening Process by Fractional Factorial Design (FFD) 87

4.11.1 Analysis and Findings 87
4.11.2 Concluding Remarks of FFD Study 95

4.12 Optimization by Response Surface Methodology (RSM) 96

4.12.1 Concluding Remarks of CCD study 103

4.13 Validation of Empirical Model Adequacy 103

4.14 Scale Up 105

CHAPTER5 GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

106

5.3 General Conclusion 108

5.4 Recommendations for Future Works

109

REFERENCES

APPENDICES

D Operational Framework, Correlation Chart, Standard Methods and Standard Curves 123

E Data Generations for OFAT, FFD and RSM study 136

F List of Publications 142

LIST OF FIGURES

Figure No.	Title	Page
2.1	Coconut sugar	10
2.2	The stages of FOS production from microbial FTase	17
3.1	The experimental flowchart	40
3.1	The dilution method of coconut sugar	42
3.2	Viscometer	46
3.3	Scale up process	57
4.1	Sugar contents in coconut's part	59
4.2	Total sugar contents in coconut sugar	61
4.3	Spectral Analysis of coconut sugar	63
4.4	a) Microscopic image of coconut sugar before reaction	64
	b) Microscopic image of coconut sugar with FTase after reaction	64
4.5	Viscosity of coconut sugar at various coconut sugar concentration and temperature limit	66
4.6	Viscosity of coconut sugar at various coconut sugar concentration	66
4.7	Typical Arrhenius plot for coconut sugar concentration	77
4.8	Enzyme activity of thermal stability	70
4.9	Relative activity of thermal stability	70
4.10	Enzyme activity of pH stability	71
4.11	Relative activity of pH stability	72
4.12	Spectral Analysis of FTase	73
4.13	FOS structure	74
4.14	Spectra Analysis of FOS	75

4.15	FOS production plot with coconut sugar concentration effect	77
4.16	Effect of reaction time on the production of FOS and other reducing sugars in coconut sugar	80
4.17	Effect of reaction time	81
4.18	Effect of coconut sugar concentration	82
4.19	Effect of enzyme concentration	83
4.20	Effect of temperature on the production of FOS	84
4.21	Effect of pH on the production of FOS	85
4.22	Effect of agitation speed on the production of FOS	86
4.23	The half normal plot for 24 fractional factorial design	92
4.24	Plot of interaction effect for FOS production (g/L): (a1 and a2) effect between coconut sugar concentration and temperature (b1 and b2) effect between pH and agitation speed	94
4.25	Normal probability plot of residual for FOS production	100
4.26	Plot of residual against predicted response for FOS production	100
4.27	a) Interaction graph, b) contour plot and c) 3D surface of FOS production from the model equation: effect of coconut sugar concentration and temperature	102

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE PROBLEM

There is now an array of first generation oligosaccharides available at industrial scale that are used as food ingredients due to their prebiotic properties and as they are selectively fermented by bifidobacteria, the prebiotics boost the total number of these microorganisms present in the colon, promoting a number of beneficial effects to our health. Fructo-oligosaccharides (FOS) are naturally occurring sugars that constitute one of the most established groups of prebiotic oligosaccharides in the world. It is present in trace amounts in natural foods like onion, asparagus, wheat, banana, tomato, and honey. FOS consists of sucrose molecules to which 1, 2 or 3 additional fructose units are added by a β-(2-1)-glycosidic linkage to the fructose unit of sucrose. FOS are manufactured either from sucrose by transfructosylation or from inulin by controlled enzymatic hydrolysis (Crittenden and Playne, 1996). The enzymes catalyzing the production of FOS are β-fructofuranosidase which is also called invertase and fructosyltransferase. In many recent studies, FOS has been produced by using chemical synthesis and microbial production of FOS by the action of fungal fructosyltransferase (FTase) on sucrose. Types of microbes changes, depends on the study that had been observed. However, in this study, the enzymatic reaction is retained by using commercial enzyme but the commercial sucrose utilization has been changed to the coconut sugar as the main substrate. It is mainly for cost reduction by using Malaysian natural harvest.

1.2 PROBLEM STATEMENT

There were already many researchers who studied the production of FOS. Most of them had used the conventional way which was microbial production of FTase by fermentation, while the second stage was the reaction of FTase against sucrose (substrate) to produce FOS under controlled conditions. However, FOS being product of interest, the parameters involved in the two processes must significantly be selected to obtain the maximum yields of FOS. This is more important as the main interest is to maximize the FOS yields (Sangeeta et al., 2005a). Hence, microbial production by the action of fungal fructosyl transferase (FTase) on sucrose is more feasible at industrial level. Microbial production of oligosaccharides provides a cost effective and convenient alternative to chemical synthesis (Prapulla et al., 2000). This study was retained the FOS production by using the enzymatic reaction of FTase against sucrose in batch reaction.

The utilization of sucrose for the production of sucrose is compulsory for the FOS production as FTase utilizes sucrose as the sole energy source for oligosaccharides synthesis. Most studies mainly use commercial sucrose as the substrate to react with FTase. Each production of FOS used 60% to 80% w/v sucrose concentration, which means extensive amount is needed through out the study and experiment. The alternative has to be sought for FOS production so that it is appealing to the industries in terms of cost reduction. Commercial sucrose is expensive and to achieve maximum production of FOS, a large amount of a cheaper alternative would be attractive in terms of profit margin apart from the product being beneficial to health.

In Malaysia, it is reported that the number diabetic patients is increasing daily and it is one of the most fatal diseases. The resource of sugar from sugar cane in the market endangers the patients' life due to the excessive consumption of sucrose. Besides that, the number of diseases involving the intestines has also increased. The concept of formulating foods for health benefits is a now become a popular trend. A significant driving force in the 'functional food' market place is consumer demand-the quest by consumers to optimize their health through food. Unfortunately, most of the functional foods such as prebiotics and probiotics are imported as Malaysia has not yet develop its

own industry. The price especially FOS is very expensive in the market. As the utilization of FOS is extensive, Malaysia has to find the best way to produce it and most importantly, is by using our own raw material especially from the agricultural harvest.

1.3 OBJECTIVES OF THE STUDY

The main objectives of this research are:

i) To determine the optimum parameters for producing FOS which are substrate concentration, enzyme dosage, temperature, pH and agitation speed by using enzymatic reaction from the commercially produced coconut sugar and commercial enzyme within two phases of operational frameworks which is characterization and experimental work.

ii) The experimental work which has 4 stages experimental methods and there are one factor at one time (OFAT) and screening process (Design Expert 6.0.8) for preliminary stages.

iii) To optimize the parameters obtained from preliminary stages by using Design Expert 6.0.8 and finally validation of model equation and scale up by using fabricated enzymatic reactor (10 L).

1.4 SCOPE OF THE STUDY

There are mainly four scopes in this research:

i) The characterization of materials (coconut sugar, FTase and FOS) was done before the reaction was carried out. The coconut sugar was characterized by the total sugar (sucrose, glucose and fructose) of all coconut parts, the total sugar (sucrose, glucose and fructose) of coconut sugar, moisture content, spectra analysis by Fourier Transform Infra Red (FTIR) and microscope for morphology study and viscosity of coconut

sugar varies with temperature and concentration. The characterization of FTase was analyzed using qualitative (SDS-PAGE) and quantitative (FTase activity), stability of FTase (thermal and stability) and finally spectral analysis by FTIR. While FOS will be characterized for spectral analysis by FTIR only.

ii) Determination of vital range and elimination of insignificant factor in the reaction between commercial coconut sugar and FTase which the parameters are coconut sugar concentration, FTase concentration, temperature, pH, reaction time and agitation speed, and initially with one factor at one time (OFAT) followed with a screening process (Design Expert 6.0.8).

iii) The significant factors for the reaction mixture that obtained from the preliminary stages then used to gain the optimum conditions by using Response Surface Method (RSM) also utilizing Design Expert 6.0.8.

iv) The equation obtained from the RSM in the optimization of FOS production finally will be validated and finally using scale up with optimum condition in 10 L self fabricated enzymatic reactor.

1.5 RATIONALE AND SIGNIFICANCE

In this study, the new material for FOS production has been introduced as an alternative way from the conventional method. Mainly, FOS can be produced by chemical synthesis and enzymatic reaction. Many dangerous and hazardous chemical have been used in order to produce FOS. Besides, the yield of FOS in the end of the reaction is higher and at maximum for the microbial production compared to the chemical synthesis (Prapulla et al., 2000). Recent study will approach enzymatic reaction with the utilization of commercial enzyme which is commercial FTase or Invertase. Commercial FTase can be obtained from the chemical suppliers at a reasonable price. The characteristics of the FTase that resist to temperature changes, pH changes and bacteria existence really help to facilitate the study and perhaps for

manufacturing industry in the future. FTase is mobilized enzyme that does not need other materials to keep it stable. It may reduce cost as cultured enzymes are very difficult to manage such as epoxy acrylic polymers (Ghazi et al., 2005). The selection of the substrate is very important in order to produce FOS intensively besides the utilization of commercial FTase in enzymatic reaction.

Due to the utilization of the FOS in a variety of ways for food industry, this is important to invent new solutions to reduce the production cost. In this study, coconut sugar was introduced as the new source of sucrose. Malaysia has been blessed with an abundance of agricultural harvest. One of it is coconut or Cocos Nucifera. In spite of accessibility, it is very cheap compared to its benefits. Coconut sugar extracted from coconut also has the essential benefits similar the coconut itself (Jirapeantong et al., 2007). The manufacturing of the coconut sugar has been introduced in Malaysia in the early 40's. Then it was later commercialized for the local market after independence and has been in the world market since the 80's. Presently, it is available in the market and it is brown. Most importantly is the content of sucrose in the coconut sugar is very high which about 83.7% (Apriyantono et al., 2002). The utilization of coconut sugar as a sucrose source may reduce about 50% of the production cost compared to when using the commercially produced sucrose.

FOS which is prebiotic and functional food can reduce hyperglycemia and finally prevent diabetes. Besides, significant benefits from FOS those posses interesting functional and physiological attributes like low sweetness, non-cariogenicity, low caloric value, hypolipidemic and hypocholestrolemic properties are suitable for diabetics patients. (Katapodis and Kalogeris, 2003). Besides, FOS was reported as being one of the prebiotics that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited numbers of bacterial species, so it is sufficient for patients who suffer from or to prevent colon cancer. Therefore, FOS is currently used in many food products. Even, the addition of FOS has already been manipulated in infant milk to improve feed efficiency, reduce diarrhea and as the most simple sugar that can easily absorb in the ileums.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The production of FOS has been an interest at the industrial level for the world market. Mainly, the microbial production of FOS is enzymatic reaction has been selected rather than chemical synthesis which is acknowledged as being more feasible, economical and more convenient. Basically, this method requires the two-stage experimental work. The first stage refers to the production of enzyme which is FTase from microbes and finally FTase will be used for the second stage is the reaction with substrate (sucrose) to produce FOS (Sangeetha et al., 2005a). In this study, the enzymatic reaction will remain but the process will be united to one process as the microbial production of FTase is no longer implemented but is substituted with the utilization of commercial FTase that is directly purchased from supplier. Besides, commercial coconut sugar also has been introduced as the new source of sucrose for FOS production. These new approaches will thoroughly be studied and discussed in this study for the new method beyond industrial application for FOS production that we know is really important in human's health and life.

The application of FOS is extensive especially for health and medication. The possible health benefits associated to these compounds have led to their popularity as food ingredients as well as they also being promoted as alternative sweeteners for diabetic formulations. Average daily consumption of FOS has been estimated to be 1 to 4 gram in US and 3 to 11 grams in Europe. The most common sources of FOS are wheat, honey, onion, garlic, and banana (Flamm et al., 2001). Table 2.1 illustrates the natural resources of FOS.

All these characteristics were discovered and as a result researches and FOS production were increased all over the world. Research work is being carried out all over the world for the past two decades in term of production, properties, analytical aspects and nutritional benefits of FOS. Many review articles describing the occurrence, preparation, properties and application of FOS (Crittenden and Playne, 1996; Yun, 1996; Slavin, 1999) have been published. Flamm et al. (2001) have critically reviewed the composition and source of FOS, its physiological effects upon consumption and its relation to the dietary fiber. All of these interesting topics will be detailed in the following text.

2.2. SUCROSE AS CRUCIAL SUBSTRATE FOR FOS PRODUCTION

In order to use FOS extensively, any probability to reduce the production cost has been studied. Authors have screened an array of substrates like apple pomace as substrate (Hang et al., 1995), cereal bran (Prapulla et al., 2002a), corn products (Prapulla et al., 2002b), by-products of coffee and tea processing industries (Sangeetha et al., 2003a), sugar cane bagasse and cassava bagasse for the production of FTase by *Aspergillus oryzae* under SSF conditions (Sangeetha et al., 2004b). In addition, alternate sources of sucrose like jaggery and sugarcane juice were used for the production of FOS using FTase from *Aspergillus oryzae* (Sangeetha et al., 2003b).

Table 2.1: Natural resources of FOS

Source	% FOS
Barley	0.15
Tomato	0.15
Onion	0.23
Banana	0.30
Brown sugar	0.30
Rye	0.50
Garlic	0.60
Honey	0.75

This has resulted in value addition to the agricultural by-products in preparing a high value product like FOS. Coconut sugar is also one of Malaysia's agricultural products that is rich with sugar contents especially sucrose. In this study coconut sugar will be the raw material as the new source of sucrose and its ability to produce FOS will be determined in this study.

For this study coconut sugar has been introduced for alternative source of sucrose. Coconut sugar was selected as a novel substrate in this study because it contains more than 80% of sucrose (Apriyantono et al., 2002) besides it is really cheap which about RM 1.80 per kg compared to the commercial sucrose which is priced at RM 349.99 and the cost maybe reduced by 150%. Apart from reducing the production cost, coconut sugar is long lasting and only expired after eight months if it is kept in the sterilized bottle in the refrigerator with temperature below 5^{o}C.

2.2.1 Advantages of Coconut Sugar as Substrate

In our country, we are blessed with extensive amounts of agricultural harvest. Coconut or Cocos Nucifera provides a nutritious source of flesh, juice, milk, and oil that has fed and nourished populations around the world for generations. On many islands coconut is a staple diet. Nearly one third of the world's population depends on coconut to some degree for food and economy. Coconut is highly nutritious as it is rich in fiber, vitamins, and minerals. Modern medical science is now confirming the use of coconut in treating many health problems of the previous conditions.

Coconut is easily available and cheap compared to its numerous benefits. Coconut sugar extracted from the coconut also has the essential benefits like the coconut itself (Jirapeangtong et al., 2008). Coconut sugar is the next big thing after virgin coconut oil was introduced in the market about three years ago. The raw coconut sugar granule is dark brown like the moscuvado sugar from sugar cane. It tastes similar to cane sugar but smells like burnt coconut flesh. The sugar particles look rough, but feel soft in one's hand, melt easily in the mouth and tastes slightly sweet.

The manufacturing of the coconut sugar has been introduced in Malaysia in the early 40's. Then it was commercialized to the local market after independence and been in the world market in the 80's. This pure and simple cane sugar alternative is produced from the sweet juices from the blossoms of tropical coconut palm sugar. Traditional sugar farmers climb high into the canopy of swaying coconuts and harvest the sweet nectar by gently slicing the flower. Once collected, the nectars are kettle-boiled into a thick caramel and ground into a fine crystal.

Coconut sugar has long been a staple for south East Asian culinary heritage and herbal medicine. Coconut Sugar is naturally low in Glycemic Index (GI), which has the benefits in weight control and improves glucose and lipid levels in diabetics (type 1 and type 2). It also has a nutritional content far richer than any other commercially available sweeteners. Coconut Sugar is especially high in Potassium, Magnesium, Zinc and Iron and is a natural source of the vitamins B1, B2, B3, B6 and C. It is a 100% Organic, unprocessed, unfiltered, and unbleached natural sweetener and contains no preservatives (Apriyantono et al., 2002)

This coconut sugar is produced by smallholder farmers. 100% of the money from growing, harvesting and primary processing of this ingredient stays in the local community or is known as Italicise Small and Medium sized Industry (SMIS). Through aggressive product marketing and production training, smallholder sugar tappers have risen well above the poverty line and are able to earn an increase in personal income of close to 200%, while maintaining a competitive market price as the cane sugar alternative. The Food and Agriculture Organization (FAO) of the World Bank has reported that palm sweeteners like coconut sugar are the single most sustainable sweetener in the world. Coconut Sugars are not produced from the same palm species as is used for the production of Palm Oil. Coconut Palms produce an average of 50 to 75% more sugar per acre than Sugar Cane and use less than 1/5th of the nutrients for that production. Tropical palms are ecologically beneficial tree crop which grows in diverse, wild-life supportive agro-ecosystems. The crop helps restore damaged soils and requires very little water.

Presently, coconut sugar found in the market uses the finest technology and it is brown. Most importantly is the content of sucrose in the coconut sugar is very high which is about 83.7% (Apriyantono et al., 2002). Furthermore the utilization of coconut has expanded into a new discovery which utilizes the contents of sucrose found in it. The innovation was to produce FOS through reaction with commercial enzyme FTase or also known as Invertase. This is an extremely great idea where it may reduce the cost on purchasing the substrate thus promoting the Malaysia SMI industry besides supporting our Malaysia's ex Prime Minister Tun Abdullah Badawi call to developed agriculture in our country. Figure 2.1 below shows the coconut sugar in powder form.

2.3 THE NOVELTY OF FTASE FOR FOS PRODUCTION

Generally, enzyme is a high molecular-weight protein or protein – like substance that acts on a substrate (reactant molecule) to transform it chemically at a greatly accelerated rate, usually 103 to 107 times faster than the uncatalyzed rate. Without enzymes, essential biological reactions would not take place at a necessary to sustain life. Enzymes are usually present in small quantities and not consumed during the course of the reaction nor do they affect the chemical reaction equilibrium. Enzymes provide the alternate pathway for the reaction to occur thereby requiring lower activation energy.

Figure 2.1: Coconut Sugar

Fructosyl Tranferases (FTases) are the enzymes responsible for the microbial production of FOS. FTase produces FOS (GF_n) from sucrose (GF) in a disproportionate mode, thereby forming 1 kestose (GF_2) initially, then 1-nystose (GF_3), followed by 1-fructofuranosyl nystose (GF_4) as explained by Yun (1996). Microbial FTase is derived from bacterial and fungal sources. Several microorganisms capable of producing FTase have been screened (Sangeetha et al., 2003a). Table 2.2 shows a list of microorganisms reported to produce FTase enzyme.

In the literature, different microbial sources of FTase are reported to produce FOS with different linkages to form 1-kestose, 6-kestose and neokestose in varying yields based on initial sucrose concentration is also discussed. The uses of immobilized enzymes and cells have led to the development of effective and economic methods for large-scale production of FOS. Forced flow Membrane reactor systems, biocatalyst system with a bioreactor equipped with a microfiltration systems, have been used for production of high content FOS by removing the released glucose and unreacted sucrose from the reaction mixture resulting up to 98% FOS. The use of mixed enzyme system of Fructosyl Transferase and Glucose Oxidase or Glucose Dehydrogenase, could produce highly concentrated FOS up to 90 to 98%. Nano-filtration for removing glucose resulted in FOS of 90% concentration. The purified enzyme was found to produce kestose and nystose unlike the crude enzyme which produced GF5 and GF6 oligosaccharides (Sangeetha et al., 2005).

2.3.1 Bacterial FTases

Bacterial strains have been reported to produce inulinases but FOS producing enzymes are very rare in bacterial strains. A transfructosylating enzyme, which produces FOS from sucrose, has been isolated from *Bacillus macerans* EG-6 which, unlike other

Table 2.2: List of microorganisms able to produce FTase enzyme

Source	Reference
Fungal	
Aureobasuduim pullulans	Yun, 1996
Aureobasidium sp	Yun, 1996
Aspergillus japonica	Yun, 1996
Aspergillus niger	Yun, 1996
Aspergillus sydowi	Yun, 1996
Calviceps purpuria	Yun, 1996
Fusarium oxyporum	Yun, 1996
Penicilium frequentants	Yun, 1996
Penicilium spinolosum	Yun, 1996
Phytophtora parasitica	Yun, 1996
Scopulariopsis brevicaulis	Yun, 1996
Saccharomices cerevisae	Yun, 1996
Penicilium citrinum	Hayashi et al., 2000
Plant	
Agave Americana	Yun, 1996
Agave vera cruz	Yun, 1996
Asparagus officinalis	Yun, 1996
Allium cepa	Yun, 1996
Chicorium intybus	Yun, 1996
Crinum longifolium	Yun, 1996
Sugar beet leaves	Yun, 1996
Helianthus toberosus	Yun, 1996
Lactuva sativa	Yun, 1996
Lycoris radiate	Yun, 1996
Taraxicum officinale	Yun, 1996
Bacterial sources	
Artrobacter sps	Yun, 1996
Bacilus macerans	Park et al, 2001

FTases, produced selectively GF5 and GF6 fructooligosaccharide. The final yield of FOS was reported to be 33% when 50% sucrose was used as substrate (Park et al., 2001). The ethanol producing bacteria *Zymomonas mobilis* has been reported to produce a levansucrase capable of producing FOS and levan. The extracellular levansucrase that precipitated along with levan after ethanol treatment of culture fluid has been used as a biocatalyst for FOS production in sugar syrup. The yield of FOS was found to be 24 to 32%, which constituted a mixture of 1-kestose, 6-kestose, neokestose and nystose. Glucose content was found to increase during all 24 h of reaction. The

presence of ethanol (7.0%) in sucrose syrup limited the enzyme's FOS forming activity to 24% during the first 24 h of incubation. Fructan syrup produced from sucrose by using levan-levansucrase sediment as biocatalyst was reported to have satisfactory taste, reduced energetic value and therefore, may be used as a source of prebiotics (Beker et al., 2002). *Lactobacillus reutri* strain 121 has been reported to produce 10 g L^{-1} FOS (95% kestose and 5% nystose) in the supernatants when grown in sucrose containing medium. FTase isolated from the strain when incubated with sucrose, produced FOS as well as inulin. After 17 h of incubation with sucrose, 5.1 g L^{-1} FOS and 0.8 g L^{-1} inulin were synthesized (Van Hijum et al., 2002).

2.3.2 Fungal FTases

Several fungal strains, especially of *Aspergillus sp*, are known to produce extracellular or intracellular FTase. *Aspergillus niger* AS 0023 has been reported to produce an intracellular FTase which yielded 54% FOS using 50% sucrose as substrate (L'Hocine et al., 2000). Purification and partial characterization of fructosyl transferase and invertase from the cells of *Penicillium citrinum* have been reported (L'Hocine et al., 2000) to produce a syrup containing neofructooligosaccharides wherein the efficiency of FOS production was more than 55% using 70% sucrose as substrate. Production of FOS from sucrose catalyzed by *β*-Fructofuranosidase (FTase) was achieved by (Chien et al., 2001) with the use of mycelia of *Aspergillus japonicus* immobilized in gluten. One gram of mycelia-immobilized particles having a cell content of 20% (w/w) was incubated with 100 ml sucrose solution with an initial concentration of 400g L^{-1}. After a reaction period of 5 h, the FOS yield was 61% of the total sugars. The reaction velocity increased with the cell content in the gluten matrix and a maximum value was obtained when the cell content was as high as 20% (w/w).

The authors have reported *Aspergillus oryzae* as a novel source of extracellular FTase (Sangeetha et al., 2003c). The cultural conditions and reaction parameters have been standardized to get FOS yield of 58% (Sangeetha et al., 2002). Culture fluid, cells and culture broth homogenate of *A. oryzae* CFR 202 and *A. pullulans* CFR 77 have also been used for FOS production to get up to 60% FOS (Sangeetha et al., 2004a). Table 2.3 shows the summary of the FOS yields obtained using FTase from various

microorganisms. In reference to the table, the highest FOS yield obtained using FTase was from *Aspergillus Japonicus* (61%) followed by the commercial FTase and FTase from *Aspergillus Aculeatus* at 60.4% and 60.1% respectively.

Table 2.3: FOS yields obtained using FTase from various microorganisms

Source	Substrate sucrose (g/L)	FOS yield (%)	Reference
Commercial FTase	450	60.4	Hang et al., 1995
Aspergillus aculeatus	600	60.1	Ghazi et al., 2007
Aspergillus niger AS 0023	500	54	L' Hocine et al., 2000
Penicilium citrinum	700	55	Hayashi et al., 2000
Aspergillus japonicus	400	61	Chien et al., 2001
Aspergillus oryzae CFR 202	600	58	Sangeetha et al., 2002
Aureobasidium pullulans CFR 77	550	60	Sangeetha et al.,2004a
Aureobasidium Pullulans cells	360	46.1	Shin et al., 2003
Bacillus macerans EG-6	500	33	Park et al., 2001
Zymomonus mobilis	500-600	24-32	Beker et al., 2002

2.3.3 Commercial FTase (Invertase)

There are many kinds of commercial FTase in the market. They are generally produced by bacteria and fungal. For this study, the commercial FTase has been purchased from Sigma Aldrich that has been cultured from baker's yeast. The characteristics of commercial FTase are almost the same with other bacteria FTase. However, the commercial FTase is more resistant to its surrounding conditions. In this study, the commercial FTase purchased were made from baker's yeast, perhaps it will prove the facts that FTase is more resistant to the temperature changes, pH changes and existence of other microbes. Commercial FTase was chosen as it is easy to obtain, posses good specifications and it is easy to manage the reaction for FOS production.

Commercial FTase is mobilized enzyme, the enzyme is colorless. When the enzyme was subjected to thermal treatment, the fluorescence of tyrosine and tryptophan decreased slowly, while after high-pressure treatment, these aromatic residues become more exposed to the aqueous solvent during unfolding, giving rise to a large decrease in fluorescence in the 330 to 340 nm region. Moreover, in the latter case, an enhancement of light scattering intensity showed changes in protein-protein interactions.

2.3.4 Cell recycling for the production of FTase

A recycling cell culture system was developed for repeated production of FTase and FOS by reusing the pellets of *A.oryzae* CFR 202. The system is cost effective compared to those using immobilized enzymes or cells and it can be used for getting many folds more FTase and FOS than using the conventional fermentation process (Sangeetha et al., 2005).

2.4 THE TYPE OF PROCESS IN FOS PRODUCTION

FOS can be produced from the reaction of FTase on the substrate which is sucrose. FTase can be directly purchased or cultivated from many kinds of microbes while sucrose is the only substrate that has been used for FOS production. Other process that can produce FOS is by using chemical hydrolysis on the long chain of inulin. The chain bond will be split into smaller chain and the molecules that are newly form is FOS. The details of the process are as follows:

2.4.1 Chemical synthesis in production of FOS

There are two types of process that was used to produce FOS which is chemical synthesis and enzymatic reaction. Chemical synthesis was believed to firstly founded by Japanese researchers and it was the first method published to produce FOS. The syntheses of FOS from chemical reactions have variety ways for example by Blecker et al. (2009) the FOS can be synthesized from the process of acid hydrolysis of inulin. The hydrolysis process uses free inulases in laboratory scale. The FOS can be synthesized from the inulin as FOS has shorter chain which can be obtained by breaking the bond

from a longer inulin chain. The chemical structures of both FOS and inulin are almost similar but FOS consists only 10 chains of other saccharides besides fructose. The acid hydrolysis beginning from the initial fructose release rate is found to be roughly proportional to the inverse of the average polymerization degree in number. A pseudo first order kinetic is found with respect to the fructosyl chain end concentration and to the proton concentration. Arrhenius plot is found to reasonably fit the data in a relatively wide temperature range (7 ^{o}C to 130 °C). The results allow the estimation of the fructose release rate in many foodstuff processing conditions.

2.4.2 Enzymatic reaction in production of FOS

Microbial production of FOS is enzymatic reaction that is very popular in the literature. The novelty of the best enzymes and methodology depend on the yields of the FOS produced. Basically, this method requires two-stage experimental work which the first stage refers to the production of enzyme FTase, from microbes and finally FTase will be used for the next stage which is reaction with sucrose to produce FOS. The details will be discussed as following. Different fermentative methods have been used for the production of FOS. SSF has been used for the production of a value added product FOS utilizing various agro industrial byproducts. As shown in Figure 2.2 is the production of FOS from microbial production. The figure demonstrated in lab scale and the application of microbial FTase on the production of FOS.

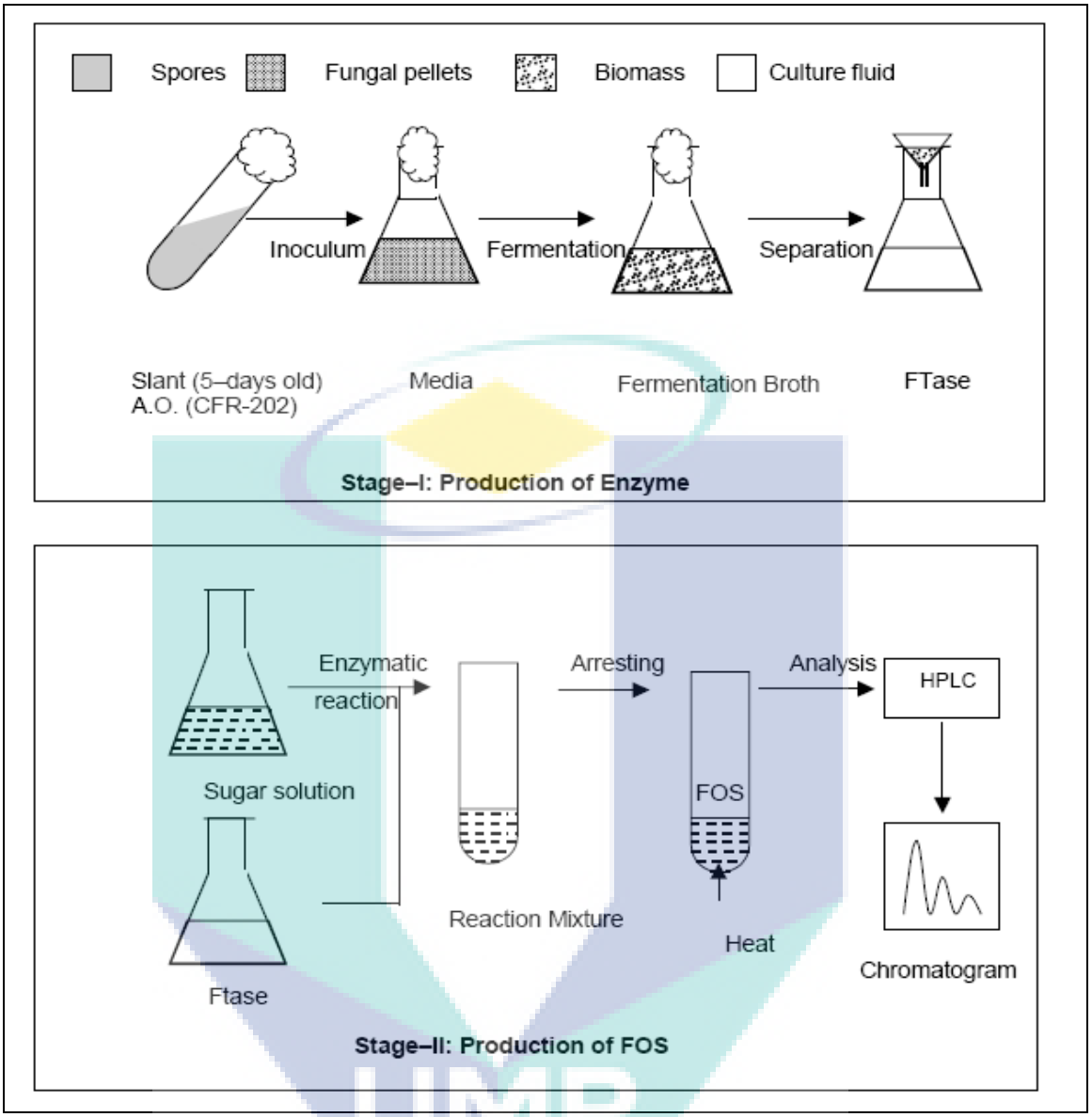


Figure 2.2: The stages of FOS production from microbial FTase

Referring to the figure, the productions of FOS from microbial FTase consists of two stages which the first stage is the production of FTase and the next stage is the production of FOS from the cultured FTase in the stage 1. This is mainly the conventional method of the utilization of microbial FTase in order to produce FOS.

2.4.2.1 Microbial Production of FOS

FTases from different microorganisms have been reported to produce FOS with different linkages to form 6-kestose, 6-kestose and neokestose. Microbial production of oligosaccharides has been extensively reviewed by Prapulla et al. (2000). Enzymes derived from microorganisms like *Aspergillus phoenicis*, *A. japonicus*, *A. niger*, *Fusarium oxysporum*, *Scopulariopsis brevicaulis*, *Penicillium frequentens*, *Penicillium rugulosum*, *Aureobasidium pullulans* and *Arthrobacter* sp. have been reported to produce FOS from sucrose. *S. brevicaulis* has been found to produce only 1-kestose. More detailed discussion on this has been reported (Prapulla et al., 2000).

Mineral salts in the fermentation media have been found to improve FOS production by microbes. The effect of salt concentrations on the synthesis of FOS has been studied by Vigants et al. (2000). It has been reported that 0.6 M NaCl concentration led to an increase of FOS production by 3.5-fold by *Z. mobilis* 113S during fermentation in a medium containing 10% sucrose. Sorbitol was also produced as a fermentation by-product in the presence of mineral salts. In a medium with high (65% w/w) sucrose content, the salts had an inhibitory effect on FOS production by lyophilized *Z. mobilis* cells. The enhanced production of FOS and formation of orbital have been concluded to play an osmoprotective role to *Z. mobilis*.

2.4.2.2 Fermentative Methods of Microbial Production of FOS

There are two methods of FTase production by fermentation which are Submerged Fermentation (SmF) and Solid State Fermentation (SSF). Production of enzymes by SSF has potential advantages over SmF with respect to simplicity in operation, high productivity fermentation, less favorable for growth of contaminants and concentrated product formation. SSF requires less space capital and operating costs, simpler equipment and easier downstream processing compared to SmF. In addition, it permits the use of agro-industrial residues as substrates, which are converted into bulk chemicals and fine products with high commercial value. There are many reports on FTase production by SmF using various microorganisms. Prapulla et al. (2000) have discussed FTase production by SmF in detail.

2.4.3 Continuous Production of FOS

Semi batch production of fructooligosaccharides from sucrose by immobilized cells of *Aureobasidium pullulans* has been reported by Yun et al. (1990). They have discussed the use of immobilized system for repeated production of FOS in a stirred tank reactor (STR). They reported that the semi batch process was superior to a continuous process in a STR. The main reason for the poor yields of FOS in stirred tank reactor is due to the product inhibition by glucose. From the point of process development, it is well known that a continuous process offers many advantages over a batch or semi-batch process. Thus an efficient system of a packed bed reactor for the continuous production of FOS has been reported by Yun et al. (1992). Under the optimum conditions, the reported reactor productivity was 180 g/L h. They have also reported that the initial activity was maintained for more than 100 days and the reactor was also scaled up to 1000 L.

Authors have developed a two-stage continuous process for the maximization of FOS production (Sangeetha et al., 2005). The uses of immobilized enzymes and cells have led to the development of effective and economical methods for large-scale production of FOS. This technique has imparted operational stability to the enzyme thereby resulting in continuous production of FOS. The optimized conditions were then scaled-up to 15 L of FTase in a fermentor and then at 10 L level of FOS production using a specially designed reactor. The results matched with the shake flask level studies. The feasibility of developing maximization programme for a continuous two-stage process was demonstrated. This can be suitably adopted to commercialize the continuous process for the production of FOS. A method for the continuous production of FOS was studied by Chien et al. (2001) immobilizing the mycelia on gluten particles and packing it into a column reactor. FOS yield of 173 g h^{-1} L^{-1} of reaction volume was achieved at a flow rate of 0.8 ml min^{-1}. The mass fraction of FOS increased from 0.2 to 0.54 (w/w) as the flow rate decreased from 1 to 0.1 mL min^{-1}, which corresponded to an increase in the residence time from 0.35 to 3.5 h. The immobilized preparation was reported to be stable in long term operation since gluten was found to be adequate as the base material to immobilize mycelia-associated enzymes. However, the half-life of the enzyme was found to be 34 days.

A forced flow membrane reactor system for transfructosylation was investigated by Nishizawa et al. (2000) using several ceramic membranes having different pore sizes. *β*-Fructofuranosidase from *A. niger* ATCC 20611 was immobilized chemically to the inner surface of a ceramic membrane activated by a silane coupling reagent. Transfructosylation took place while sucrose solution was forced through the ceramic membrane by cross flow filtration and the yield of FOS was reported to be 560 times higher than that is reported in a batch system. The half-life of the immobilized enzyme on the membrane was estimated to be 35 days by a long-term operation. Sheu et al. (2002) have reported a complex biocatalyst system with a bioreactor equipped with a microfiltration (MF) module to produce high-content FOS in a continuous process initiated by a batch process. The system used mycelia of *A. japonicus* CCRC 93007 or *A. pullulans* ATCC 9348 with b-fructofuranosidase activity and *Gluconobacter oxydans* ATCC 23771 with glucose dehydrogenase activity. Calcium carbonate slurry was used to maintain pH at 5.5 and gluconic acid in the reaction mixture was precipitated as calcium gluconate. Sucrose solution with an optimum concentration of 30% (w/v) was employed as feed for the complex cell system and high content FOS was discharged continuously from a MF module. The complex cell system was run at 30 ^{o}C with an aeration rate of 5 vvm and produced more than 80% FOS with the remainder being 5 to 7% glucose and 8 to 10% sucrose on a dry weight basis, plus a small amount of calcium gluconate. The system was operated for a 7-day continuous production process with a volumetric productivity of more than 160 g L^{-1} h^{-1} FOS. The complex cell system with both b-fructofuranosidase and glucose dehydrogenase activities was proved to be as effective as a two enzyme system. Since the enzyme activities were retained up to 6 days, the complex cell system might be more economical than two-enzyme system (Sheu et al., 2002).

2.4.4 Yield of FOS Production

FOS is produced by removing the liberated glucose and unreacted sucrose from the reaction mixture resulting in up to 98% FOS. Industrial production of FOS carried out with microbial FTases has been found to give a maximum theoretical yield of 55 to 60% based on the initial sucrose concentration. The FOS yield does not increase beyond this value because glucose liberated during the enzymatic reaction acts as a competitive

inhibitor (Yun, 1996). To enhance the FOS conversion by removing the liberated glucose, the use of mixed enzyme systems has been recommended by many authors.

Studies were carried out on mixed enzyme systems using a commercial enzyme, with glucose oxidase and catalase, and mycelia of *A. japonicus* CCRC 93007 and *A. niger* ATCC 20611 with *β*-fructofuranosidase activity to produce high yields of FOS. The reaction was performed in an aerated stirred tank reactor maintained at pH 5.5 by slurry of $CaCO_3$. Glucose (an inhibitor of *β*-fructofuranosidase) produced, was converted by glucose oxidase to gluconic acid, which was then precipitated by slurry of $CaCO_3$ to calcium gluconate in solution. The system produced more than 90% (w/w) FOS on a dry weight basis, the remainders were glucose, sucrose and a small amount of calcium gluconate (Sheu, et al., 2001). Nishizawa et al. (2001) has achieved higher yields of FOS with a simultaneous removal of glucose using a membrane reactor system with a nano-filtration membrane, through which glucose permeated but, not sucrose and FOS. FOS percentage of the reaction product was increased to above 90%, which was much higher than that of the batch reaction product (55 to 60%). Studies have been carried out by Crittenden and Playne (2002) to remove glucose, fructose and sucrose present in food grade oligosaccharide mixtures using immobilized cells of the bacterium *Z. mobilis*. Unpurified fructo, malto, isomalto, gentio and inulin oligosaccharides containing total carbohydrate concentrations of 300 g L^{-1} were added to immobilize cells, in 100 ml batch reactors. Glucose, fructose, and sucrose present in the mixtures were completely fermented within 12 hours without any pH control or nutrient addition. The fermentation end products were ethanol and carbon dioxide without any degradation of the oligosaccharides in the mixtures. A minor amount of sorbitol was also produced as a fermentation by-product. The methods using mixed enzyme systems and mixed cultures have facilitated the removal of the residual sucrose as well as the inhibitory by-product glucose, thereby improving the final FOS yields. The use of mixed enzyme system of fructosyltransferase and glucose oxidase for the production of high content fructooligosaccharides has been investigated by Yun and Song (1993). They have reported that by using 10 units of fructosyltransferase of *Aureobasidium pullans* KFCC 10524 (Sangeetha et al., 2004a) and 10 units of glucose oxidase (E.C.1.1.3.4) from *A. niger* with a stated activity of 25,000 units/g per gram of sucrose, produced highly concentrated FOS up to 90% was obtained. Yun et al. (1994) have

reported the production of high content FOS using a mixed enzyme system of *β*-fructofuranosidase and glucose oxidase. Under the optimized conditions, high content FOS up to 98% was obtained. Complete consumption of released glucose and unreacted sucrose by the mixed enzyme system resulted in high content FOS.

They have reported that there was a significant difference in sugar composition in the FOS produced by the mixed enzyme system when compared to that produced by fructosyltransferase/furanosidases. The content of nystose was higher in the former. Barthemeuf and Pourrat (1995) have reported the use of crude fructosyltransferase from a new strain of *Pencillium rigulosum* isolated in their laboratory for the production of high content FOS. They have reported that the crude enzyme from *Pencillium rigulosum* to be a mixed enzyme system of fructosyltransferase and glycosidase. Under optimized conditions they were able to obtain a yield of 80% FOS. The FOS produced had a high concentration of fructofuranosyl nystose. Studies were carried out on mixed enzyme systems using a commercial enzyme, with glucose oxidase and catalase, and mycelia of *A. japonicus* CCRC 93007 and *A. niger* ATCC 20611 with *β*-fructofuranosidase activity to produce high yields of FOS. The reaction was performed in an aerated stirred tank reactor maintained at pH 5.5 by slurry of $CaCO_3$. Glucose (an inhibitor of *β*-fructofuranosidase) produced, was converted by glucose oxidase to gluconic acid, which was then precipitated by slurry of $CaCO_3$ to calcium gluconate in solution. The system produced more than 90% (w/w) FOS on a dry weight basis, the remainder was glucose, sucrose and a small amount of calcium gluconate (Sheu et al., 2001).

Nizhizawa et al. (2001) have achieved higher yields of FOS with a simultaneous removal of glucose using a membrane reactor system with a nano filtration membrane, through which glucose permeated, but not sucrose and FOS. FOS percentage of the reaction product was increased to above 90%, which was much higher than that of the batch reaction product (55 to 60%). Studies have been carried out by Crittenden and Playne (2002) to remove glucose, fructose and sucrose present in food grade oligosaccharide mixtures using immobilized cells of the bacterium *Z. mobilis*. Unpurified fructo, malto, isomalto, gentio and inulin oligosaccharides containing total carbohydrate concentrations of 300 g L^{-1} were added to immobilize cells, in 100 mL

batch reactors. Glucose, fructose, and sucrose present in the mixtures were completely fermented within 12 h without any pH control or nutrient addition. The fermentation end products were ethanol and carbon dioxide without any degradation of the oligosaccharides in the mixtures. A minor amount of sorbitol was also produced as a fermentation by-product. The methods using mixed enzyme systems and mixed cultures have facilitated the removal of the residual sucrose as well as the inhibitory by-product glucose, thereby improving the final FOS yields. Sangeetha et al. (2004c) have reported the use of whole cells of *Aspergillus sp.* 27H, a soil isolate for the production of FOS. The organism was found to possess both hydrolytic and the transfructosylating activities. Under optimized conditions they were able to obtain a maximum concentration of FOS of 376 dm^{-3} corresponding to a value of 600 to 620 g kg^{-1} of FOS solids in the reaction mixture by 6 h of reaction.

A complex enzyme system in a bioreactor with a micro filtration facility using both the mycelia with *β*-fructofuranosidase activity and bacterial cells with dehydrogenase activity. Table 2.4 illustrated the summary of the methods used by many literature sources in order to produce FOS.

Table 2.4: The method for FOS production from many literature sources

Method	**FOS Yield**	**Reference**
Enzymatic production of FOS from sucrose by using commercial FTase	70%	Hang and Woodams,1995
FOS production by *Aspergillus sp. N74* in mechanically agitated airlift reactor	70%	Sanchez et al., 2008
FOS production by the colonization of *Aspergillus Japonicus*	69%	Mussato et al., 2009
FOS and *β*-fructofuranosidase production by *Aspergillus Japonicus* immobilized in liguocellulosic materials	64 to 69.4%	Mussato et al., 2009
Immobilization of FTase from *Aspergillus acueletus* on epoxy-activated Sepabeads EC for FOS production	61.5%	Ghazi et al., 2005
Immobilization of *Aspergillus Japonicus* by entrapping cells in gluten for FOS production	61%	Chien et al., 2001
Production of FOS by the mycelia of *Aspergillus Japonicus* immobilized in Calcium Alginate	61%	Cruz et al., 1998
Maximization of FOS production of two stage continue process by using *Aspergillus Oryzae* CFR 202	58%	Sangeetha et al., 2005
Immobolization of Pectinex Ultra SP-L for FOS production	57%	Tanriseven et al., 2005

FOS production using two mobilized microorganisms in an internal loop airlift agitator	55%	Lin et al., 2008
FOS production using FTase from *Aspergillus oryzae* CFR 202	50 to 53%	Sangeetha et al., 2004a
FOS production using FTase from *Aureobasidium pullulans* CFR 77	50 – 54%	Sangeetha et al., 2004a
FOS production using FTase from recycling culture of *Aspergillus Oryzae* CFR 202	53%	Sangeetha et al., 2004b
Production of FOS from molasses by Aureobassidium pullulans cells	46%	Shin et al., 2003
Optimization of FOS production from plantation white sugar	26%	Toharisman et al., 2009
FOS production by FTase from *Aspergillus aculeatus*	22.3%	Nemukula et al., 2008

2.5 PARAMETERES OF FOS PRODUCTION USING ENZYMATIC REACTION

Hang and Woodams (1996) had studied the optimization of using the reaction between commercial FTase and sucrose syrup. Five parameters had been observed which are time course, substrate concentration, enzyme concentration, temperature and pH level. All the reported data are average values of duplicate. In this study, the agitation speed has been added while other five parameters remained but the substrate concentration which is sucrose syrup has been changed to coconut sugar concentration as the new substrate was introduced in this study.

2.6 CHARACTERIZATION AND PURIFICATION OF RAW MATERIALS.

Characterization of raw materials has been studied before all the parameters were to take place. Coconut sugar will be characterized due to its total sugar contents

which are sucrose, glucose and fructose, moisture contents, morphology by SEM and functional group by FTIR. Referring to only established literature that studied about coconut sugar by Apriyantono et al. (2002) coconut sugar contains 91.4% of total sugars and the main sugars detected were glucose, fructose and sucrose as shown in Table 2.5. The details of each characterization were thoroughly discussed in CHAPTER 3 and CHAPTER 4.

Purification and characterization of an enzyme is a necessary step to improve the understanding of its mode of action. Many authors have reported the purification and characterization of FTases from various sources.

Table 2.5: Change of coconut sap weight, pH and composition during preparation of coconut sugar

Parameter (Heating time), (min)	**0**	**22.5**	**45**	**67.5**	**90**
Weight (g)	2637.5	1926.0	1254.4	762.3	355.4
pH	6.40	6.35	6.10	6.05	n.a
Moisture content (%)	87.07	81.39	71.12	53.69	6.95
Sucrose (g)	345.0 (100%)b	344.2 (99.8%)	342.9 (99.4%)	319.8 (92.7%)	288.7 (83.7%)
Glucose (g)	45.7 (100%)b	41.3 (90.3%)	32.4 (70.8%)	20.7 (45.3%)	12.6 (27.7%)
Fructose (g)	35.8 (100%)b	23.2 (64.9%)	21.9 (61.2%)	13.9 (38.8%)	12.8 (35.7%)

FTases have been found to differ in their molecular weight and properties from one source to another. Park et al. (2001) have purified FTase from *B. macerans* EG-*6*, 63.5 fold by ammonium sulfate precipitation (20 to 60%), *CM Sepharose* CL 6B and fast protein liquid chromatographies on Resource Q, Phenyl Superose HR 5/5 and Mono S. The purified enzyme had a molecular mass of 66 kDa by SDS PAGE. The enzyme was stable at the pH range of 5 to 7 and had an optimum pH at 5. The optimum temperature for enzyme activity was at 50 ^{o}C. An important feature of this purified FTase is that the oligosaccharide composition in each product was significantly different based on the use of the enzyme obtained from each purification step.

The purified enzyme was found to produce kestose and nystose unlike the crude enzyme which produced GF5 and GF6 oligosaccharides (Park et al., 2001). Purification of FTase from the crude extract of *A. niger* AS 0023 has been detailed by L'Hocine et al. (2000) by successive chromatographies on DEAE Sephadex A-25, Sepharose 6B, Sephacryl S-200, and concanavalin A-Sepharose 4B columns. FOS yield was increased by 8% when purified enzyme was used. On native and SDS PAGE, the enzyme migrated as polydisperse aggregates yielding broad and diffused bands, which showed that the enzyme is a typical glycoprotein. FTase on native PAGE migrated as two enzymatically active bands with different electrophoretic mobility, one around 600 kDa and the other from 193 to 425 kDa. On SDS PAGE, these two fractions yielded one band corresponding to a molecular weight which range from 81 to 168 kDa.

The optimum pH and temperature for FTase were found to be 5.8 and 50^{o}C, respectively. Studies on the effect of metal ions showed that FTase was completely inhibited with 1 mM Hg_2C and AgC (L'Hocine et al., 2000). Wang and Rakshit (2000) have partially purified four fractions of an enzyme with transferase activity from *A. foetidus* NRRL 337. After ammonium sulphate precipitation and DEAE cellulose column chromatography, the purification folds of the fractions were 64, 25, 29 and 43. The optimum temperature was 60 ^{o}C and pH stability was in the range of 4 to 6. The pH optima, heat sensitivity and kinetic parameters for the four fractions were however, not the same. The details for all the experiments are in CHAPTER 3.

Table 2.6: Characteristics of FTase purified from various microbial sources

Source of FTase	Purification fold	Molecular weight (kDa)	Optimum		Stability		Reference
			pH	Temperature	pH	Temperature	
Bacillus macerans EG-6	63.5	66	5.0	50 ^{o}C	5.0 – 7.0	20 – 50 ^{o}C	Park et al., 2001
Athrobactor oxydans 117-21	95.5	54	6.5	45 ^{o}C	5.0 – 11.0	20 – 40 ^{o}C	Jang et al., 2003
Microbacterium laevaniformans ATCC 15953	45.6	64	6.0	30 ^{o}C	5.0 – 7.0		Park et al., 2003
Aspergillus niger ATCC 20611	51.6	340	5.0-6.0	50 – 60 ^{o}C	4.5 – 10.0	Up to 60 ^{o}C	Hirayama et al., 1989
Arthrobactor sp K^{-1}	405.3	52	6.5-6.8	55 ^{o}C	5.5 – 10.0	Up to 40 ^{o}C	Fujita et al., 1990, 1994
Streptococcus salivarius ATCC 25975	34.5	125.4	6.0 – 7.0	37 – 40 ^{o}C	-	-	Song and Jacques, 1999
Microbacterium sp AL-210	98.8	45	7.0	40 ^{o}C	7.0 – 8.0	Up to 40 ^{o}C	Cha et al., 2001
Aspergillus niger AS0023	78.5	81-168	5.8	50 ^{o}C	4.5 – 11.0	30 – 50 ^{o}C	L Hocine et al., 2000
Aspergillus foetidus	25	-	4.5	60 ^{o}C	4.0 – 6.0	Up to 40 ^{o}C	Wang and Rakshit, 2000

2.7 RESPONS SURFACE METHODOLOGY (RSM)

Optimization process in this study using statistical approach response surface methodology (RSM) to solve the complexity involved in this enzymatic reaction. RSM is a collection of statistical and mathematical techniques useful for developing, improving and optimizing process (Sharma et al., 2008). The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions (Chen et al., 2005) and (Karacan et al., 2007). RSM has been largely used for optimizing the media for enzyme production or citric acid production (Lotfy et al., 2007).

This methodology can be used in developing suitable treatment technology considering the effects of operational conditions on the reaction process or to determine a region that satisfies the operating specifications (Myers, 2002) and (Ravikumar et al., (2005). Recently, several studies describe the use of RSM for optimization of process parameters (pH, substrate concentration, biosorbent dose etc.) for bioreaction of enzymes (Can et al., (2006) and (Kiran et al., (2007) or dyes (Mohana et al., 2008) from synthetic solutions. However, there have been very few studies to optimize the media components for metal/dye removal (Mohana et al., 2008). Kiran et al., (2007) modelled the growth and copper uptake capability of *Candida utilis* as a function of sucrose and Cu (II) ion concentration in the bioaccumulation medium. The authors claim that this model can be used to find growth rate and Cu uptake (%) in mixtures containing unstudied concentrations of sucrose and Cu (II). Such studies are therefore very useful to predict nutrient supplementation for effective metal removal.

However, to the best of our knowledge, no study has been reported on application of RSM for optimization of FOS production from natural coconut sugar as substrate. Therefore, in order to evaluate the effects of the parameters and to optimize the relative dose of these media components, response surface methodology was employed in the present study.

2.8 APPLICATION OF FOS

FOS has a number of interesting functional properties that make them important food ingredients. The functional properties like for the use as prebiotics, dietary fiber, role in absorption and defense/Immunity, lipid metabolism control of diabetics have been discussed. The nutritional and health benefits of FOS have been the subject of many reviews in the recent years (Flamm et al., 2001; Flickinger et al., 2003). They have been extensively reviewed the nutritional responses to the presence of inulin and oligofructose in the diets of domesticated animals. The discovery of the significance of FOS has resulted the increment of researches and FOS production of the world. This phenomenon occurred after realizing that humans need FOS for their health enhancement. Below are the numbers of FOS application and benefits that have been revealed by the reviews. The summary of the application of FOS as tabulated in table 2.6.

2.8.1 Role of FOS in the Control of Diabetes

It has been reported by Luo et al. (2000) that the daily consumption of 20 g of FOS would decreased basal hepatic glucose production in healthy subjects without any effects on insulin stimulated glucose metabolism. When the effect of chronic ingestion of FOS on plasma lipid and glucose concentrations, hepatic glucose production and insulin resistance in type 2 diabetics was evaluated, it was found that FOS did not modify fasting plasma blood glucose and insulin concentrations or basal hepatic glucose production. Also, serum triacylglycerol, total and HDL cholesterol, free fatty acid, apolipoproteins A1 and B concentrations were not modified by the chronic ingestion of FOS. Purification of FOS by removing glucose and sucrose present in the mixture would increase its market value leading to development of food products aimed at diabetics. One of the main therapies proposed for type 2 diabetes is diet therapy that can control hyperglycemia, hyperlipidemia and insulin resistance. In this context, low calorie sweeteners like FOS have special significance because of their beneficial effects on lowering blood glucose. FOS has been claimed to lower fasting glycemia and serum total cholesterol concentrations, possibly via effects of short chain fatty acids produced during fermentation. Jerusalem artichoke, a member of the sunflower family that

produces edible tubers is a good source of inulin, a naturally occurring fructan. Roberfroid and Delzenne (1998) suggest that inulin-type fructans may be helpful for Non Insulin Dependent Diabetes. Yamachita et al. (1984) observed a noticeable reduction of cholesterolmia in diabetic subjects receiving a diet supplemented in fructooligosaccharides. Levrat et al. (1991) demonstrated that even low levels of inulin are effective in decreasing plasma cholesterol in rats. FOS was not found to significantly affect fasting concentrations of serum total cholesterol, HDL cholesterol, LDL cholesterol, serum triacylglycerols, serum free fatty acids, serum acetate, or blood glucose. The researchers concluded that 20 days of dietary supplementation with FOS had no major effect on blood glucose, serum lipids, or serum acetate in patients with type 2 diabetes. The effects are favorable in diabetes mellitus (Kaufhold et al., 2000).

2.8.2 FOS as Prebiotic

Prebiotics are non-digestible food ingredients that selectively stimulate the growth and/or activity of potentially health-enhancing intestinal bacteria. Since FOS is not hydrolyzed by the human digestive enzymes, it undergoes fermentation in the colon and encourages the growth of beneficial bacteria in the colon. This in turn discourages the growth of potentially putrefactive microorganisms in the colon resulting in a healthy gut environment. FOS has been demonstrated as an effective prebiotic through both in vivo and in vitro assessments. Durieux et al. (2001) have investigated the prebiotic effect of FOS by studying the metabolism of two types of chicory fructooligosaccharides (Fibruline Instant and Fibrulose F 97) by *Bifodobaterium longum*, *B. infantis* and *B. angulatum*. Chromatographic analysis of the medium after 120 h revealed consumption of all the fructose oligomers present in the commercial chicory FOS by all the strains. Maximum measurable degree of polymerisation of the substrates before fermentation was 41. Biomass production was highest with *B. infantis* (1.4 and 1.7 g dry wt L^{-1}) for its cultivation in a medium supplemented with Fibruline instant and Fibrulose F 97 respectively as substrate. These results indicate that Chicory FOS can be used as prebiotic (Durieux et al., 2001). A comparative evaluation of the fermentation properties of prebiotic oligosaccharides has been carried out by Rycroft et al. (2001). Populations of predominant gut bacterial groups were monitored over 24 h batch culture through fluorescent in-situ hybridization. Short chain fatty acid and gas

production was also measured. All prebiotics increased the numbers of bifidobacteria and most decreased clostridia. The oligosaccharides were found to differ in their fermentation characteristics. Fructooligosaccharides produced the highest populations of lactobacilli with least flatulence. Studies were carried out by Perrin et al. (2001) to compare the physiological behaviour of *Bifidobacterium infantis* ATCC 15697 growing on synthetic oligofructose. The studies were carried out in regulated and non-regulated batch cultures on semi-synthetic media. Differences between the carbohydrate utilization patterns were determined. Glucose was the preferred substrate for growth and biomass production, whereas fructose was the best for lactate and acetate production. With sucrose, biomass production reached the level obtained with glucose, where as with FOS and fructose more metabolites were produced. In a mixture of FOS, the shorter saccharides were used first and fructose was released in the medium (Perrin et al., 2001). Studies were carried out on two commercial strains of *Bifidobacterium spp* (Bf-1 and Bf-6) cultured anaerobicallyat 37 ^{o}C for 48 h in 12% (w/w) reconstituted Nonfat Dry Milk (NDM) containing 0, 0.5, 1.0, 3.0 or 5.0% (w/v) FOS. Growth and activity of the cultures in the presence of FOS were determined. Viability of each strain was assessed after 4 weeks of refrigerated storage at 4 ^{o}C. Growth promotion, enhancement of activity and retention of viability was more when *Biifidobacterium* Bf-1 (67%) and Bf-6 (45%) were grown in the presence of FOS. The effects of FOS increased with its increasing concentration and were maximal at 5% (w/v) (Shin et al., 2000). The effect of ingesting a low dose of FOS (5 g/day) by healthy human subjects on the faecal microflora especially bifidobacteria was investigated and compared to the ingestion of a placebo-sucrose (Rao, 2001). In a place controlled study design, faecal samples were collected from healthy human subjects, who were not on any medication, and immediately enumerated for bifidobacteria, bacteriodes, coliforms, total anaerobes and total aerobes. Faecal samples were collected from the subjects after administering FOS (5 g daily for 3 weeks). Samples subjected to microbial enumeration showed that ingestion of sucrose (5 g per day) was without effect on all faecal bacteria enumerated, whereas consumption of FOS (5 g per day) for 11 days resulted in close to one log cycle increased in bifidobacteria numbers. No further increase was observed after the next 10 days. At 2 weeks after termination of FOS ingestion, bifidobacteria numbers had decreased to almost that of the period before treatment. Increase in the numbers of bacteriodes and total anaerobic bacteria also occurred but not aerobic bacteria. Kaplan

and Hutkins (2000) screened twenty-eight strains Lactic Acid Bacteria (LAB) and bifidobacteria for their ability to ferment FOS on MRS agar. Twelve of 16 *Lactobacillus strains* and seven of eight bifidobacterial strains tested were able to ferment the substrate. It was found that like glucose, FOS was equally a good substrate in supporting growth. When individual oligosaccharides like GF2, GF3 and GF4 were used, their utilization was found to be minimal and the pH decreased to only 6.0 and none of the strains were able to use GF4. The tolerance and threshold dose of FOS that significantly increased fecal bifidobacteria were assessed and the optimal dose for increased bifidobacterial counts without significant side effects such as flatulence was reported to be 10 g/day. Supplementation with FOS led to an increase in LAB after 2 weeks without changing anaerobic bacterial levels. LAB is considered to be immunomodulatory and it directly or indirectly influenced the Gastrointestinal Tract (GIT) and systemic defense functions. Corresponding with this, supplementing the diet with FOS that would increase the densities and metabolic capacities of the LAB enhances the defense mechanisms of the host, increases resistance to various health challenges and accelerates recovery of the gastrointestinal tract after disturbances (Kolida et al., 2002).

2.8.3 FOS as Dietary Fiber

Dietary fibre consists of remnants of edible plant cell polysaccharides and associated substances resistant to hydrolysis by human alimentary enzymes, which may benefit health through a wide range of physiological effects. FOS is stored carbohydrates found in a number of vegetables, fruit and whole grains. They resist digestion and absorption in the stomach and small intestines of humans, as shown by their full recovery at the end of the ileum of healthy or ileostomised volunteers. Studies in patients with a conventional ileostomy by Cherbut (2002) have shown that mean excretion of FOS at the end of ileum was about 90% of the ingested dose. Thus, they enter the large intestine where they will be available for fermentation, as demonstrated by increased breath hydrogen. Increased lactate concentration has been found in colonic and fecal contents of rats, fed with FOS. Fermentation is complete and no residue has been found in human stools. They also improve laxation. Their bulking capacity comprises between 1.2 and 2.1 g of stool per gram of ingested substrate, resulting

mainly from the increase in microbial biomass in the colon. In addition, due to their fermentation properties, they also affect the intestinal epithelium that may strengthen mucosal protection and reduce the risk of gastrointestinal diseases. Therefore, FOS has been found to fit well within the concept of dietary fiber (Cherbut, 2002).

2.8.4 FOS and Mineral Absorption

Colonic fermentation of FOS leads to the decrease in pH in the colon and this facilitates the absorption of mineral ions from the intestine, mainly calcium and magnesium. This has been indicated by long term beneficial effects on bone health such as accumulation of bone mineral content in growing rats or prevention of bone loss in ovariectomised rats. The addition of 5% FOS prevented bone loss significantly in the femur and lumbar vertebra in the presence of dietary calcium (1%). At 0.5% calcium, 10% oligofructose was needed to significantly increase bone mineralization.

The effect may be due to enhancement of passive and active mineral transport across the intestinal epithelium, mediated by an increase in certain metabolites of the intestinal flora and a reduction in pH (Ahrens and Schrezenmeir, 2002). The effect of FOS on protein digestibility and mineral absorption was studied by Gudieal-Urabano and Goni (2002) in rats fed with diets containing 5 g kg^{-1} FOS, 5 g kg^{-1} cellulose/ FOS (1:1) or 5 g kg^{-1} cellulose as a source of dietary fiber. Addition of cellulose/FOS or FOS to the diet did not significantly modify the daily food intake and food efficiency.

However, FOS fed group showed significant decrease in body weight gain compared to cellulose fed groups. Faecal excretion was significantly lower when there was FOS intake, despite there being no significant difference in cellulose fed groups. Intake of FOS produced an increase in caecal content and an enlargement of the ceacal wall. This trophic effect could be attributed to the short chain fatty acids produced from the anaerobic fermentation of FOS by intestinal bacteria. Cellulose/FOS enhanced apparent absorption and apparent retention of Ca, Mg, Zn and Fe. FOS fed rats experienced an increase in apparent absorption and apparent retention of Mg compared to cellulose fed rats. FOS intake at the lowest dose was enough to provide a desirable

effect on mineral bioavailability in rats without any modification of nutritional parameters (Gudieal-Urabano and Goni, 2002).

2.8.5 Role of FOS in Defense Functions

FOS is known to prevent the colonization of human gut by pathogenic microorganisms because it encourages the growth of beneficial bacteria. This effect is attributed to the low pH environment created during fermentation of FOS in the colon and due to the secretion of antibiotic like substances by the beneficial bacteria. Studies have shown that supplementing the diets of chickens, pigs and rats with oligofructose and other Non-Digestible Oligosaccharides (NDOs) reduces fecal densities of *Salmonella* (Letllier et al., 2000). Supplementing the diet of mice with inulin and oligofructose reduces the densities of Candida in the small intestine of mice 7 days after infection. Mice infected systemically with virulent strains of *Listeria monocytogenes* and *Salmonella typhimurium* after being fed a diet with inulin and oligofructose (at 100 g/kg) had lower mortality than mice fed a diet with cellulose as the source of fiber (Buddington et al., 2002). FOS stimulates higher rates of colonocyte proliferation than cellulose and other NDOs without increasing the total amount of mucosa. Feeding mice with diets supplemented with inulin and oligofructose increased the activities of natural killer cells and phagocytes and enhanced T-lymphocyte functions compared to mice fed with diets of cellulose or lacking in fiber. These results are consistent with the observations of heightened resistance to systemic infections of *Listeria* and S*almonella,* the lower incidence and growth of tumors after exposure to carcinogens and transplanted tumor cells, and are in agreement with enhanced innate and acquired immune functions provided by *Lactobacillus* and other LAB. Supplementing diets with FOS should increase production of SCFA, and particularly butyrate, and it can be predicted to strengthen mucosal defenses and enhance responses to health challenges.

2.8.6 Anticancerous Effect of FOS

Studies with inulin and FOS have shown reduction of chemically induced aberrant crypts and prevention of colon cancer. According to Zobel et al. (2002), in rats, a prebiotic effect resulting in the proliferation of bifidobacteria (with the major

metabolites lactate or acetate) as well as of other bacteria could be responsible for the observed anticancer effects. Dietary treatment with inulin/oligofructose (15%) incorporated in the basal diets for experimental animals resulted in:

i) reduction of the incidence of mammary tumors induced in Sprague Dawley rats by methylnitrosourea

ii) inhibited the growth of transplantable malignant tumors in mice

iii) decreased the incidence of lung metastases of a malignant tumor implanted intramuscularly in mice.

It is reported that the dietary treatment with FOS/inulin significantly potentiated the effects of subtherapeutic doses of six different cytotoxic drugs commonly utilized in human cancer treatment (Taper and Roberfroid, 2002).

2.8.7 Additional Health Effect of FOS

Roberfroid and Slavin (2000) have reported that feeding rats with FOS (10%) for a few weeks decreased uremia in both normal and nephrectomized rats. Dietary FOS enhanced fecal nitrogen excretion and reduced renal excretion of nitrogen in rats. This occurs because these fermentable carbohydrates serve as energy source for the intestinal bacteria, which during growth, also requires a source of nitrogen for protein synthesis.

2.8.8 Applications of FOS in Food Formulations

Inulin and oligofructose are ingredients that deliver a number of important nutritional benefits as well as contribute functional properties that enhance shelf life and taste profile of various food products like nutritional bars (Izzo and Niness, 2001). Examples of the use of FOS in food products include the following:

i) Light jam products: FOS can be used as the sole sweetening agent and reduces 34% calorie reduction compared to the sucrose standard.

Organoleptic characteristics of the products are claimed to be very similar, with the test sample having a lower sweetness and a softer texture.

ii) Ice cream: FOS can be used with inulin to replace all the sugar and reduce the fat content and give excellent mouthfeel characteristics. Since the freezing point depression is less with oligofructose than with sugar, the texture can be harder.

iii) Confectionery applications: Hard candies, gums, and marshmallows can be made while achieving significantly reduced energy values (Murphy, 2001).

CHAPTER 3

METHODOLOGY

3.1 OPERATIONAL FRAMEWORK

This research is conducted in order to determine the optimum reaction parameters which can produce high yield of FOS from coconut sugar and FTase. The flowchart of experimental procedures were carried out in two main phases as shown in Figure 3.1 while the details of has been described in operational framework in Appendix A1. This aim would be best achieved by carrying out the following experimental procedures.

3.1.1 Phase 1: Characterizations of Materials

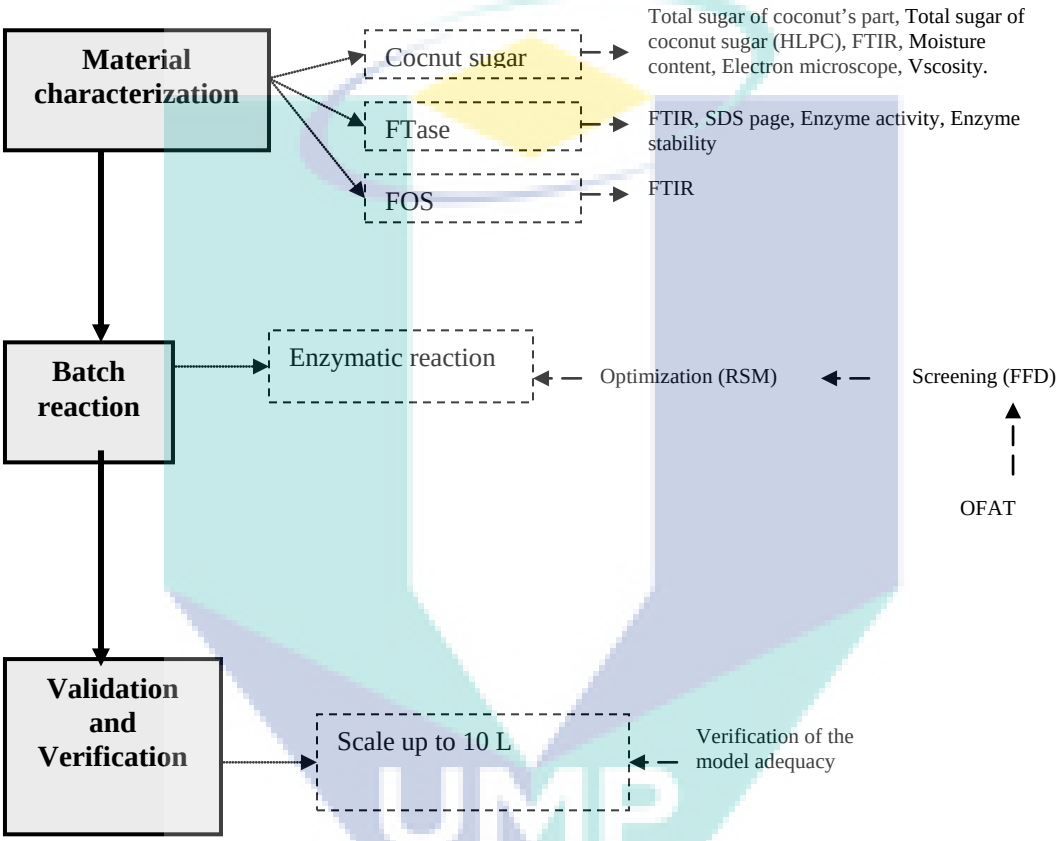
The objective of this phase is to determine the coconut sugar, FOS and FTase characteristics (functional group of coconut sugar, FOS and FTase). Since this study employed reaction process, better understanding on the characteristics or raw materials and product is very important in order to elucidate the relationship between raw materials (coconut sugar and FTase) and product (FOS).

3.1.2 Phase 2: Reaction Process in Batch System

The objective of this phase is to determine the optimum substrate concentration, enzyme concentration, reaction time, agitation speed, temperature and pH which were found to be significant parameters that would influence FOS production. In order to acquire the most significant parameters that attributed to greater FOS concentrations, the fractional factional design (FFD) was employed in this study. These significant

parameters will be applied in the response surface method (RSM) in gaining the high yield of FOS in batch system. Finally, the optimum parameters were then applied into the large scale enzymatic reactor.

Figure 3.1: The experimental flowchart of this research



3.2 PREPARATION OF THE MATERIALS FOR ENZYMATIC REACTION FOR FOS PRODUCTION

The preparation of materials has been done to assist the experiments. The appropriate storage has been selected for the best sample environment to keep. Refrigerator, Schott bottles, apparatus and equipment for all the processes were facilitated by UMP laboratory. All the preparations were made as required in each stage of the process for example, six different concentration of coconut sugar was needed in OFAT stage which were 300 g/L, 400 g/L, 500 g/L, 600 g/L, 700 g/L and 800 g/L. Besides coconut sugar and enzymes, buffer solutions also played an important role in the reaction. The preparation of each material will be thoroughly detailed on the following subtopics.

The equipment that been used in this study were HPLC, FTIR, SEM, Viscometer and Glucose Analyzer for analysis, waterbath shaker and enzymatic reactor for reaction medium, and oven for the drying purposes. All the equipment must be in hygienic condition without any living bacteria in it. This is because enzymatic reaction will be compromised with the existence of any organisms.

3.2.1 Coconut Sugar

The most important raw material of this research is coconut sugar. The selection of the raw material was based on the high contents of sucrose which is more than 80%. The coconut sugar has been purchased at the market in cube-shape at price RM2.30 per kg. Coconut sugar is coconut tree nectar that has been cooked until it is concentrated and brown. Coconut sugar ready for market has a shelf life for 6 months if it is kept in the refrigerator at -5 ^{o}C.

The coconut sugar is in solid phase and in cube form and in order to facilitate the reaction the coconut sugar must be in liquid phase. The coconut sugar must be diluted due to the requirement. The dilution method of coconut sugar is as Figure 3.2.

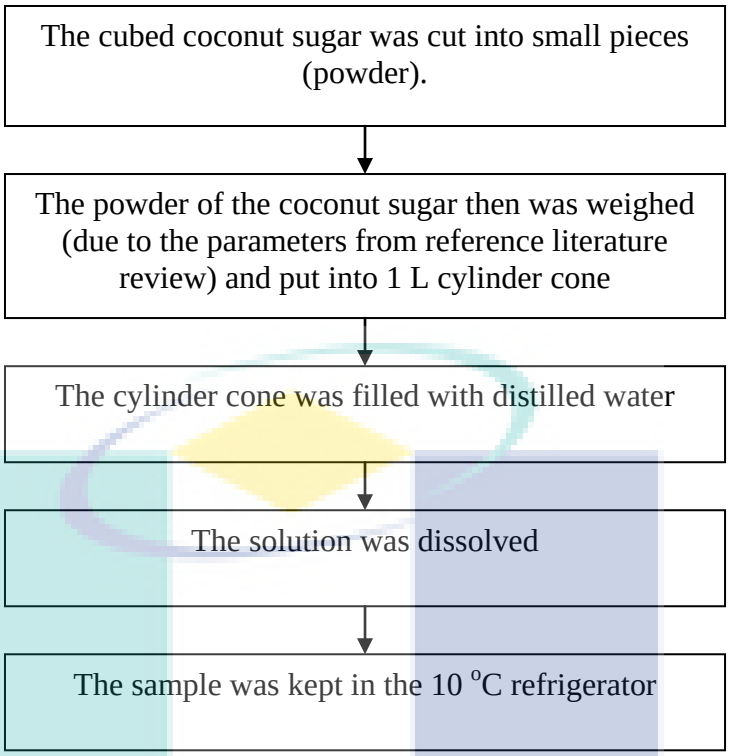


Figure 3.2: The dilution method of coconut sugar

3.2.2 FTase and FOS

Commercial FTase or Invertase was used as it can convert sucrose into FOS. FTase that was used in this study is a commercial enzyme preparation from baker's yeast. While FOS is from chicory and both were purchased from Sigma Aldrich. The enzyme (237 U/mg) arrived in powder form. It must been diluted before it reacted with sucrose in the reaction. The dilution preparation of enzyme based on the parameters as assayed on the literature review. The dilution material that was used is distilled water. The dilution method is also like dilution of coconut sugar but in this case there was no need to cut the FTase in small pieces as it was already in powdered form.

The diluted solution will be kept in the Schott bottles in the refrigerator at -5 °C. It is to avoid from the breeding of bacteria which may affect the reaction and experiments results.

3.2.3 Buffer

The selection of buffer was made in reference to the requirement of pH of the study based to the literature. Acetate Citrate buffer was chosen as the reaction optimized in pH ranging from 5.0 to 6.5.

3.3 CHARACTERIZATIONS OF MATERIALS

3.3.1 Coconut Sugar

Coconut sugar is the main raw material that is being used in this study. It is due to its high sucrose content and as the main substrate for the FOS production; the sucrose will react with FTase and finally would produce FOS. Coconut sugar will be characterized for six criteria. There are total sugar contents in all coconut parts (coconut milk, coconut water and coconut sugar), the sugar contents in coconut sugar only, moisture contents, spectral analysis for functional group study by FTIR, structure analysis by microscope with camera, and finally are viscosity study of coconut sugar.

The characterization of coconut sugar has been done to elucidate the study and to better understand of its behavior. For example is the Fourier Transform Infra Red (FTIR) study is very helpful to screen whether the coconut sugar consist any other strange compound like C-X that will effect the FOS production when reacted with the FTase that is very sensitive with the environmental conditions.

3.3.1 1 The total sugar contents in coconut's part

The initial stage of characterization is to determine the best part of the coconut that would be essential to substitute the commercial sucrose in order to produce FOS intensively. The parts of coconut that were being studied were coconut milk, coconut water and coconut sugar. The main criteria that it should posses are the high content in sucrose. The determination of sugar contents has been done by analyzing all the samples using HPLC. Sucrose, glucose and fructose have been analyzed and according to Apriyantono et al. (2002) of which coconut sugar contains more than 80% of sucrose

followed by glucose and fructose. Due to this study, coconut sugar has been proceeded to be the raw material of the study as discussed in CHAPTER 4.

3.3.1.2 Total Sugar Contents in coconut sugar

The total sugar contents that have been analyzed in coconut sugar are sucrose, glucose and fructose. All the sugars have been analyzed by using HPLC. A direct method of measuring reducing sugar is by HPLC. Sucrose, fructose and sucrose were easily separated on an ion-exchange column (e.g., HPX-87C, Biorad, Richmond, VA, USA) which was connected to a refractive index detector. The column temperature was kept constant at 85 °C. Water was used as the mobile phase at a flow rate of 0.6 $mlmin^{-1}$ (Yun, 1996). The total sugar content in coconut sugar has been compared to the analysis of total sugar in coconut sugar by Apriyantono's study.

3.3.1.3 Moisture Content

The second character that has been studied is its moisture content. It is important to know the moisture content of coconut sugar because coconut sugar has contents water. The moisture content can be determined using two methods. The first method is to dry the coconut sugar by direct sunlight and the second method is to dry the coconut sugar by using an oven. The second method was selected because the exact temperature was needed for calculation. The vacuum oven method was used to determine moisture content. Sample portions were weighed and put on the glass empirical (Tsukakoshi et al, 2009). The vacuum oven was used to obtain the dry matter of the samples. The drying condition was at 60 ^{o}C for 3 hours. After drying, the samples were cooled for 1 h, and then the residues were weighed. As shown in Eq. (3.1) is the calculation method for moisture content.

$$\frac{W_1 - W_2}{W_2} \times 100 = a\% \qquad (3.1)$$

W_1 = sample weight before drying

W_2 = sample weight after drying

a= moisture content

While the drying rate will be

$$\frac{W1-W2}{3\times 60\,\text{min}} = b\,{}^{g}\!/_{\text{min}} \qquad (3.2)$$

W_1 = sample weight before drying

W_2 = sample weight after drying

b = rate of drying

g =gram

min = minutes

3.3.1.4 Spectral Analysis

The third character that has been studied is the functional group of coconut sugar. It has been determined by using Fourier Transformed Infra Red (FTIR). System 2000 FTIR from Perkin Elmer, USA has been used in the analysis. The film casting technique on sodium chloride (NaCl) disks was adopted in this study. The samples were prepared in dimethyl formamide (DMF). The concentration was maintained around 0.5%. The film thickness is not so critical in this type of analysis; however, too thick or too thin a film may generate bad quality spectra with poor resolution. To avoid this type of problem, a lead spacer of 0.01 mm was placed on the NaCl disk. The FTIR spectrum was collected after background correction. The peak intensity was measured from the absorbance value after base line correction (valley to valley). The corrected height in absorbance units was noted (Chakraborty et al., 2007).

3.3.1.5 Morphology Study

The next character of coconut sugar that has been studied is its morphology. The internal structure of coconut sugar can be visualized by using Microscope with camera. The morphology has been studied due to the comparisons between the mixture of coconut sugar and FTase before the reaction and the mixture of coconut sugar and

FTase after the reaction. The reaction has been carried out in the acetate buffer pH 5.5, 55 ^{o}C, 3 hours and 150 rpm for the reaction pH, temperature, time and agitation speed respectively. The difference of the morphology has been studied and each feature has been snapped by the camera that has been equipped in the microscope.

3.3.1.6 Viscosity

The final criterion for coconut sugar characterization is its viscosity. Viscosity was measured using Brookfield model DV III ULTRA Programmable Rheometer 500 concentric cylinder rotational viscometer with MVDIN sensor with internal radius of the outer cylinder, 19.36 mm radius and 58.08 mm length of the inner cylinder. The viscometer was connected to a PC with VT500 version 1.3 software. The PC automatically was increased the rotational speed of the sensor so that shear stress vs shear rate curve was obtained for shear rates from 5 to 100 s^{-1}. The viscometer was also connected to the waterbath that may control the temperature needed for the study. The temperature can be controlled by the controller on the waterbath (Yanniotis et al., 2006). Figure 3.3 illustrates the equipment that has been used for the study of viscosity.

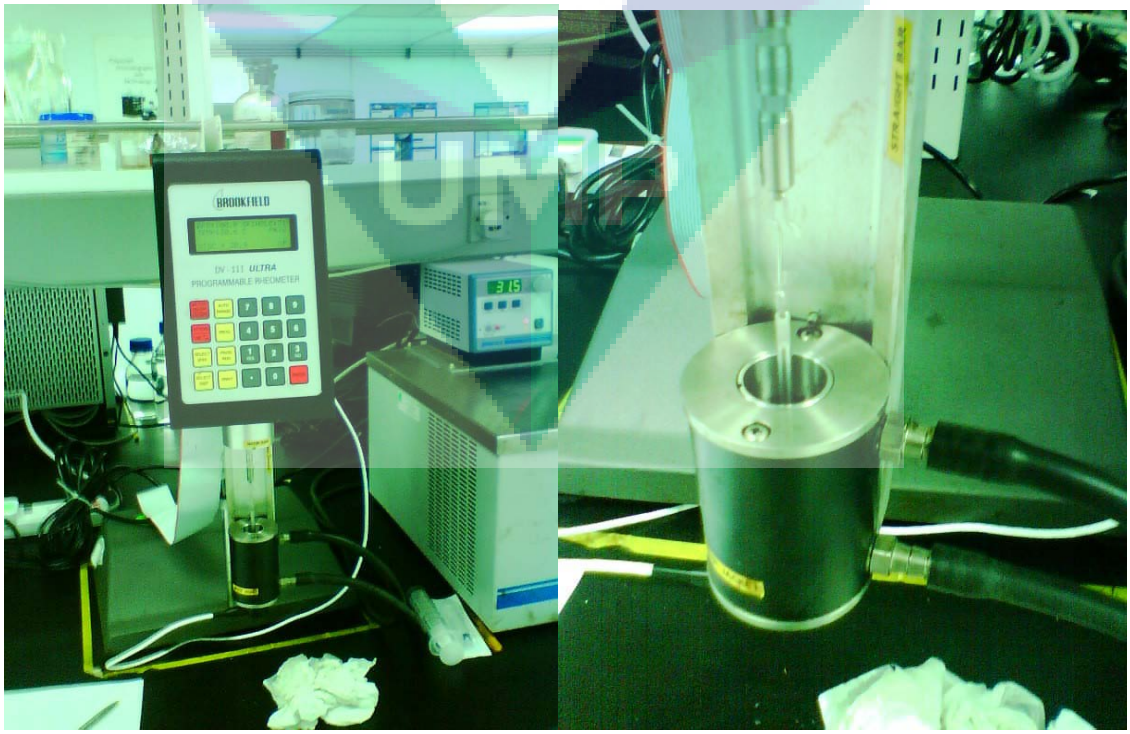


Figure 3.3: Viscometer

Viscosity was measured at 25, 30, 35, 40, 45, 50, 55 and 60 ^{o}C at the initial coconut sugar concentration, 65% w/v, 70% w/v, w/v and 75% w/v. All the experiments were run in duplicate and the average values were reported.

3.3.2 Characterization of FTase

Enzymes are proteins and as such, they are isolated from all kinds of biological material which is mainly from microorganisms. Synthetic enzymes, synzymes, are no doubt interesting from the scientific point of view, but for common use only natural enzymes are available. The majority of biological materials from which enzymes are isolated are huge mixtures with other proteins, nucleic acids and lower molecular weight substances. Therefore, the isolation and purification procedures are often complicated. Hence there was a need to use a very effective separation technique, because the resulting enzyme preparations, especially those for analytical and medical use, should be highly purified.

The purified enzyme preparation is then characterized, so that its properties are clearly described. Main characteristics of the enzyme are isoelectric point, molecular weight, Michaelis constant, optimum pH, optimum temperature, specificity of enzyme action, number of subunits, isoenzymes and purity of the preparation.

In this study, FTase has been characterized due to its crucial characters which are qualitative and quantitative. The quantitative character is to determine the molecular weight of FTase Molecular weight which can be determined either by gelpermeation chromatography or by electrophoretic methods and the qualitative character was studied by determining the enzyme activity. Its activity was determined by using glucose analyzer.

3.3.2.1 Molecular Weight Determination

According to Laemli (1970), the molecular weight of FTase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The FTAse was boiled for 5 minutes and centrifuged prior to be loaded onto gel. The 5x sample

buffer (10 ml) containing 0.8 mL 1 M Tris-HCL (pH 6.8), 2.3 mL 10% SDS, 4.8 mL 50% glycerol, 0.6 mL distilled water, 0.5 mL 2-mercaptoethanol and 1 mL 1% bromophenol blue. The polyacrylamide gel can be divided into two elements, which are stacking gel for the protein samples concentration before separation and resolving gel for the sample preparation. The resolving gel was mixed well, poured between two plates and overlaid with water to keep the gel surface flat and left to polymerize for an hour. A distinct interface will occur between the resolving gel and water after the gel had polymerized. The water was rinsed off with fresh distilled water and then stacking gel was prepared. Subsequently, the stacking gel was poured on top of the resolving gel. A comb was gently inserted into the top of stacking gel in order to avoid bubbles trapped at the ends of the teeth. The gel was supposed to polymerize for 30 minutes. After the gel was found to be polymerized, the gel was connected to electrode assembly of Bio-Rad Mini Protean II Gel System and inserted into eloctrophoresis tank, which filled with 1x Tris-glycine electrophoresis buffer. Then the comb was detached from the stacking gel and the molecular weight markers and FTase solution were introduced into well using Hamilton syringe. The electrophoresis was performed on a vertical slab gel using 15% acrylamide gel at constant voltage of 150 V for 2 hours at room temperature. The gel was stained with 1% Coomassie Brilliant Blue R-250 to detect the protein band.

3.3.2.2 Enzyme Activity

One unit (1U) was defined as the amount of enzyme activity required to produce 1umol of FOS/min under the following conditions: a) pH 5.5, b) temperature 55 ^{o}C c) reaction time 1 hour, d) reaction mixture consisting of the following composition: 7.5 ml of sucrose 800 g/L, 2.3 ml of citrate buffer (pH 5.5) and 0.2ml of enzyme sample. The enzyme reaction was stopped by heating at 100 ^{o}C for 15 min and the released FOS from the enzyme reaction was measured by HPLC (Shin et al., 2003). Eq. (3.3) is the calculation method of FTase activity.

$$A = \frac{\%OD_{decrease} \times Y_p \times D_f \times 10^3}{MW \times t_i} \quad (3.3)$$

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

$\%\ OD_{decrease}$ = $\frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100\%$

Y_p = mg of FOS equivalent to 100% $OD_{decrease}$ of the standard curve

D_f = dilution factor

t_i = incubation time

MW = molecular weight of FOS, 180.1559 g/mol

3.3.2.3 Stability of FTase

The stability of FTase has been studied due to the effects of thermal and pH on the FTase ectivity. Besides, the storage condition also has been studied to determine FTase stability affected by the storage time. The enzyme activity for the study of FTase stability has been determined by measuring the amounts of FOS produced with time. The relative enzymes activity was calculated by comparing the activity in the presence of sucrose with that without any sucrose (Li et al., 2010).

3.3.2.3.1 Thermal stability

The mixture of 0.2 mL of FTase in 0.1 M of acetate buffer pH 5.5 were incubated at different temperatures (30 ^{o}C to 70 ^{o}C) for 1h and then heated up to 100 ^{o}C for 20 min. The relative activity was determined by comparing the activity with that without thermal incubation (Li et al., 2010).

3.3.2.3.2 pH stability

The mixture contained of 0.2 mL of FTase in 0.1 M of acetate buffer pH 5.5 at different pH (4.0 to 8.0). The 0.2 mL of the mixture was incubated at different pH conditions (pH 4.0 to 8.0) for 1 h. Then the residual enzyme activity was measured at 55 ^{o}C, pH 5.5. The relative activities of FTase after incubation under different pH

conditions were determined by comparing the activity with that without incubation (Li et al., 2010).

3.3.2.4 Spectral Analysis

The final character that has been studied is the functional group of FTase. It has been determined using Fourier Transformed Infra Red (FTIR). System 2000 FTIR from Perkin Elmer, USA has been used in the analysis. The film casting technique on sodium chloride (NaCl) disks was adopted in this study. The samples were prepared in dimethyl formamide (DMF). The concentration was maintained around 0.5%. The film thickness is not so critical in this type of analysis; however, too thick or too thin a film may generate bad quality spectra with poor resolution. To avoid this type of a problem, a lead spacer of 0.01 mm was placed on the NaCl disk. The FTIR spectrum was collected after background correction. The peak intensity was measured from the absorbance value after base line correction (valley to valley). The corrected height in absorbance units was noted (Chakraborty et al., 2007).

3.3.3 Characterization of FOS

3.3.3.1 Spectral Analysis

The character that has been studied was the functional group of FOS. It has been determined by using Fourier Transformed Infra Red (FTIR). System 2000 FTIR from Perkin Elmer, USA has been used in the analysis. The film casting technique on sodium chloride (NaCl) disks was adopted in this study. The samples were prepared in dimethyl formamide (DMF). The concentration was maintained around 0.5%. The film thickness is not so critical in this type of analysis; however, too thick or too thin a film may generate bad quality spectra with poor resolution. To avoid this type of problem, a lead spacer of 0.01 mm was placed on the NaCl disk. The FTIR spectrum was collected after background correction. The peak intensity was measured from the absorbance value after base line correction (valley to valley). The corrected height in absorbance units was noted (Chakraborty et al., 2007).

3.4 STANDARD OF FOS PRODUCTION

The standard reaction mixture contained 0.5 mL of 10% w/v enzyme and 1 mL of 700 g/L coconut sugar concentration in 0.2 mL sodium acetate buffer pH 5.5. The reaction was incubated at 55 ^{o}C for 5 hours. Control samples were prepared in the same manner except no enzyme was added. Then the enzyme was denatured by heating it for 20 min in boiling water, and then analyzed by using HPLC (Ghazi et al., 2005).

3.5 FOS ANALYSIS

The supernatant from the 100 times diluted sample that had been centrifuged by using microcentrifuge for 30 mins and 6000 rpm had been separated by using filtration. HPLC Agilent 1300 series with Refractive Index Detective have been used and following the standard assay which analysis have been done using this equipment with quaternary pump 9 Delta 600, waters) coupled with a Lichrosorb – NH_2 column (250 x 4.6 mm) (Merck, Spain). The mobile phase was acetonitrile:water (75:25 v/v), conditioned with helium and used at a flow rate 0f 0.7 ml/min. The column temperature was kept constant at 25 ^{o}C. A differential refractometer (model 9040, Varian) was used and set to a constant temperature of 30 ^{o}C. The data obtained were analyzed using Millenium software, using external standards for calibration in the range 0 to 100 g/L (Ghazi et al., 2005).

3.6 ONE FACTOR AT ONE TIME (OFAT)

OFAT studies were carried out to determine specific levels of each parameter. Any insignificant parameters for the production of FOS will be eliminated and not included at the next stage which is the screening process and optimization by using the Design Expert program. The significant of the parameters was based on the quantitative factor of FOS production. All the results of the experiments have been analyzed by using HPLC as mentioned on the method 3.5.3.

In the time course study, experimental conditions were in the same as the standard of FOS production except that the reaction mixture was incubated at 55 ^{o}C for various periods of time where the times ranged from 1 to 10 hours.

In the substrate concentration study, experimental conditions were the same as the standard of FOS production except that different substrate concentration was used where the coconut sugar concentration ranged from 300 to 800 g/L.

In the enzyme concentration study, experimental conditions were the same as the standard of FOS production except that different enzyme concentrations were used where the enzyme concentration ranged from 0 to 0.15 g/L.

In the temperature study, experimental conditions were the same as the standard of FOS production except that the reaction mixture was incubated at different temperature where the temperature ranged from 20 ^{o}C to 80 ^{o}C.

In the pH study, experimental conditions were as the same method as the standard of FOS production except that the pH ranges of pH 4.0 to pH 6.5 was used. The buffers 0.2 M used were: acetate (pH 4.4 to pH6.0) and phosphate (pH 6.0 pH 7.0).

In the agitation speed study, experimental conditions were as the same method as the standard of FOS production except that in the speed range of 50 rpm to 300 rpm. All the reported data were average values of triplicate samples.

3.7 Determination of Kinetic Parameters in Batch System

The characterization of enzymatic reactions involved the determination of K_m and V_{max}. The K_m and V_{max} values for the FTase was determined by incubating 0.5mL of 10% w/v enzyme and 1.5 mL of 700 g/L coconut sugar concentration in 0.2 mL sodium acetate buffer of pH 5.5 at 55 ^{o}C for 5 hours. The kinetic parameters were determined using the Michealis Menten equation. The production rate of FOSs, *v* (μg/mL.min), versus the initial substrate (coconut sugar concentration), [S] (mg/mL),

was fitted to the following Michealis Menten Eq. (3.4) (Mu et al., 2006; Pallud et al., 2006),

$$v = \frac{V_{\max}[S]}{K_m + [S]} \tag{3.4}$$

Where V_{max} is the maximum reaction rate ((μg/mL.min) and K_m is the Michealis Menten constant (mg/mL).; In Michealis Menten kinetics, V_{max} value corresponds to the condition where every enzyme molecule present is saturated with substrate. K_m value represents the affinity between substrate and enzyme. The Michealis Menten equation was further derived into Eq. (3.5) according to which a linear plot between 1/*v* and 1/[*S*] was attempted. The latter relationship is called Lineweaver-Burk plot.

$$\frac{1}{v} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} \tag{3.5}$$

The intercept on the y-axis is equal to $1/V_{max}$ and the slope Km/Vmax. It is equal to Eq. (3.6)

$$y = mx + c \tag{3.6}$$

Where m denotes to the linear slope while c is to the intercept on the y- axis
Both intercept and slope were determined by linear regression.

3.8 EXPERIMENTAL DESIGN

Experimental design was employed in order to obtain the optimum FOS production. In particular, the experimental design analyzed the obtained data statistically which was found to be simplified and reduced the experimental period without influencing the worthwhile data compared to the experiments that were solved by conventional method (Haaland, 1989; Montgomery, 1991). The conventional method that has been widely applied is known as one factor at one time (OFAT). Moreover, the

statistically experimental design could also determine the interactions between factors that might be revealed in the employed experiments data.

In general, there were three steps in designing an experimental design which are screening, optimization and verification process. However, if there are only two factors studied, the screening process was unnecessary to carry out. The experimental design was carried using Design Expert Software (State Ease Inc. Statistics made easy, Minneapolis, MN, US, Version 6.0.8)

3.8.1 Screening process using Two Level Half Fractional Factorial Design (FFD)

The main purpose of the screening process is to determine the significant factors that could effect FOS production. Since FOS production in batch process consists of six parameters, the screening process has to be performed prior to identifying the optimum condition for FOS production. The factors studies for FOS production in batch system were pH, temperature, agitation speed, reaction time, coconut sugar concentration and FTase concentration. The analyses were done by observing the concentration of FOS as response in the design by using HPLC. The experiment was done in duplicates. The factors and level for screening process as been tabulated in Table 3.1.

Table 3.1: Factors and levels for the fractional factorial design

Factors	**Unit**	**Levels**	
		-1	+1
A (*x*1) Coconut sugar	g/L	600	800
concentration	U/mL	5	20
B (*x*2) Enzyme concentration	^{o}C	50	70
C (*x*3) Temperature	pH	4	6.5
D (*x*4) pH			

3.8.2 Optimization Process using Central Composite Design (CCD)

A central composite design (CCD) will be used for this optimization process which allowed determination levels of various parameters to be carried out with the interrelation between each parameter which evolved simultaneously (Montgomery, 1991; Shiow-Ling et al., 1997; Ibrahim et al., 2005). In order to describe the nature of the response surface in the optimum region, a central composite design (CCD) with two levels were performed. The design consists of a 2^k factorial design augmented by 2^k axial (star) points and n_o center points, where α is the distance of the star point from the center. The axial distance was chosen to be 2.0 to make this design rotatable.

Where Y is the predicted response, β_o is the offset term, β_1 is the linear effect, β_{ii} is the squared effect, and the β_{ij} is the interaction effect. The quality of the fit of the second order polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance was checked by F-test. The F-test was also used to evaluate the significance models. Table 3.2 summarized the factors and level for optimization study.

Table 3.2: Independent variables and concentration levels for response surface study

Factors	**Unit**	**Levels**	
		-1	**+1**
A (*x*1) Coconut sugar concentration	g/L	600	800
C (*x*3) Temperature	^oC	50	70

3.8.3 Validation of Model Empirical Equation

The final equation obtained in the CCD for optimization finally been verified for the validation purpose. The maximum yield of FOS been selected in the analysis at Design Expert after all the FOS concentration was inserted when all experiments have been done. The maximum limit of the significant parameter will be given and the

experiment has been run due to the data given. Table 3.3 depicts the detail of the verification process

Table 3.3: The details of verification process

Run Factor		FOS Concentration		
Substrate Concentration	**Temperature**	**Predicted**	**Residual**	**Error**
746.33	54.43	232.37	8.72	3.62%
750.00	55.00	231.29	2.93	1.25%
680.00	55.00	231.29	9.61	3.99%

The FOS concentration obtained from the experiment must be validated against FOS concentration that obtained from the software which was 238.294 g/L with desirablity of 0.956.

Table 3.4: The details of confirmation run

Run Factor		FOS Concentration		
Substrate Concentration	**Temperature**	**Predicted**	**Residual**	**Error**
746.33	54.43	232.37	8.72	3.62%

3.9 SCALE UP

The final stage of all the experimental works was scaled up into industrial scale. The objective of this process is to confirm that the study is suitable to be applied to the industry for the worldwide market.

The experiment had been carried out in a batch system by using 10 L self-fabricated enzymatic reactor. The reaction mixture that contains coconut sugar solution, FTase and buffer solution with the total volume is 10 L will be filled in the reactor, with the condition that had already been optimized from the OFAT, FFD and CCD. Figure 3.4 illustrates the scale up process for 10 L.

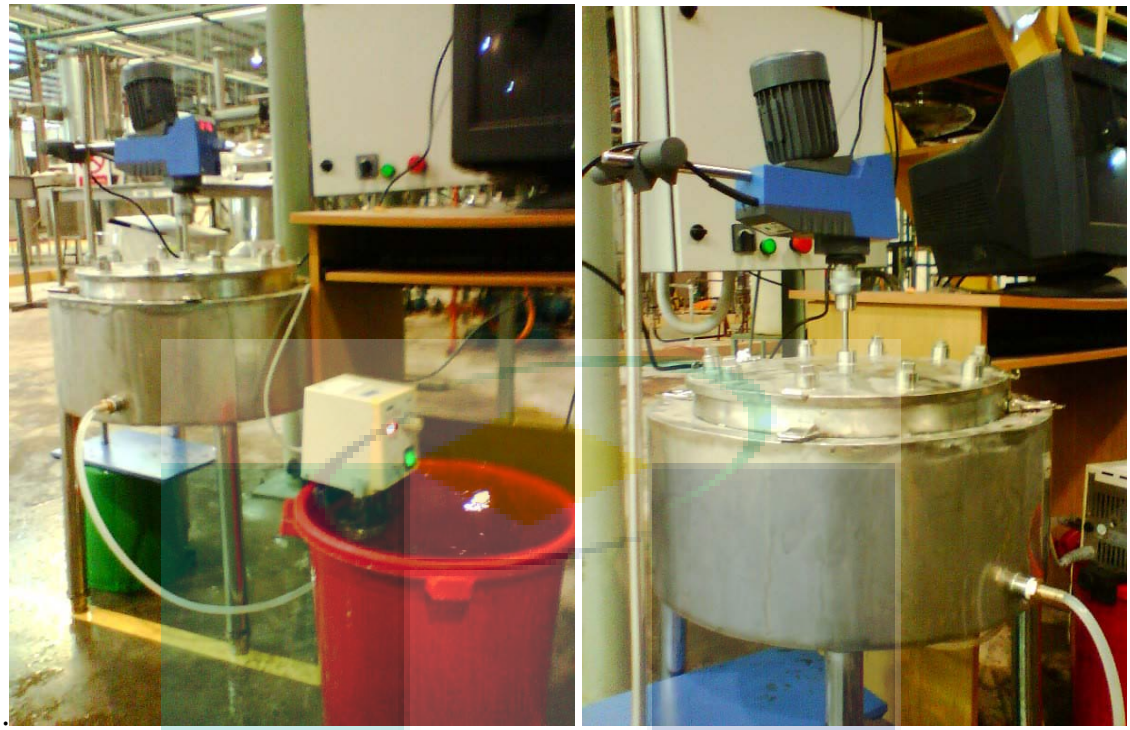


Figure 3.4: Scale up process

CHAPTER 4

RESULTS AND FINDINGS

4.1 CHARACTERIZATION OF RAW MATERIALS

Coconut sugar and FTase are the main raw materials of the projects. The assessments of both characteristics have been done based on the literature and information gained from many resources. The importance of this study is to earn the important approximate values or features that will facilitate the experiments in order to gain the best condition on FOS production. The results from the characterization had been observed and ultimately been discussed in this chapter.

Coconut sugar is the main raw material that initializes the characterization. Its quality that fulfills the requirement to be one of the sucrose sources have been studied based on six characterizations. Before all the characterizations were done for coconut sugar, all other coconut parts, that is coconut water, coconut milk and coconut sugar had been characterized for the total sugar (sucrose, glucose and fructose) contents. The objective of the study is to determine the best part of the natural coconut that qualifies the requirement to be a new source of sucrose. Then, the highest contents of sucrose will be proceeded to be raw material for the production of FOS, which according to the Apriyantono et al., (2002) coconut sugar shows the highest results of sucrose with 81.3%. Total sugar contents, moisture contents, morphology by electron microscope with camera, viscosity by using viscometer and spectra analysis by FTIR are the elements that have been studied for coconut sugar characterization.

The second raw material that plays an important role in this study is FTase. In this study, FTase had been characterized due to its molecular weight, enzyme activity,

enzyme stability and its functional group. The molecular weight will be determined by using SDS-PAGE method described in the previous chapter, the enzyme activity will be determined by using HPLC, while FTase functional group by using FTIR.

The last material that was studied in this research is the product, Fructo-oligosaccharides (FOS). FOS had been characterized for its group and that had been purchased from chemical supplier (SIGMA ALDRICH). The equipment that had been used is FTIR for analyzing purpose.

4.1.1 Characterization of Coconut Sugar

4.1.1.1 Total Sugar Contents in Natural Coconuts Parts

The objective of this study is to determine the total sugar contents (sucrose, glucose and fructose) in all coconut part that is coconut milk, coconut water and coconut sugar. All these parts of the coconut had been analyzed by using HPLC. It was to determine the best part of the coconut that contains the highest sucrose. Figure 4.1 illustrates the contents of sucrose, fructose in the natural coconut parts which are coconut milk, coconut water and coconut sugar.

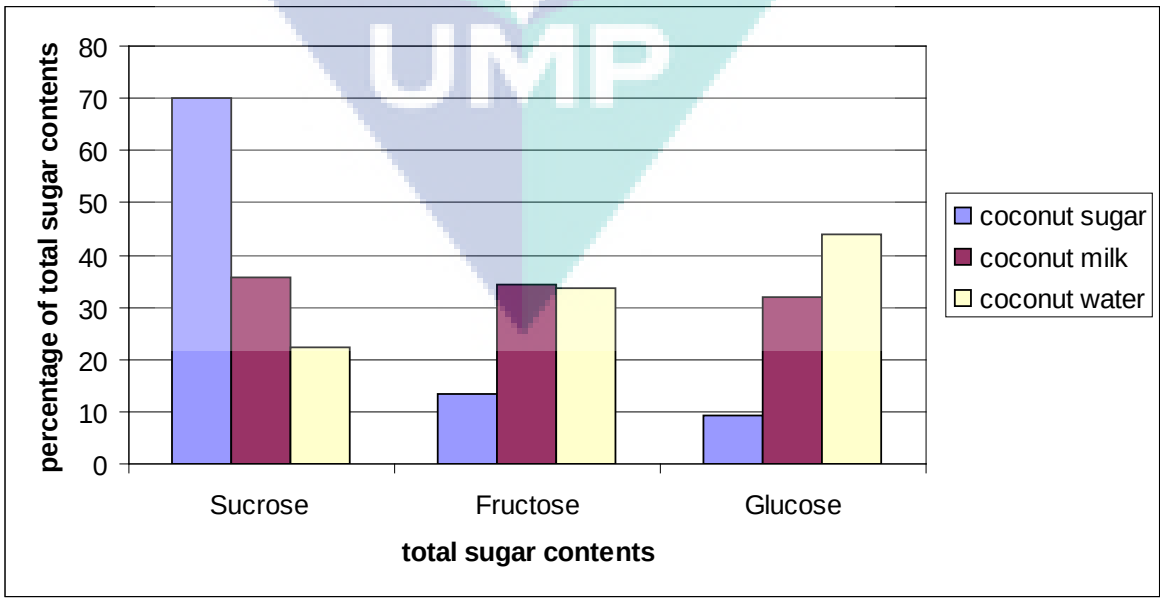


Figure 4.1: Sugar contents in coconut's part

Based on this figure, the highest content of sucrose in coconut parts is in the coconut sugar with about 71%. This study indicated that coconut sugar was the most suitable to become raw material of the study as the pronounce content of sucrose was needed to be a new source of sucrose. The coconut milk also possessed intense contents of sucrose which about 38% and followed by coconut water which had less content of sucrose but really high content of glucose. While coconut milk seems to have a more balance of sucrose, glucose and fructose (3.5:3.4:3.1) of the content of total sugar percentage.

The selection of coconut sugar as the raw material is really necessary as it has high contents of sucrose among other coconut parts. From Sangeetha et al. (2005), to produce FOS, high consumes of sucrose is needed at a range of about 40 to 50% w/v of sucrose in the reaction mixture with FTase in acetate buffer with pH 5.0 to 6.0, 50 ^{o}C to 70 ^{o}C and about 3 to 7 hours of reaction pH, temperature and time respectively.

4.1.1.2 Total sugars of Coconut Sugar

The determination of sugars like sucrose, glucose and fructose had been done in reference to the Apriyantono et al. (2002) that coconut sugar consisted of mainly sugars at amounts of total sugar is 91.4%. The main sugars detected were glucose, fructose and sucrose. The results for coconut sugar obtained from this study were similar to Apriyantono's study but the values of the sugars were different. The analysis results as shown in Figure 4.2.

The analysis for sucrose, glucose and fructose had been studied by using HPLC. Total sugars content in coconut sugar is 93% which constituted 71% of sucrose, 13% of glucose and 9% of fructose and 7%. Others here constituted for pyrazines and acid organic as according to Apriyantono et al. (2002) study. The high content of sucrose in the coconut sugar was the main reason for the selection as alternative source for sucrose in FOS production. The findings were slightly different with Apriyantono et al. (2002) study where all the values is quite less which is 91.4% of total sugar that constituted of 83.7% of sucrose. It is mainly because of the two different harvest products

Apriyantono's study was using Indonesia coconut sugars while for this study the local products had been used. Besides, the process of the coconut sugar manufacturing of these two countries also differs. In Malaysia the coconut sugar produced by the industry is done by machine while in Indonesia the conventional method still applies, which is by using bare hands and traditional cooking tools and methods.

In relation to the findings, the commercialized products of FOS from coconut sugar can be measured. If 71% of sucrose in coconut sugar, it means that 1 kg of coconut sugar consist about 710 grams of sucrose. If 1 kg of coconut sugar priced at RM1.80 so, 1 kg sucrose from coconut sugar priced at RM2.53 while the commercial sucrose is at RM349.99 for 1 kg. The production cost is estimated to reduce to not more than 150%. The value is really high and the FOS may produce intensively by Malaysia harvest products. And if 800 g/L of sucrose can produce about 300 g/L of FOS which priced 1 gram for RM90.00 so 800 gram of sucrose from coconut sugar which priced RM2.04 will have returned for RM2700 which the production profits is about RM2697.06.

So, this study is really valuable if the FOS can be successfully been produced from the coconut sugar.

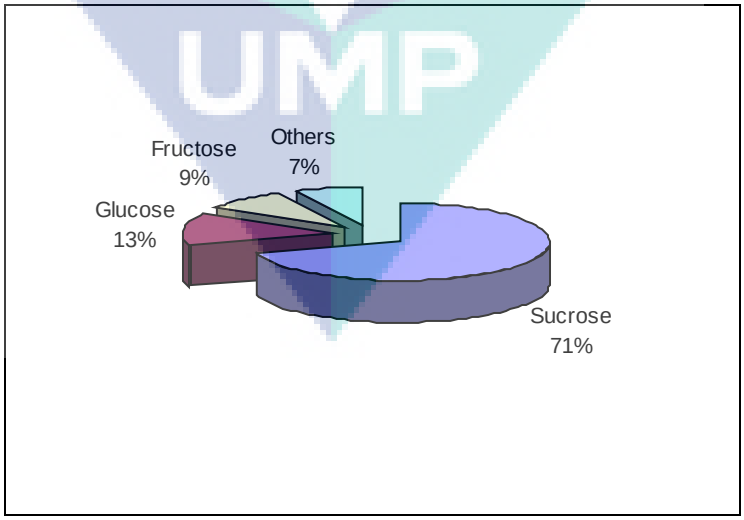


Figure 4.2: The total sugar contents in coconut sugar.

4.1.1.3 Moisture Contents in Coconut Sugar

The moisture content is very important in enzymatic reaction where water content in the coconut sugar can be determined. From the study, there was only about 7.255% of water content in the coconut sugar after being dried for 3 hours and at 65 ^{o}C. Coconut sugar is more watery at room temperature and easily melts especially in large size particles. It is indicated that coconut sugar is easier to dissolve at low heat or temperature. Conveniently, this condition may facilitate the reaction that leads to a more accurate and faster during sample preparation. Coconut sugar is also believed to be really high in carbohydrates or total sugar contents which amount to 93%, 7.255% of water contents and only about 3.745% of others such as organic acid and pyrazines (Apriyantono et al., 2002).

4.1.1.4 Spectral Analysis of coconut sugar

The approach of Fourier-transformed Infrared Spectrophotometry (FTIR) in the characterization was widely used in many literatures. Infrared spectroscopy can be used efficiently by polymer and rubber technologists for identification of polymer, polymer–blend ratio calculation, raw material evaluation, study of reaction mechanism and microstructure determination. Coconut sugar is also a polymer that degrades from many types of sugar contents which has C-OH bond. As shown in Figure 4.3 the FTIR spectrum of coconut sugar

The ratio of coconut sugar stretching at 1000 cm^{-1} and that of the C-OH bond .The transmissions percentage of the bonding in coconut sugar was 105. Based on the Figure 4.3 first peak detected is O-H content or maybe water in coconut sugar. The moisture content in coconut sugar was quite high which around 7 to 8 % as discussed previously was. The highest and complex peak are shown, beginning from resolutions at 1000 cm^{-1} and it is C-OH group or more precisely the polysaccharides group and it is referred to the spectra analysis from the existed library. This comparison tells that coconut sugar functional group is a long chain of sugar family and it is intense with sucrose contents.

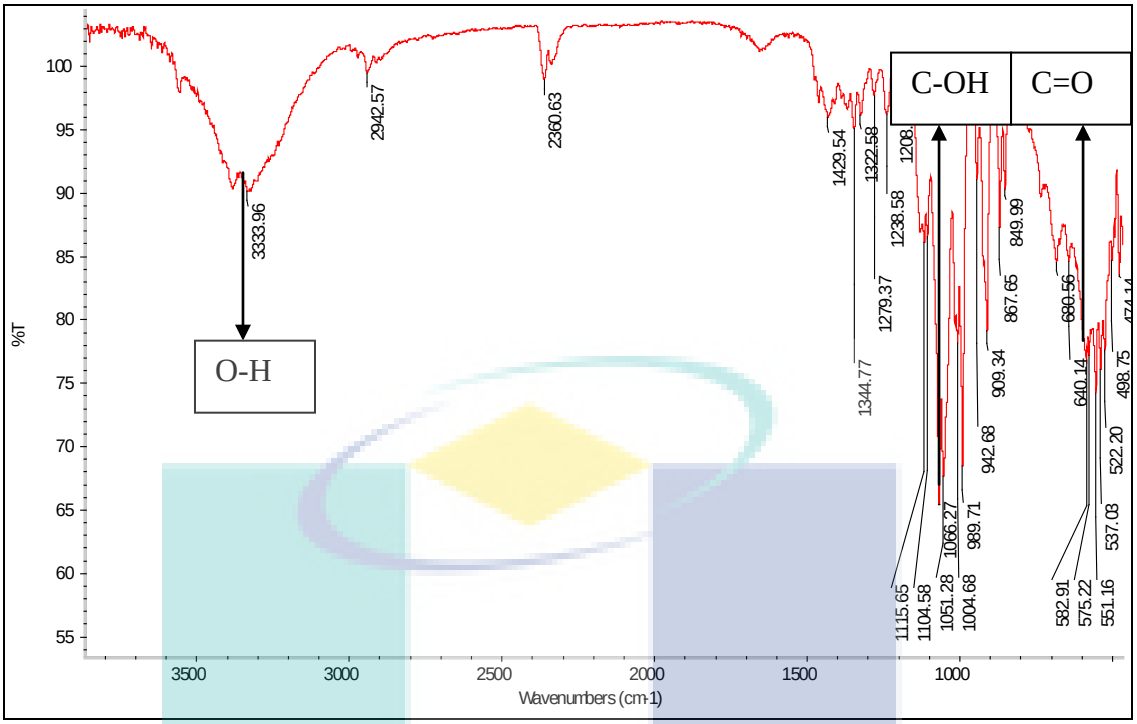


Figure 4.3: FTIR spectra of coconut sugar

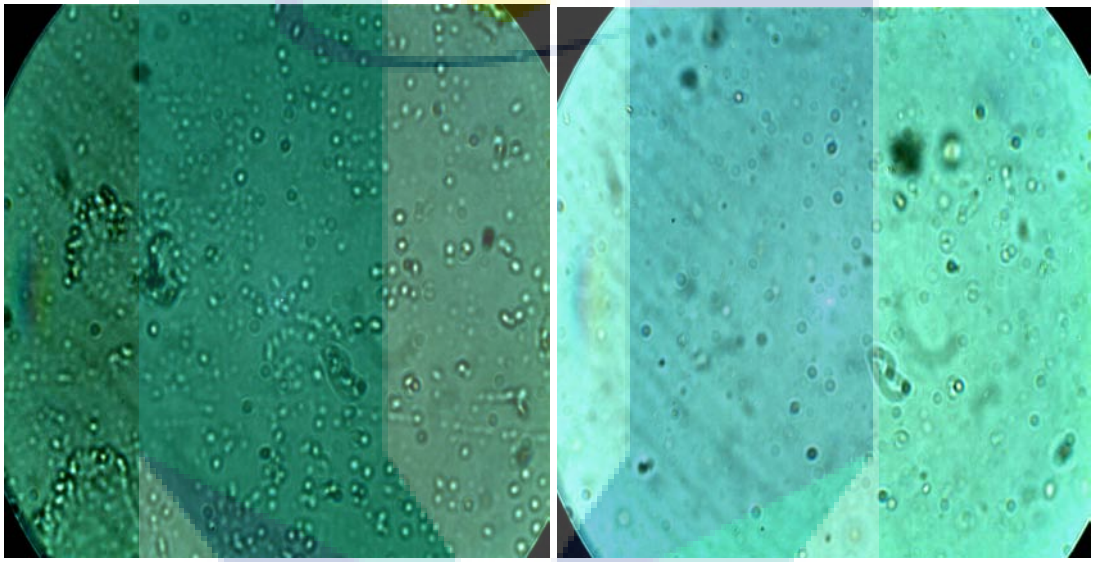
The FTIR spectra analysis study strongly approves that sucrose; glucose and fructose are consisted in coconut sugar. While, there is also the presence of other compound besides sugar, it is clearly demonstrated on the second highest peak which is at wavenumber below than 500cm^{-1} and it is compounds that consisted of C=O group like phenol, alcohol or others may contains in the coconut sugar. According to Apriyantono et al. (2002) study, there are pyrazines and organic acid in the coconut sugar. Even the value is too little, but it still can be detected from the spectral analysis by using FTIR

4.1.1.5 Morphology Study

The characterization of coconut sugar was preceded with the morphology and structure study by the vision of electron microscopic with camera. Table 4.1 is the conditions that had been set for the study and following are the images obtained as illustrated in Figures 4.4 (a) and (b).

Table 4.1: Electron microscopic with camera conditions

Microscope parameter	Setting
Lenses resolution	40µm
Light intensity	highest
Lense spaces	12 mm
Light spectrum	Blue



(a) (b)

Figure 4.4: a) Microscopic image of coconut sugar before reaction and b) Microscopic image of coconut sugar with FTase after reaction

Figure 4.4 shows the results of electron microscopic analysis. There were two samples that had been studied for internal morphology and structure. Figure 4.4 (a) illustrates the morphology of coconut sugar solution before any enzyme was added. Based on the figure, all the molecules in the coconut sugar that was hypothesis to be sucrose, which is the largest particles among others, were extending closely to each other in the structure. The molecules were extending everywhere and it also showed that the bonding of each molecule is a long chain and it is complex. There were no other

strange compounds in the solution so it means that there were no other microbes or microorganisms that might disturb the reaction study.

Figure 4.4 (b) illustrates the morphology of reaction mixture at 5 hour and 55 ^{o}C of reaction time and temperature respectively between coconut sugar and FTase. Clearly showed how the enzyme engulfing the entire sucrose molecule. The vision can be viewed by reducing the size of the entire molecule that had been discussed earlier at Figure 4.4 (a). The formation of FOS can also be illustrated by this figure which can be seen by the existence of new molecules around the sucrose molecule. The utilization of the equipment with the camera also facilitated the study to view the difference between these two figures. First figure shows the more concentrated solution compared to the second figure. The other difference can also demonstrated by the molecules (round shape) that seemed many in numbers but reduced after reaction and finally the obvious changes of this figure is the first figure had the molecules (round shape) that bonded to each other with a long chain but in the second figure the bonding had been split and the single molecule seemed to be increasing.

4.1.1.6 Viscosity

Viscosity of coconut sugar concentration affected with temperature has been illustrated in Figures 4.5, 4.6. While the Arrhenius plot for thermodynamic study in the coconut sugar concentration and its viscosity depicted in Figure 4.7.

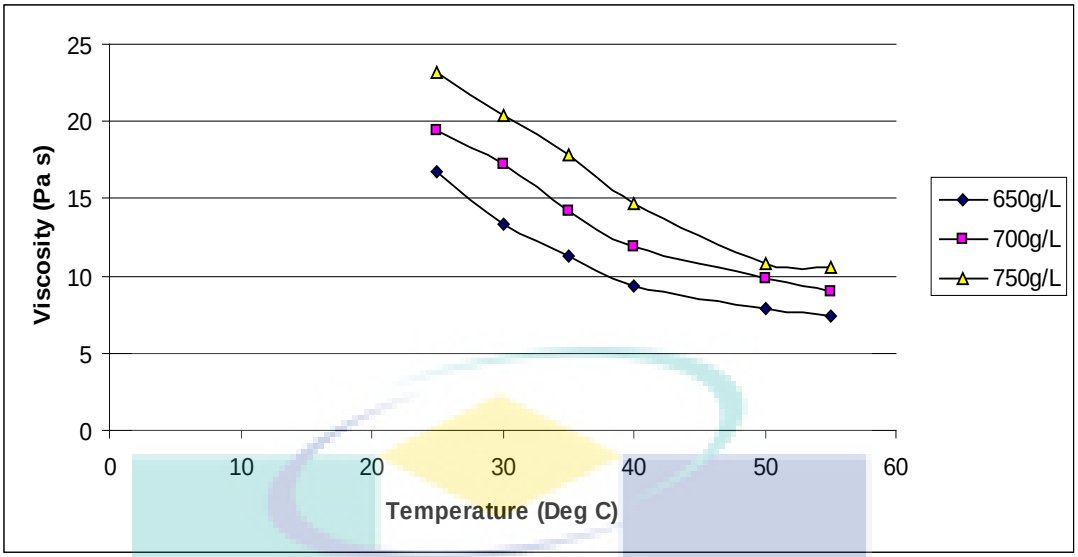


Figure 4.5: Viscosity of coconut sugar at various coconut sugar concentrations and temperature limit

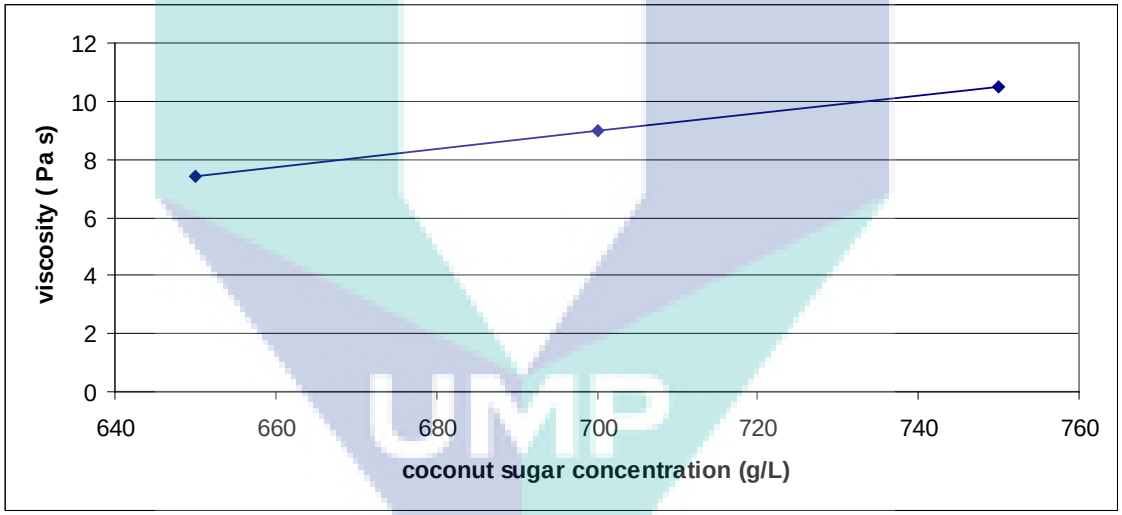


Figure 4.6: Viscosity of coconut sugar at various coconut sugar concentrations

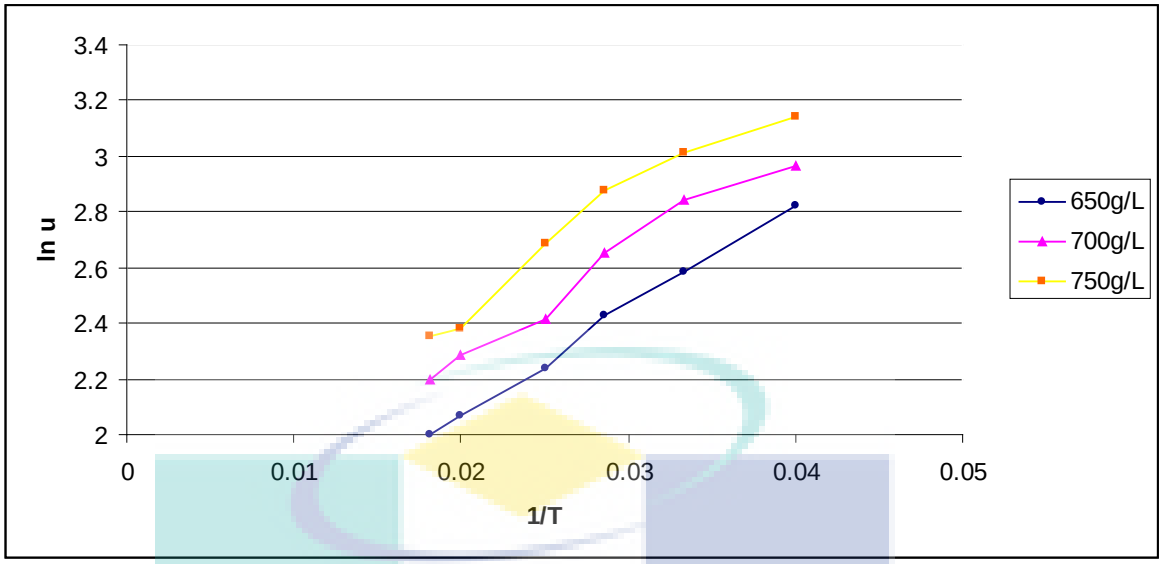


Figure 4.7: Typical Arrhenius plot for coconut sugar concentration at various temperatures

Viscosity values are shown in the Figures 4.5 and 4.6. As expected, viscosity decreases substantially as temperature increases and coconut sugar decreases as shown in the Figure 4.6. The effect of concentration is pronounced for coconut sugar concentration up to 750 g/L, below this level the effect of viscosity is weak. The effect of temperature is more pronounced for temperature below room temperature which is about 25 ^{o}C and the effect is much less between 50 ^{o}C to 60 ^{o}C.

Figure 4.6 shows the viscosity values at reaction temperature of 55 ^{o}C. At this temperature the pronounce effect took place at 80% w/v of coconut sugar concentration. At 65% w/v the effect of concentration is less.

A plot of ln μ vs 1/T produced a linear relationship for all samples indicating that the Arrhenius equation (Eq. 4.1) can be applied to describe the variation of viscosity of coconut sugar concentrations with temperature

$$\mu = \mu_o \exp (E_a/RT) \qquad (4.1)$$

where μ is the viscosity (Pa.s), μ_o a constant (Pa.s), Ea the activation energy (kJ/mol), R the gas constant (0.00831434 kJ/mol K) and T the temperature (K) (Yanniotis et al., 2006). The best fitting straight line through all the experimental points are shown in the Figure 4.7, where the logarithm of viscosity is plotted vs 1/T for coconut sugar concentration. Similar plots were obtained for all the samples with R^2 ranging between 0.9951 and 0.9999. The activation energy and the constant μ_o were obtained from these regression lines at each coconut sugar concentration. The activation energy decreases as the coconut sugar concentration increases indicating that the viscosity is more sensitive to temperature changes at low coconut sugar concentration. From the study, it is learn that at coconut sugar concentration of 750 g/L and at 55 ^oC would give the best point for the reaction to occur as the activation energy is high and at the lowest point of viscosity.

4.1.2 Characterization of Enzyme (FTase)

4.1.2.1 Molecular Weight

Molecular weight of FTase has been determined by SDS-PGE method as described in CHAPTER 3. FTase that has been used in this study is commercial FTase that has been manufactured by a microbiology industry. As other commercial enzymes, FTase also has much resistance and less sensitivity of surrounding factors such as temperature changes, pH changes and existence of other microbes. This leads to a large of molecular weight value which ranges from 100 to 500 kDa. From the experimental work, the molecular weight of FTase obtained is 145 kDa. This number is quite large from the cultured enzyme which is commercial FTase stability and pH had also been studied in this part of experimental work. It is to prepare the best conditions on the enzymatic reaction experimental work.

4.1.2.2 Enzyme Activity Assay

FTase activity was assayed due to its activity on the substrate after been prepared in the reaction mixture. The activity of FTase had been measured by using HPLC after the reaction mixture which consisted 1.5ml 60% w/v sucrose on 0.1 M citrate buffer (pH 5.5) and 0.5 mL 10% FTase. The reaction was carried out at 55 ^oC for

1hour using a water bath. Then, the reaction was terminated by keeping the reaction mixture in boiling water bath for 15min. Glucose released at the end of the reaction was estimated by using HPLC. One unit of FTase activity was defined as the amount of enzyme that released 1 μmol per mL per minute under the above mentioned reaction conditions. From the HPLC results, glucose detected was 3.591 g/L with retention time of 2.21 minutes. Calculated from the enzyme activity formula, the enzyme activity of FTase is 401 U/mL.

4.1.2.3 Stability of FTase

The stability of FTase can be determined for the effects of FTase due to its reaction temperature and pH.

4.1.2.3.1 Thermal Stability

The effect of temperature on enzymatic reactions is very complex. If the enzyme structure would remain unchanged as the temperature increases, the rate would probably follow the Arrhenius temperature dependence. The relative inactivity of FTase for thermal stability had been determined by incubating the mixture of FTase in acetate buffer solution for different temperatures range (30 ^{o}C to 70 ^{o}C). Then 0.2 ml of the sample will be added with 1.5 ml of sucrose and incubated for 1h at 55 ^{o}C. The result of the enzyme activity is illustrated in Figure 4.8.

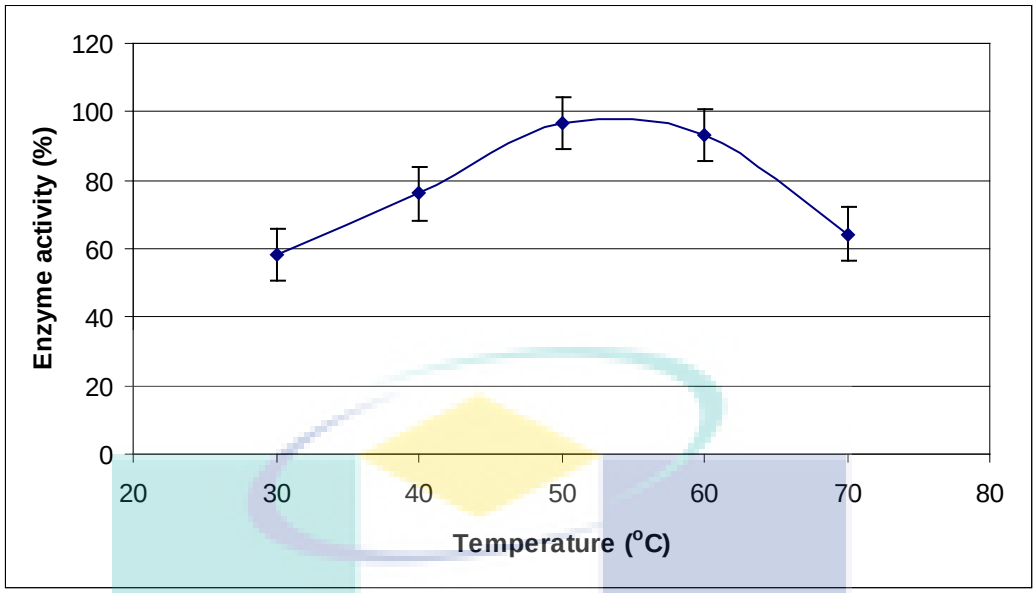


Figure 4.8: Enzyme activity of Thermal stability

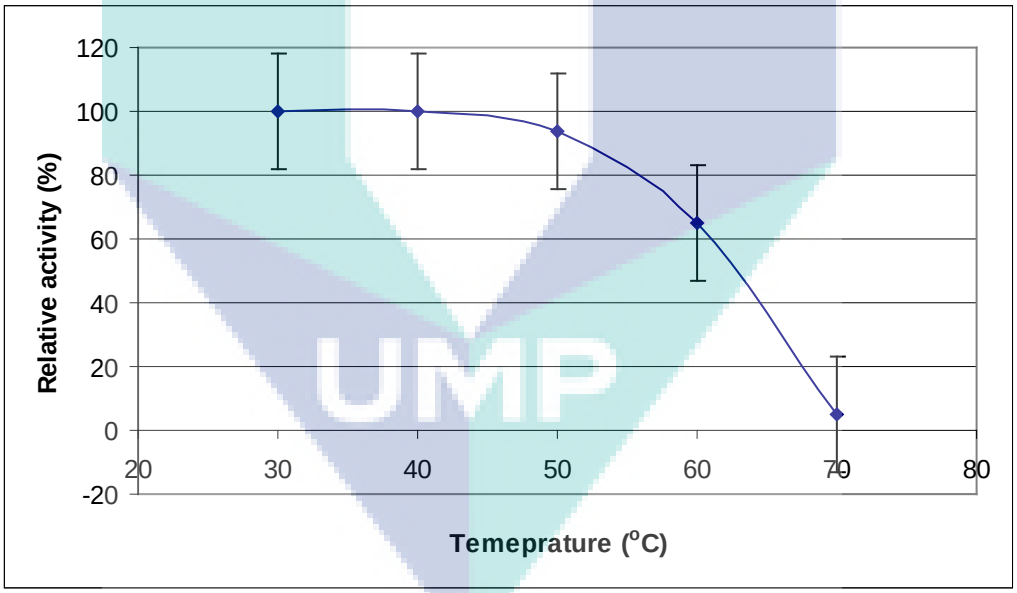


Figure 4.9: Relative activity of Thermal stability

Temperature gives the intense effect to the FTase stability where it was active and stable measured at 50 ^{o}C to 60 ^{o}C due to the relative activity. At higher than 65 ^{o}C FTase is inactivate and led to the decrease of the FOS production. Exhibition of FOS can also be shown by the figure when the temperature was between 30 ^{o}C to 50 ^{o}C

where the relative activity increased until it reaches the optimum value at 55 ^{o}C. According to the relative activity (Figure 4.9), the FTase activity was most stable until 50 ^{o}C but slowly became inactivated when the temperature increased.

4.1.2.3.2 pH Stability

The stability of enzymes affected by pH level is very important as it is leads the reaction either to reversible or irreversible process. If the pH level affected the reaction, it would mean that the reaction is reversible process and the reaction is in good condition. From the Figure 4.10, FTase is stable at pH 5.0 to 6.0. The optimum pH of the FTase measured with sucrose as substrate is at pH 5.5. No significant activity was found at pH values below 3.5 and above 7. The pH stability was assayed in the presence of high concentration of a carbohydrate, to mimic the operational conditions of FOS synthesis. As shown in Figure 4.11 the enzyme retained more than 90% of its initial activity after 5 hours incubation at neutral and moderately acidic pH values (from 4.5 to 6.5).

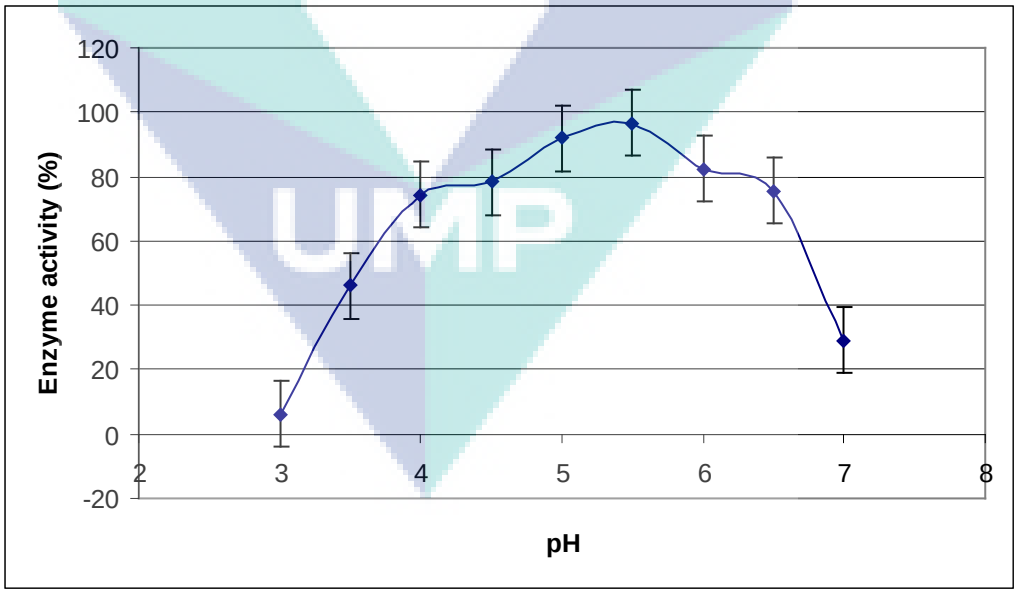


Figure 4.10: Enzyme activity of pH stability

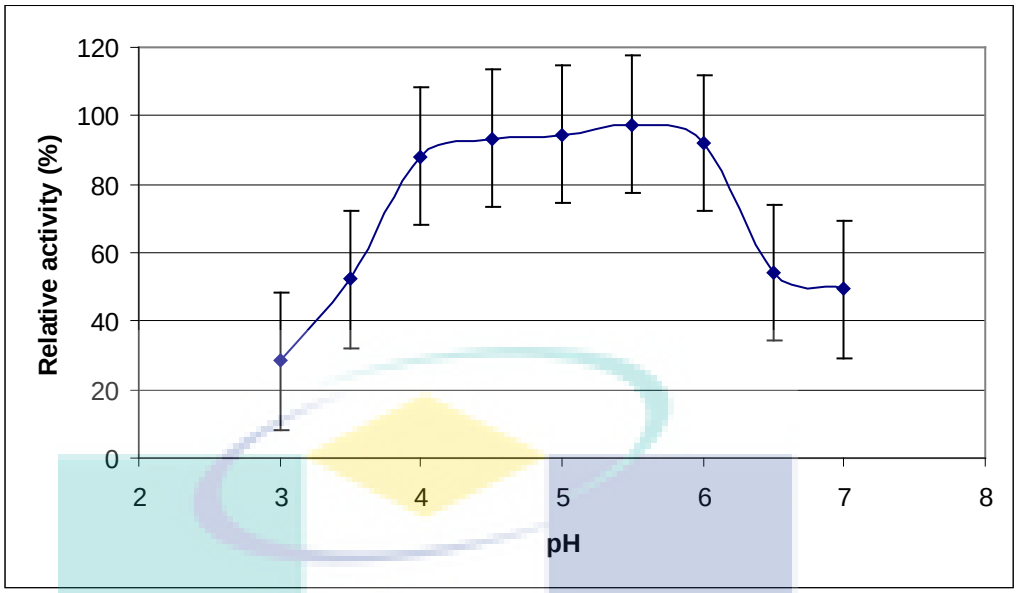


Figure 4.11: Relative activity of pH stability

4.1.2.4 Spectral Analysis of FTase

The second approach of Fourier Transformed Infrared Spectrophotometer (FTIR) in the characterization is to determine the functional group in FTase. FTase is an enzyme that is cultured from fungal and has been manufactured widely in the chemical industry. Like other bacterial enzymes, FTase consists of protein compound that facilitate the chemical reaction when using biological substrate. As shown in Figure 4.12 is the FTIR spectrum of FTase.

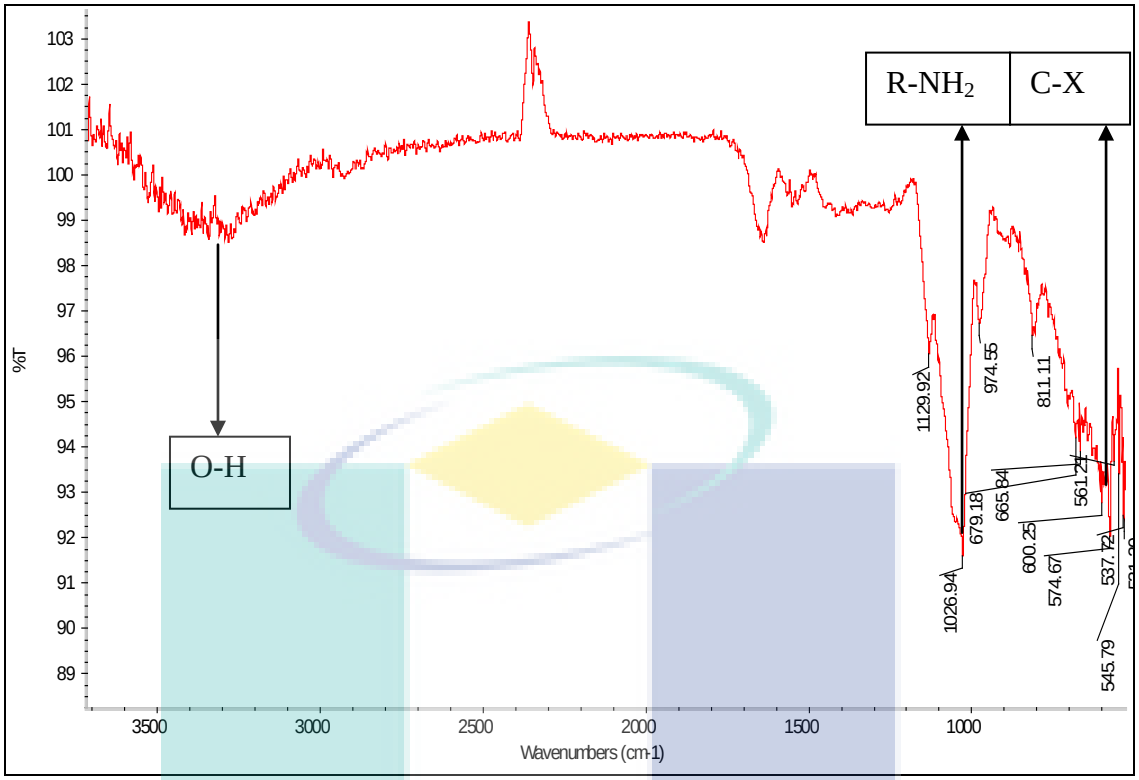


Figure 4.12: FTIR spectra of FTase

The ratio of FTIR stretching started from 3300 cm^{-1} and it is an R-NH_2 bond. The transmissions percentage of the bonding in FTase was 102. The first peak found in the FTase was water content and it was probably the moisture content after FTase had been taken out from the fridge and the following peak was the highest at wavenumber 1200cm^{-1} which indicated protein compound in this enzyme itself. Protein was structured from more than 30 amino acids compounds. So the complex bonding of protein was clearly demonstrated by the spectra and indicated by the instability of the noise. The spectral analysis also demonstrated the existence of H-NMR compounds which is shown on the second highest peak at 500 cm^{-1} where more than 550 of spectrum was found. This was probably the proteins compound that consisted in the FTase itself. Other than this, it was the second highest peak at 1100 cm^{-1} where the spectrum more than 1000 was found and it is the C-H bond. This is probably amina that commonly compound existed in the complex protein like enzyme. Finally FTase also possesed aromatic compound and it is illustrated clearly from the peak with more than 1100 spectrum and this was the reason of the scent that existed from this enzyme. This

comparison indicates that FTase functional group is long chain of polymers of many kinds of protein compounds.

4.1.3 Characterization of Fructo-oligosaccharides (FOS)

4.1.3.1 Spectral Analysis of FOS

The third approach of Fourier-transformed Infrared Spectrophotometer (FTIR) in the characterization is for the product is Fructo-oligosaccharides. FOS is also a polymer that degrades from fructose and other types of sugar contents which is C=H bonded. As shown in Figure 4.13 is the FOS structure while Figure 4.14 depitcted FTIR spectrum of FOS.

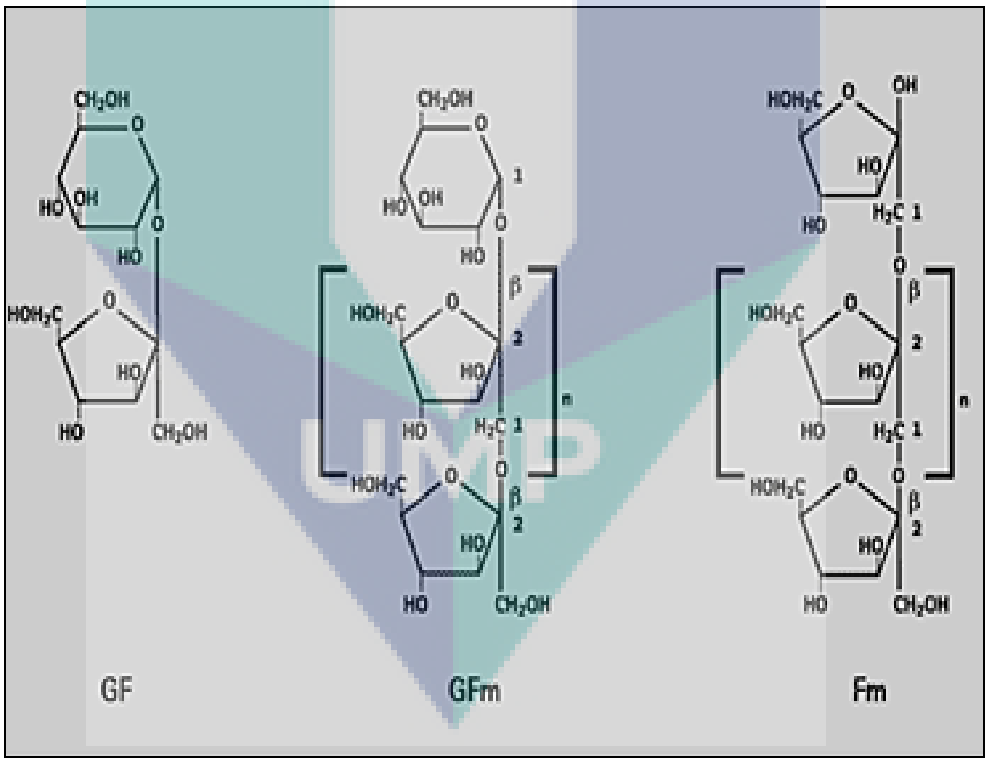


Figure 4.13: Fructo-oligosaccharides structure

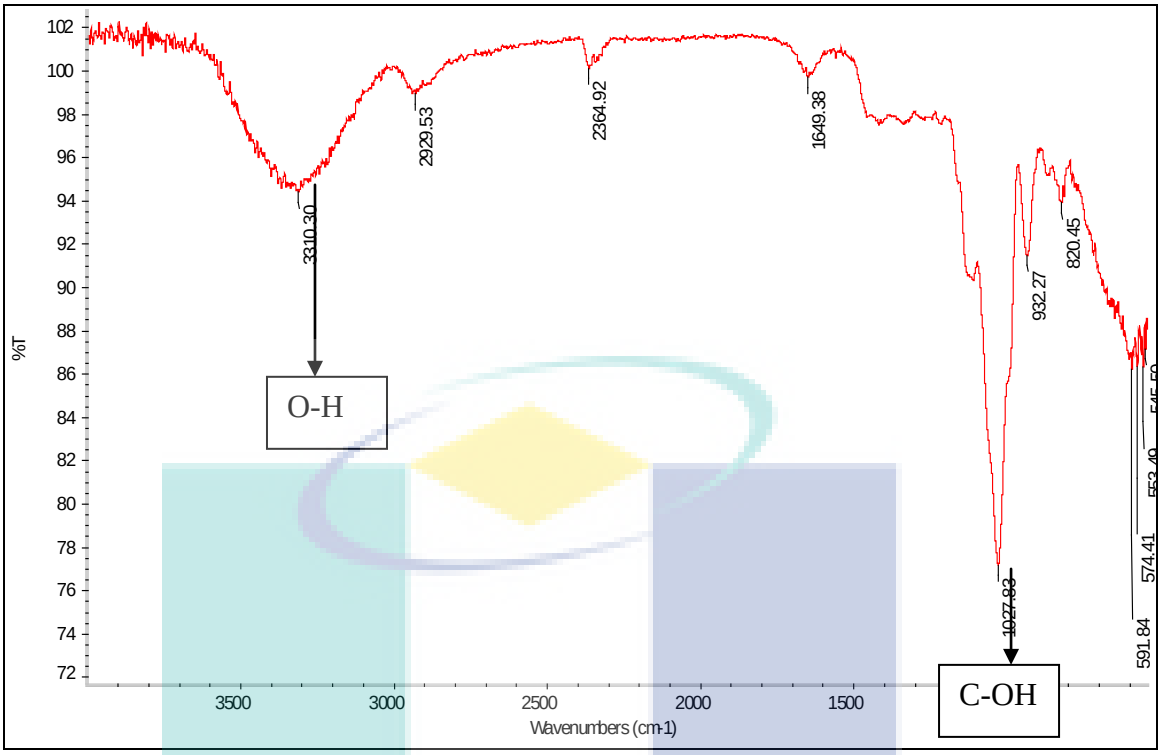


Figure 4.14: FTIR spectra of FOS

The ratio of coconut sugar stretching at 500cm^{-1} and that is C-OH. The transmissions percentage of the bonding in coconut sugar was 102. From the figure 4.13 FOS spectra analysis observed that the bonding consisted simpler spectral analysis compared to the coconut sugar spectra analysis and it is proved according to the FOS structure as shown in figure 4.12. It was indicated that FOS was one of the simple sugars that easily is digested in the ileums. This spectrum also shows that FOS contents were stable and the peaks showed the contents of water and sugar compounds. Referring to the existed library in the software, the closest ratio in regards to FOS is cellophane and isomaltose powder which are formed with sugar bonded (C-H) as illustrated in the Figure 4.13. This comparison indicates that FOS functional group has a few chain of sugar family that is also known as oligosaccharides.

4.2 KINETIC PARAMETERS IN BATCH SYSTEM

This study is mainly to determine the K_m and V_{max} values which are the parameters that affected enzymatic reaction in batch systems.

Table 4.2: The experimental value of FOS production from various substrate concentration and the values of 1/[S] and 1/v

ID	FOS(g/L)	1/[S]	1/v
300	53.20	0.0033	0.0187
400	74.50	0.0025	0.0134
500	125.60	0.0020	0.0079
600	183.24	0.0017	0.0054
700	222.84	0.0014	0.0044
800	228.40	0.0013	0.0043

The experimental work had been done to obtain the FOS production from various substrate concentration in batch reactor at 55 ^{o}C, pH 5.5, 5 hours and 150 rpm of the reaction temperature, pH, time and agitation speed respectively. The reaction occurred with the presence of FTase as the reaction enzyme in the acetate buffer. Table 4.2 shows the values obtained from the HLPC analysis and value of 1/[S] and 1/v had been calculated. This procedure is the steps to calculate the K_m and V_{max} value as described in CHAPTER 3. The next step after gaining the each value is linear graph plotting as shown in Figure 4.15. This is to find the slope as in the following methods:

The Michealis Menten equation (Eq 4.2) was further derived into following equation according to which a linear plot between 1/*v* and 1/[*S*] was attempted. The latter relationship is called Lineweaver-Burk plot.

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} \qquad (4.2)$$

The intercept on the y-axis is equal to $1/V_{max}$ and the slope is K_m/V_{max}. It is equal to linear a equation as shown at Eq 4.3.

$$y = mx + c \qquad (4.3)$$

and the calculation is as below:

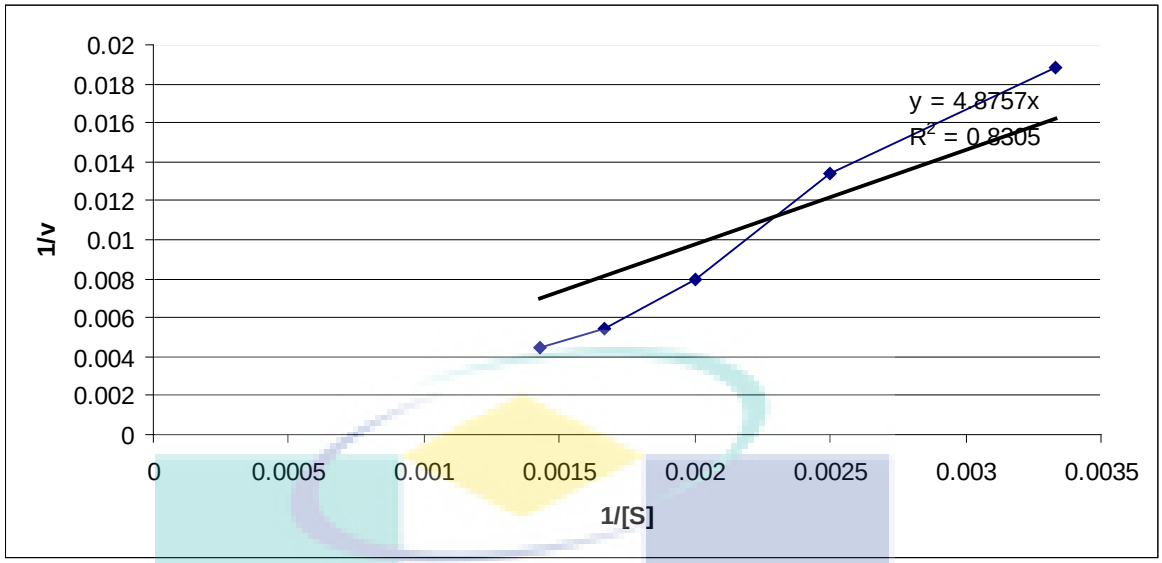


Figure 4.15: FOS production plot with coconut sugar concentration effect

$$M = \frac{y2 - y1}{x2 - x1} = \frac{0.0162 - 0.007}{0.033 - 0.0014} = 0.2911$$

$$0.2911 = \frac{K_m}{V_{max}} \quad \text{so} \quad K_m = 0.2911\ V_{max}$$

and the intercept of y axis is y = 0.001

$1/V_{max} = 0.001$ so $V_{max} = 1000$

$K_m = 291.1$

The value of K_m and V_{max} for the enzymatic reaction in batch system for the production of FOS from coconut sugar with FTase presence is 291.1 and 1000 respectively. The enzymatic reaction of FTase and coconut sugar is significant where the value of V_{max} is higher than Km. As a conclusion, the lower value of K_m will lead to high affinity of enzyme and substrate where the binding of those is really strong and engulfing. Thus the higher value of V_{max} shows that this reaction would produce a high concentration on the production of FOS.

4.3 ONE FACTOR AT ONE TIME (OFAT)

One Factor at One Time (OFAT) study includes six parameters that affected the production of FOS by enzymatic reaction from natural coconut with FTase. They are reaction time, coconut sugar concentration, enzyme concentration, temperature, pH and agitation speed. The importance of OFAT study is to select the significant range of parameters obtained from many literatures and resources. The majority of the previous study focused on the microbial production of FOS and FTase where they using commercial sucrose as the main substrate while many novel FTase sources were studied due to the maximum yield of FOS. In this study, all the parameters gained from the related literatures were accumulated then tested whether it is significant or insignificant for the same process which is microbial production and enzymatic reaction but substitution of reaction raw materials which is coconut sugar and commercial FTase.

OFAT is very important to determine the significant range of the effect factors. All the range of limits obtained from the literatures will be tested due to the insignificancies due to the data and the graph of FOS production. For the study of the effects of reaction time in the FOS production the range gained from the literatures is range 1 hour to 10 hours. While for the concentration effects on the production of FOS is 200 g/L to 800 g/L and 0.01% to 0.15% of coconut sugar concentration and enzyme concentration respectively. Temperature and pH also play important roles for producing FOS as the reaction involving enzyme as the catalyst to the reaction. Enzyme is very sensitive to environment conditions so, the best parameter for the condition has to be determined in preparation for the best environment for the reaction. The range that has to be observed is 20 ^{o}C to 70 ^{o}C and pH 4.0 to 6.5 of reaction temperature and pH respectively. Finally, the parameter that had been studied from the information gained is the speed of the reactor agitator. This study utilized lab scale water bath shaker for the reaction to occur so the agitation speed is counted as reaction parameters. The range that had been studied is 50 rpm to 300 rpm.

The selection of the significant parameters was calculated on FOS production standard deviation. The larger the number for the standard deviation means the more significant the factor is. While smaller significant number would indicates the

insignificant parameters that effected the FOS production from coconut sugar and commercial FTase.

4.3.1 Time Course Study on the FOS Production and Reducing Sugars

Figure 4.16 illustrates the time course of FOS production and reducing sugar in coconut sugar by fructosyltransferase at 55 ^{o}C and pH 5.5 of reaction temperature and pH respectively. The amount of FOS produced in the reaction mixture increased rapidly between 1 and 6 hours of the reaction time. While the substrate contents in the coconut sugar which is sucrose decreased as the FOS produced in the reaction.

The concentration of sucrose was initially about 500 g/L and gradually deccreased after 10 hours of reaction. Glucose and fructose concentrations of coconut sugar were uniform from beginning till the final time of reaction. Sucrose contents in coconut sugar decreased as time went by and this was the main cause for FOS concentration to decrease while FOS concentration gradually increased until it reached maximum value at 4 to 6 hours of reaction time. Other kind of reducing sugars like fructose and glucose remained in terms of concentration until the end of the experiments. From the study, it showed other kind of reducing sugar in coconut sugar did not affected FOS production. This study showed the utilization of sucrose solely in the reaction as it consumed and finally generated FOS in the end after reacting with FTase with the suitable conditions as enzyme reacted on specific substrate. It is also illustrated that this study did not respond other kinds of sugar but only fully utilizing sucrose as the main substrate of the FOS production of enzymatic reaction. The yield of FOS produced from the reaction of coconut sugar and FTase is more than 50% of sucrose consumed. The quality was quite high as this study approach the discovery of a new source of sucrose which is coconut sugar which is very cheap and comes from a local agricultural product.

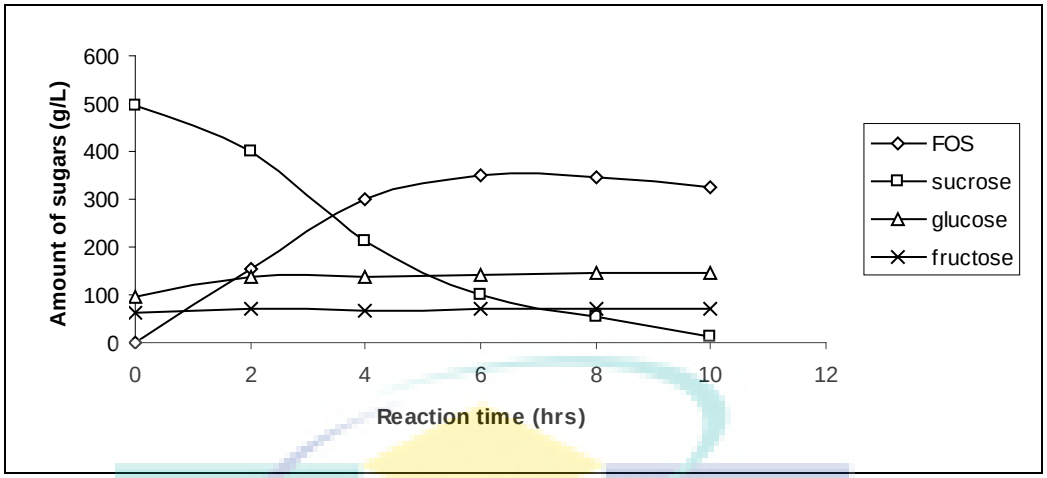


Figure 4.16: Effect of reaction time on the production of Fructo-oligosaccharides and the reducing sugars in coconut sugar

4.3.2 Effect of Reaction Time

Figure 4.17 depicted the production of FOS that started increasing gradually at 4 to 6 hours and reached the maximal value of 190 g/L at 5 h, 55 ^{o}C of reaction time and temperature respectively. This observation apparently denoted that the reaction mixture became less concentrated after 6 hours. Theoretically, the bonding of sugar in coconut sugar had been broken especially the sucrose compound that consisted in the coconut sugar itself. The sucrose started to react with the FTase in order to produce FOS. As discussed previously, the study shows that after 1 to 5 hours the reaction exhibit significant while during 5 to 6 hours the reaction slowly inhibits until 10 hours of reaction time. As conclusion, 5 hours of reaction time for FOS production from coconut sugar and FTase is the significant limit for FOS yield over 33% based on the amount of coconut sugar consumed and after 6 hours the reaction is already insignificant.

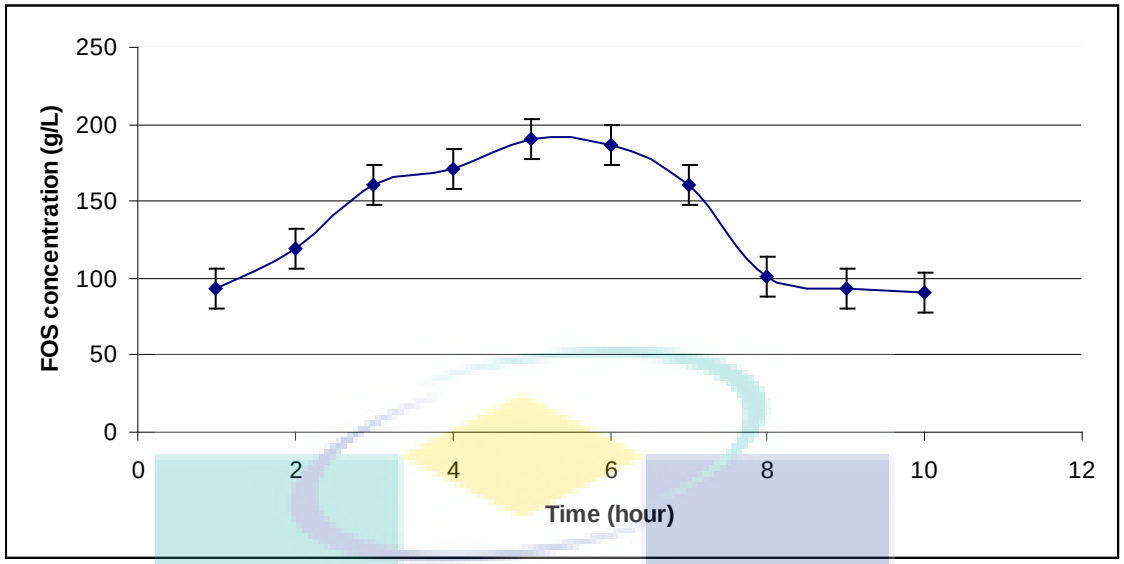


Figure 4.17: Effect of reaction time

4.3.3 Effect of Substrate Concentration

Figure 4.18 shows the effect of coconut sugar concentration on the production of FOS. The increment in concentration of coconut sugar resulted in the increment of FOS production. The maximum value of FOS produced in the reaction mixture was 197 g/L at a range between 700 g/L to 800 g/L of coconut sugar concentration at 55 ^{o}C, pH 5.5 and 5 hours of reaction temperature, pH and time respectively. This phenomenon showed that decrement of sucrose contents in the coconut sugar resulted in the decrement of FOS production.

Initially the production of FOS was less when sucrose contents in coconut sugar were also less. The FOS production was parallel to the increment of sucrose contents supplied from the coconut sugar. It was as stated in Sanggeetha et al., (2005) study that the optimum FOS production occurred at 50% of sucrose contents in the reactions with FTase. So this study answers the statement because even though the substrate had been changed in other form which is coconut sugar and not the commercial sucrose, but the sucrose content can still be measured and able to produce FOS. Based on the observation from the study, the concentration of coconut sugar played a significant

factor for FOS production 500 g/L to 800 g/L of coconut sugar concentration for the vital range and limit.

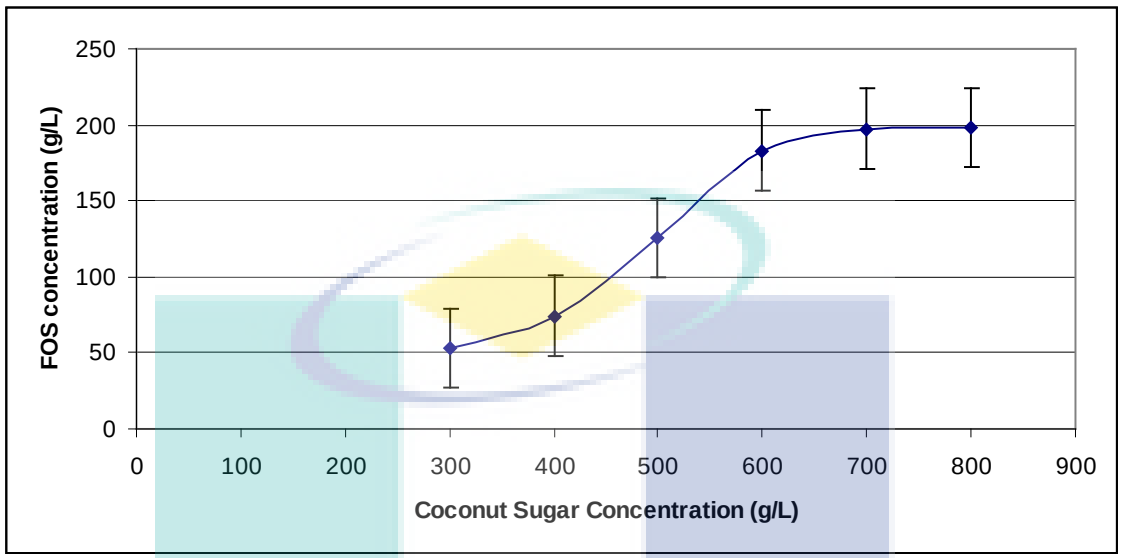


Figure 4.18: Effect of coconut sugar concentration

4.3.4 Effect of Enzyme Concentration

As shown in Figure 4.19, initially FOS was not produced in the reaction mixture without the existence of the enzyme. Commercial FTase was utilized in this study with the enzyme activity of 401U/mL. As the concentration of FTase increased, the amount of FOS produced in the reaction mixture also increased. The amount of FOS produced was directly proportional to the concentration of enzyme present until it reached the optimum value at 0.1 g/L of enzyme concentration. The concentration of enzyme played a significant factor with significant range 0.01 to 0.12. FTase is one of the most important materials in this study because FOS may not be produced by enzymatic reaction without it. So, the concentration of this enzyme played an important role in order to produce FOS.

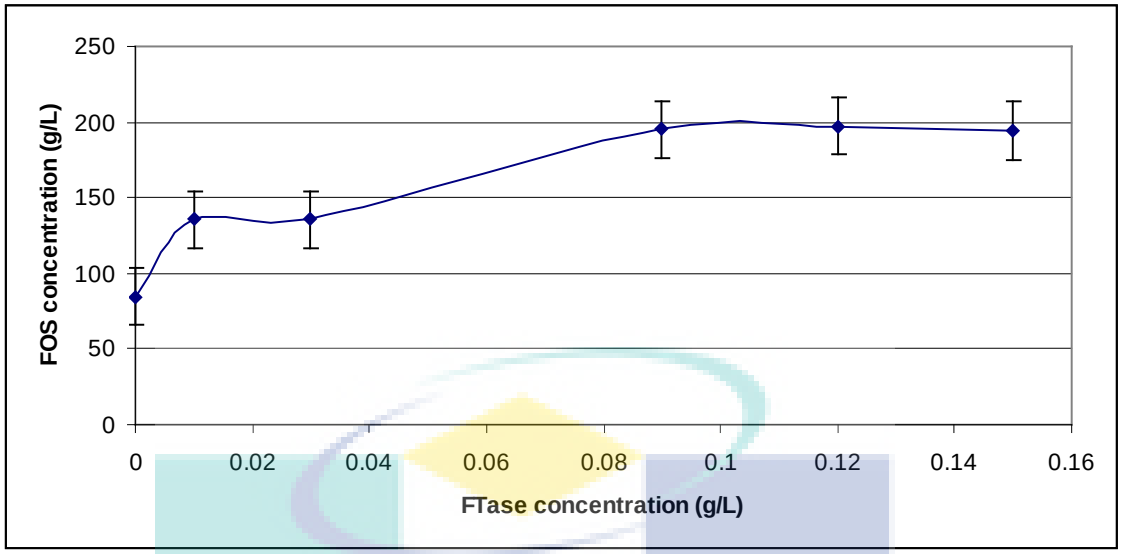


Figure 4.19: Effect of enzyme concentration

4.3.5 Effect of Temperature Changes

Temperature was found to have an intense effect on enzymatic production of FOS. Figure 4.20 shows the effect of temperature on the production of FOS from coconut sugar by FTase. The FOS produced in the reaction mixture increased as temperature increased. The optimal temperature for the FOS production was between 50 ^{o}C to 70 ^{o}C after 5 hours of reaction. It was due to the enzyme activity where FTase exhibited optimal activity between 50 ^{o}C to 70 ^{o}C. At 20 ^{o}C the enzyme hibernates and slowly awakens with the rising of the temperature and it is clearly demonstrated on the graph by the increasing FOS production until it reached the maximum value at 55 ^{o}C. FTase will be denatured at 100 ^{o}C and above. The significance of vital range is 50 ^{o}C to 70 ^{o}C due to the effect on the production of FOS.

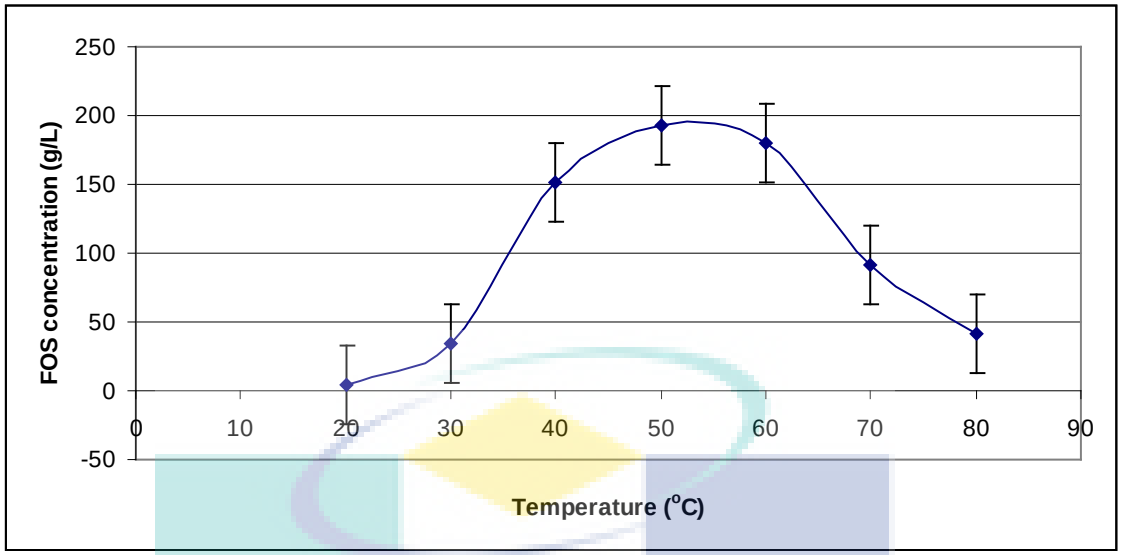


Figure 4.20: Effect of temperature changes

4.3.6 Effect of pH changes

As shown in Figure 4.21, initially, the amount of Fructo-oligosaccharides produced in the reaction mixture was directly proportional to the increment of pH until it reached the optimum value at pH 5.0 – 6.0. pH is another factor that plays intensive effects on the production of FOS from coconut sugar. It is due to the reaction surrounding that required the best condition to begin with. Similar with other enzymes FTase was active as it exhibited at the pH range of pH 5.0 to pH 6.0. Below than this level FTase used, passively reacted with the substrate until the reaction prepared the best condition for it as illustrated with the rapid increased of FOS production from pH 5.0 until pH 6.0. FTase is an enzyme that is sensitive to its environmental surrounding but in this study, the commercial enzyme utilization was approached and the same events occurred.

This strongly showed that the production of FOS was optimum in the best condition of reaction mixture which affected with the changes of pH. From the figure it is also demonstrated that the best range and limit for effect of pH is pH 4.0 to 6.0 due to the production of FOS.

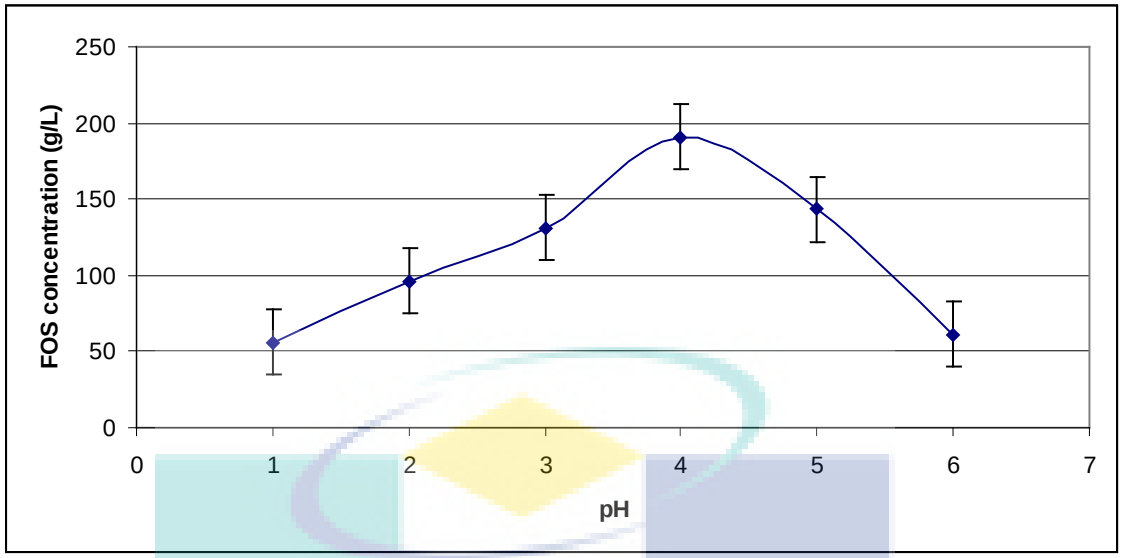


Figure 4.21: Effect of reaction pH changes

4.3.7 Effect of Agitation Speed

Figure 4.22 illustrates the agitation speed effects in the enzymatic reactor on the production of FOS. Initially, the amount of FOS produced increased as the speed of agitation increased but as it reached the optimum value at 150 rpm, the production of FOS in the reaction mixture gradually decreased. It was due to the reaction high speed agitation that confounded the enzyme. Enzyme was highly sensitive with its surrounding conditions, of which the higher speed of agitator exhibited the enzyme activity and if the speed was too fast, the enzyme can be denatured easily. The significant limit for the effect of agitation speed is on the point maximum production of FOS which is at 150 rpm.

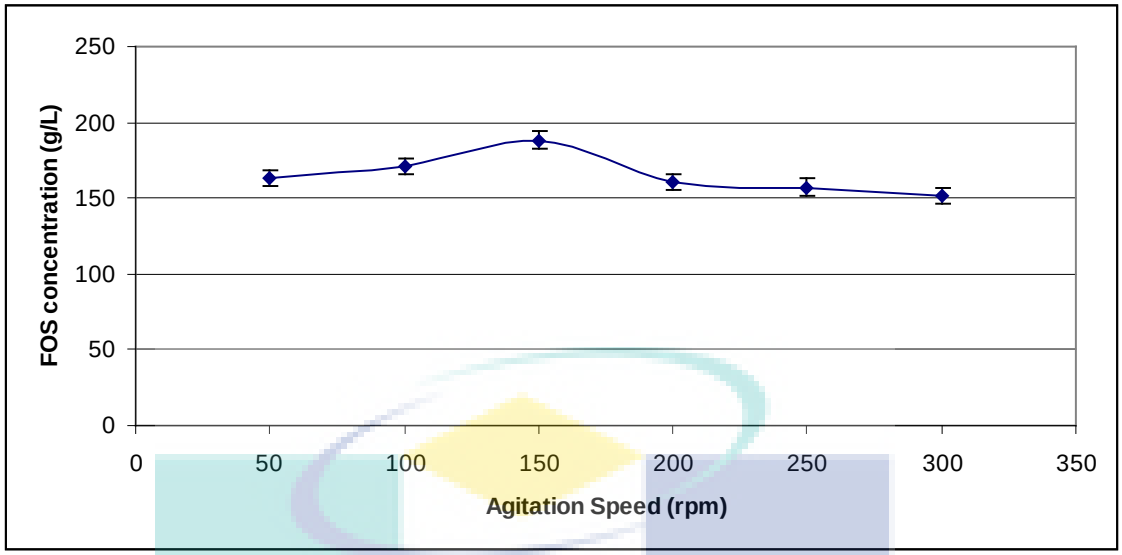


Figure 4.22: Effect of agitation speed

4.3.8 Concluding Remarks

The objective of OFAT study was to determine the best range of effect factors that were accumulated from the literatures. Table 4.3 shows the summarized information gained from the OFAT study. Thoroughly, there are six parameters found affecting the production of FOS. From the value of standard deviation calculated for each of the experimental values, there are four parameters that most effected the production of FOS from coconut sugar. This phenomenon was due to the value of standard deviation that showed the different values of range for each factor. They were substrate concentration which is coconut sugar concentration, enzyme concentration which is FTase concentration, temperature and pH. While the vital range for each reaction parameters were 700 to 800 g/L, 0.08 to 0.12 g/L, 50 to 70 ^{o}C, pH 5.0 to 6.0 for coconut sugar concentration, FTase concentration, reaction temperature and reaction pH respectively.

On the other hand, there were another two parameters that played least significance for the study which were reaction time and agitation speed is the less significant with the standard deviation only 45.72 and 13.09, respectively.

Table 4.3: Summary of OFAT study

Parameter	Standard deviation	Vital range
Reaction time	45.72	4 to 6 hours
Substrate concentration	75.10	700 to 800 g/L
Enzyme concentration	126.24	0.08 to 0.12 g/L
Temperature	81.38	50 to 70 ^{o}C
pH	67.21	pH 5.0 to 6.0
Agitation speed	13.09	150 rpm

4.4 SCREENING PROCESS BY FRACTIONAL FACTORIAL DESIGN (FFD)

4.4.1 Analysis and Discussions of FFD Study

Design Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, version 6.0.8) was used for the experimental design throughout this screening process study. A total of sixteen sets of experiments (2^4 full factorial design) and two replicates at the center point were used to demonstrate the statistical significance of the coconut sugar concentration (*A*), enzyme concentration *(B)*, reaction temperature (*C*) and reaction pH *(D)* an their effects on the concentration of response. The range and the levels of the variables investigated in this study are 600 g/L to 800 g/L, 0.08 g/L to 0.12 g/L, 50 ^{o}C to 70 ^{o}C, and pH 4 to 6.5 for factors *A*, *B* , *C* and *D* respectively. While Table 4.4 shows the experimental design and the predicted values of the screening process. Range settings for the variable factors were adjusted based on previous findings and literature (Zularisam et al., 2009).

Table 4.4: Experimental layout and results of 2^4 Full Factorial Design

Standard	Run	Block	Coded factors				FOS concentration (y) (ln y)	
			x1	x2	x3	x4	Predicted	Actual
31	1	{1}	1.0	-1.0	-1.0	1.0	3.81	3.67
22	2	{1}	-1.0	-1.0	1.0	1.0	3.56	3.67
24	3	{1}	1.0	1.0	1.0	-1.0	4.34	4.32
6	4	{1}	-1.0	-1.0	-1.0	-1.0	4.21	4.32
2	5	{1}	1.0	1.0	-1.0	1.0	3.63	3.85
14	6	{1}	-1.0	1.0	1.0	-1.0	4.04	3.85
8	7	{1}	1.0	1.0	-1.0	-1.0	4.25	4.17
11	8	{1}	1.0	1.0	1.0	1.0	4.16	4.17
23	9	{1}	1.0	-1.0	-1.0	-1.0	4.07	4.07
7	10	{1}	1.0	-1.0	1.0	-1.0	4.04	4.07
25	11	{1}	-1.0	1.0	-1.0	1.0	4.03	4.15
21	12	{1}	-1.0	-1.0	1.0	1.0	4.35	4.15
5	13	{1}	-1.0	-1.0	1.0	1.0	4.77	4.24
18	14	{1}	-1.0	1.0	-1.0	-1.0	3.73	4.24
27	15	{1}	1.0	-1.0	-1.0	-1.0	4.10	4.00
26	16	{1}	-1.0	1.0	1.0	-1.0	3.82	4.00
20	17	{1}	1.0	1.0	1.0	1.0	4.88	4.54
28	18	{1}	1.0	1.0	-1.0	-1.0	4.27	4.54
15	19	{1}	1.0	-1.0	-1.0	1.0	5.08	4.72
13	20	{1}	-1.0	1.0	1.0	1.0	4.47	4.72

2	21	{1}	-1.0	-1.0	1.0	-1.0	4.05	4.17
17	22	{1}	-1.0	1.0	-1.0	1.0	4.22	4.17
30	23	{1}	-1.0	1.0	1.0	1.0	4.77	5.04
29	24	{1}	-1.0	-1.0	-1.0	1.0	5.21	5.04
1	25	{1}	-1.0	-1.0	1.0	1.0	3.05	3.12
32	26	{1}	1.0	1.0	1.0	-1.0	3.12	3.12
3	27	{1}	1.0	1.0	-1.0	1.0	3.23	3.41
9	28	{1}	-1.0	-1.0	1.0	-1.0	3.48	3.41
4	29	{1}	1.0	-1.0	-1.0	1.0	2.53	2.76
19	30	{1}	1.0	1.0	-1.0	-1.0	3.06	2.76
10	31	{1}	-1.0	-1.0	-1.0	-1.0	3.50	3.73
16	32	{1}	1.0	-1.0	1.0	-1.0	4.06	3.73

Table 4.5: Analysis of variance (ANOVA) table (Partial sum of squares) of factorial design

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	10.02	11	0.91	11.15	< 0.0001	significant
A	1.22	1	1.22	14.88	0.0010	
B	2.857E-004	1	2.857E-004	3.496E-003	0.9534	
C	3.13	1	3.13	38.32	< 0.0001	
D	0.12	1	0.12	1.46	0.2407	
AB	0.066	1	0.066	0.81	0.3783	
AC	0.11	1	0.11	1.30	0.2673	
AD	0.28	1	0.28	3.47	0.0773	
BD	2.348E-003	1	2.348E-003	0.029	0.8671	
CD	4.37	1	4.37	53.48	< 0.0001	
ABD	0.51	1	0.51	6.25	0.0212	
ACD	0.21	1	0.21	2.62	0.1212	
Residual	1.63	20	0.082			
Lack of Fit	0.049	4	0.012	0.12	0.9719	not significant
Pure Error	1.59	16	0.099			
Cor Total	11.66	31				
Mean	0.26		R^2	0.8764		
Std. Dev.	3.97		Adjusted R^2	0.7984		

Analysis of the experimental data by a complete 2^4 factorial design was systematically conducted as an initial screening process by examining the effects and interactions of coconut sugar concentration, enzyme concentration, temperature and pH. A statistical testing using Fisher's statistical test for ANOVA was employed for the determination of significant variables where the degree of significance was ranked based on the value of *F*-ratio. As matter of fact the larger the magnitude of the *F*-value and correspondingly the smaller the "Prob > *F*" value, the more significant are the corresponding model and the individual coefficient. It was observed from ANOVA analysis (Table 4.5) that the confidence level was greater than 90% ($P < 0.1$) for removal of FOS production while the F-value and the P-value of the model were 11.15 and 0.0001, respectively, thus indicating that the estimated model fits the experimental data adequately. Furthermore, the coefficient of determination R^2 of the model was reasonably close to 1 (0.88), implying that about 88% of the variability in the data was explained by the model. It was further shown that the main effect of the coconut sugar concentration (*A*), and the temperature (*C*) and the two level interactions of *AD* and *CD* were significant model terms (factors). Other model terms especially the main effect of the enzyme concentration (*B*) were relatively less significant in influencing the FOS concentration as their confidence level was less than 95% ($P > 0.05$).

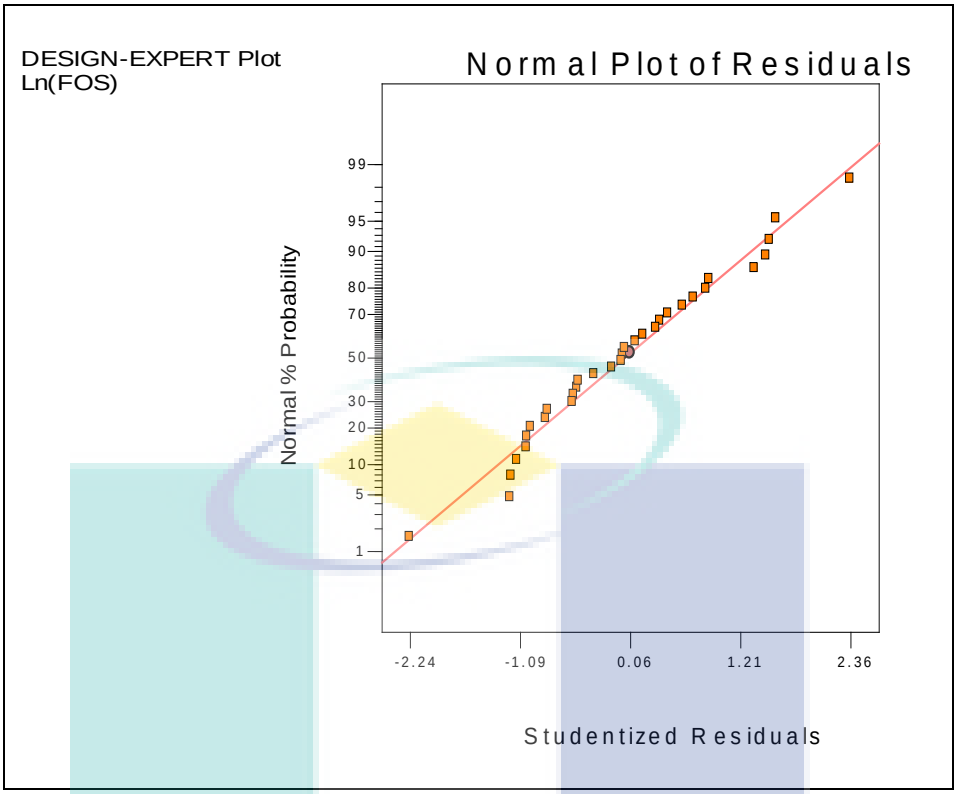


Figure 4.23: The Half normal plot for 2^4 Full Factorial Design

The significance of effects and interactions of factors on a response was further diagnosed and compared, and graphically illustrated in the half normal plot (Figure 4.23), where results of dominating effects that is likely to represent the important and influential factors were found consistent with the ANOVA analysis results. Moreover the significance of interactions between factors on the response can be best considered using the interaction analysis graph of Figure 4.24. It has been observed that the interaction effect between the coconut sugar concentration and the temperature (Figure 4.24a) depicts a remarkable improvement in FOS concentration as the interaction between the significant models with the FOS production shows an increase in the reaction temperature (50 ^{o}C to 70 ^{o}C) resulting in the increase of FOS concentration. This is because the FTase in the reaction exhibited actively by the increment of temperature. Probably, the enzyme used is not like other fungal or cultured enzyme that can be easily inhibited, as the enzyme used is a commercial enzyme that already had been modified for resistance. Similarly, the increases of the coconut sugar concentration (500 g/L to 800 g/L) also result in the increased of the production of FOS. It is because the high sucrose contents in the coconut sugar that will reacted with the enzyme and

producing FOS. When the sucrose contents getting decrease with the time flowing, the FOS concentration also resulted decreased in directly proportional with the substrate decrement. The enhancement brought about by increasing the FOS concentration appears to be greater at higher temperatures and coconut sugar concentrations, which is significantly contrary to the results presented in Figure 4.24b This is mainly because the FOS is produced in the presence of FTase, and the production increased rapidly until the FTase concentration is sufficient for the optimum production of FOS. Subsequent to this level, the excessive increase of the FTase concentration only results in the uniform production of FOS, and this is the main reason why this factor has been screened insignificant. Besides the FTase concentration, the reaction pH was also screened as an insignificant model of this study. This is probably because of the utilization of the commercial FTase in the reaction that is more resistant to the surrounding changes and exhibited actively with the changes.

Apart from the regression model obtained, the factorial design analyses can be as well adopted in screening the crucial and critical variable of the operating conditions. A variable is claimed to have a greater significant effect on the FOS production if the coefficient was relatively larger than the others, whereas the variable with a positive fitted constant has an enhancer effect on the FOS concentration compared to a negative coefficient that had the opposite effect. As can be inferred from Eq. (4.5), which includes the coefficient for each effect, the main effect of temperature (*C*) has the largest coefficient (+0.34) followed by coconut sugar concentration (*A*, +0.24), enzyme concentration (*B*, 0.013), and pH (*D*, +0.032). This result was consistent with the ANOVA analysis in Table 4.5 where the temperature (*C*) variable is shown to have the highest *F* value.

$$\textbf{Ln(FOS)} = +3.97 + 0.24(A) + 0.013(B) - 0.34(C) - 0.032(D) - 0.030\ (A \times B) - 0.013(A \times C) + 0.055(A \times D) - 5.688E\text{-}003\ (B \times C) - 6.051E\text{-}003\ (B \times D) - 0.34\ (C \times D) - 9.299 \times 10^{-4}\ (A \times B \times D) - 0.044\ (B \times C \times D) \quad \textbf{(4.5)}$$

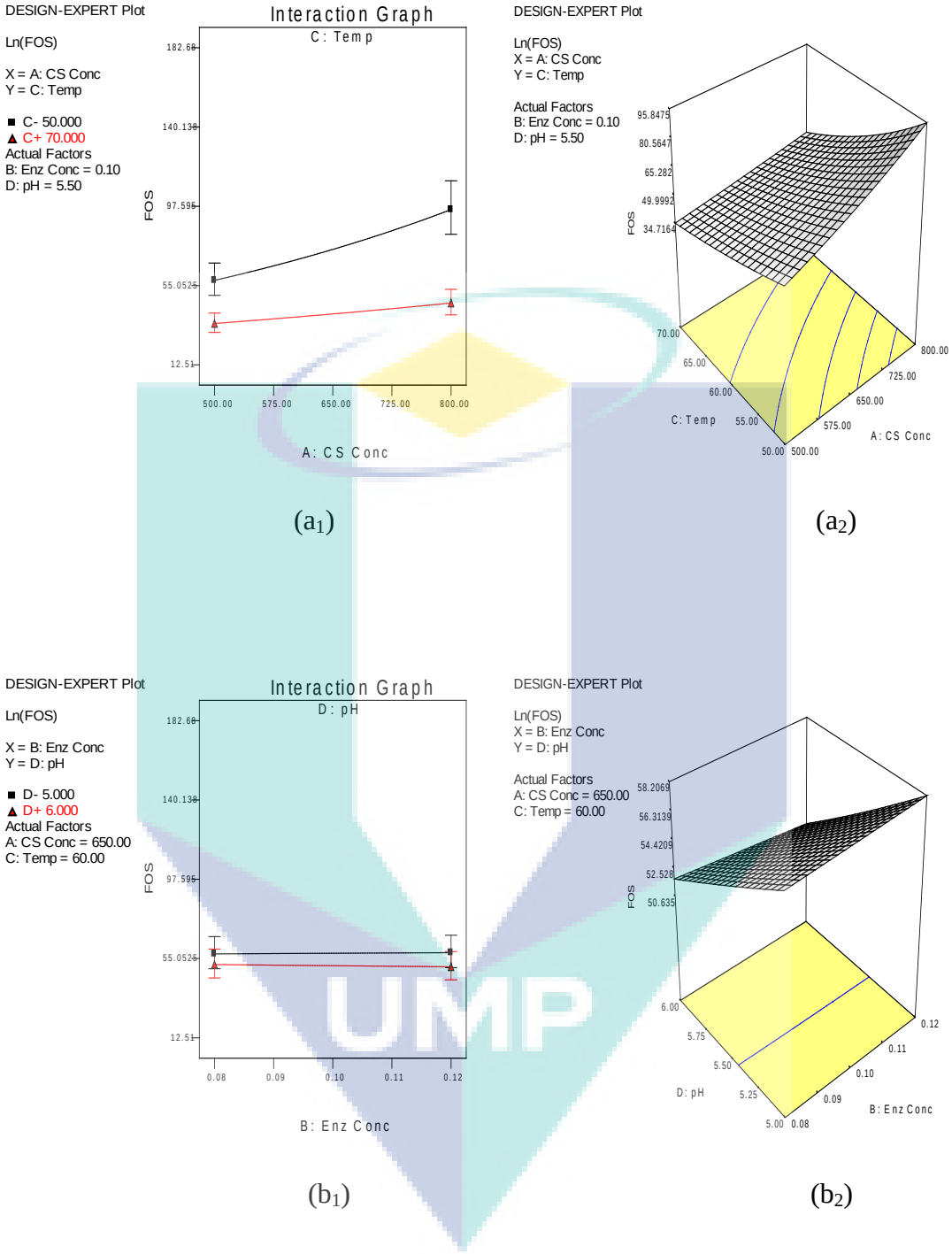


Figure 4.24: Plot of interaction effect for FOS production (g/L) : (a_1 and a_2) effect between coconut sugar concentration and temperature, (b_1 and b_2) effect between enzyme concentration and pH.

4.4.2 Concluding remarks of FFD study

The objectives of FFD study is to determined the best range of effect factors that accumulated from OFAT study. There are four parameters found affecting the production of FOS that continue from OFAT. There are coconut sugar concentration, enzyme concentration, and reaction temperature and reaction pH. The screening process have been done by the experimental work that has been design by the design expert software which is consisted of 32 different experimental with a desired value which is FOS concentration. After all the experiments have been done and all the data have been fulfill into the software there are only two parameters have been screened as significant which is coconut sugar concentration and reaction temperature. It is due to the F value which is less than 0.0001. While the vital range for each reaction parameters are 700 to 800 g/L, and 50 to 70 ^{o}C, pH of coconut sugar concentration and reaction temperature. Both of these parameters will be continuing to another stage for obtained the maximum yield of FOS which is optimization by design expert software by using Response surface method (RSM).

While there are another two parameters that have been screened to play least significant for the study which is reaction pH and enzyme concentration these parameters will be proceed to the next stage of experiment as independent variables and the limit will be approximate to the best value of FOS concentrations. There are pH 5.5 and 0.1 g/L of reaction pH and enzyme concentration respectively. Both of these values will be constant on to the every single experimental works afterword altogether with the previous insignificant factors from OFAT study which are reaction time and agitation speed.

4.5 OPTIMIZATION BY RESPONSE SURFACE METHODOLOGY (RSM)

The factorial design was further continued with the response surface methodology (RSM) developed based on the central composite design (CCD) with the FOS concentration (responses), while significant terms from the preliminary screening process were chosen as the independent factors. The CCD was conducted with a 2^3 full factorial central composite design of combinations factors at two levels (high, +1 and low, −1 levels), including six star points (axial) corresponding to an ˛ value of 2 and six replicates at the center points (coded level 0, midpoint of high and low levels). In this design, due to their relative insignificances for FOS production, the independent variable for the coconut sugar concentration is represented by variable *A* ranging from 600 g/L to 800 g/L, while the reaction temperature *B* ranged from 50 ^{o}C to 70 ^{o}C, respectively. The central composite design matrices and the experimental response of each individual experiment are shown in Table 4.6. In this design, the enzyme concentration was constantly set at the center point settings (0.01g/L) at pH 5.5 as it was found to be insignificant in the reaction (Zularisam et al., 2009). The quadratic model for predicting the optimal point was according to Eq. (4.6).

$$Y = b_0 + \Sigma b_i x_i + \Sigma b_{ii} x_i^2 + \Sigma b_{ij} x_i x_j \qquad (4.6)$$

where *Y* is the response variable, *b* is the regression coefficient of the model, and *x* is the coded levels of the independent variables. In general, the primary objective of RSM is to optimize the response (*Y*) based on the factors investigated. The Design Expert software 6.0.8 was used to develop the experimental plan and optimize the regression equation (Eq. (4.6)). The statistical significance of the second-order model equation was determined by performing Fisher's statistical test for analysis of variance (ANOVA). In particular, a good model must be significant based on *F*-value and *P*-value as opposed to the Lack of Fit (insignificant). Moreover, the proportion of variance exhibited by the multiple coefficient of determination R^2 should be close to 1 as this would demonstrate a better correlation between the experimental and the predicted values.

Table 4.6: Experimental layout and results of 2^3 full factorial Central Composite Design (CCD)

Standard	Run	Block	Factor variables		FOS concentration (y) (ln y)	
			Coconut sugar concentration (g/L)	Temperature (^oC)	Predicted	Actual
1	15	{1}	750.00	62.00	131.57	128.54
2	16	{1}	750.00	62.00	131.57	126.44
3	4	{1}	800.00	50.00	123.89	125.21
4	18	{1}	750.00	55.00	123.89	125.79
5	2	{1}	700.00	50.00	103.75	100.99
6	13	{1}	750.00	48.00	103.75	99.99
7	7	{1}	800.00	60.00	68.98	67.95
8	19	{1}	750.00	55.00	68.98	69.91
9	12	{1}	820.00	55.00	155.29	154.21
10	21	{1}	750.00	55.00	155.29	150.23
11	9	{1}	680.00	55.00	125.57	128.36
12	6	{1}	700.00	60.00	125.57	112.11
13	20	{1}	750.00	55.00	107.80	103.93
14	3	{1}	800.00	50.00	107.80	103.93
15	10	{1}	680.00	55.00	49.89	47.81
16	17	{1}	750.00	55.00	49.89	44.21
17	1	{1}	700.00	50.00	236.48	224.22
18	8	{1}	800.00	60.00	236.48	234.22
19	5	{1}	700.00	60.00	236.48	215.68
20	14	{1}	750.00	48.00	236.48	240.90
21	11	{1}	820.00	55.00	236.48	241.40

Table 4.7: ANOVA for Response Surface Quadratic Model [Partial sum of squares] Response: FOS concentration

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Model	77063.58	5	15412.72	190.30	< 0.0001	significant
A	1086.84	1	1086.84	13.42	0.0023	
B	5829.20	1	5829.20	71.97	< 0.0001	
A^2	23804.16	1	23804.16	293.91	< 0.0001	
B^2	64203.21	1	64203.21	792.72	< 0.0001	
AB	894.43	1	894.43	11.04	0.0046	
Residual	1214.87	5	80.99			
Lack of Fit	189.54	3	63.18	0.74	0.5486	not significant
Pure Error	1025.33	12	85.44			
Cor Total	78278.45	20				
Std. Dev.	9.00		*R*2	0.9845		
Mean	134.31		Adjusted *R*2	0.9793		

The two significant variables, coconut sugar concentration and temperature, were further optimized using the response surface methodology and the results on the effect of variable factors on the FOS concentration, i.e., the response variables. In this design, the enzyme concentration factor was set at the center point settings (0.01g/L) due to its low significance for the FOS production, while the pH was maintained at 5.5. A fit summary output analysis indicated that the quadratic model was statistically significant to represent the production of the FOS response. The adequacy of a quadratic model was examined by *F* test, "Prob > *F*," and the determination coefficient R^2.

As can be inferred in Table 4.7, the computed *F* and the Prob > *F* were 190.30 and <0.0001, respectively, which implied that the model was highly significant with low probability. The results obtained adequately suggest that the present mathematical model was a good prediction of the experimental results, and as a matter of fact the terms in the model have a significant effect on the response. In a similar manner, the multiple correlation coefficient R^2 was calculated to be 0.9845, indicating a good agreement existed between the experimental and the predicted value as well as depicting that 98.45% of the variability in the response could be well explained by the model while only 1.55% of the total variation was poorly described by the model. Moreover, the "Lack of Fit" value was found insignificant (Prob > *F* = 0.5486), which denoted that the model was desirably fit. The second-order effect of temperature (B^2) and coconut sugar concentration (A^2) was found to have the largest effect on the FOS concentration, and this was followed by the main effects of temperature (*B*) and coconut sugar concentration (*A*). Moreover, the two-level interactions between coconut sugar concentration and temperature (*AB*) were found to be responsible for the FOS production. The multiple regression equations (Eq. 4.7) for FOS production using coconut sugar concentration (*A*) and temperature (*B*) as the main variable are as follows: The final empirical model in terms of actual factors is:

FOS conc = - 21795.96450 + 31.35618 (CS conc) +379.50266 (Temperature)
- 0.019464 (CS conc)2 - 3.19653 (Temperature)2 - 0.042295
(CS conc x Temperature) (4.7)

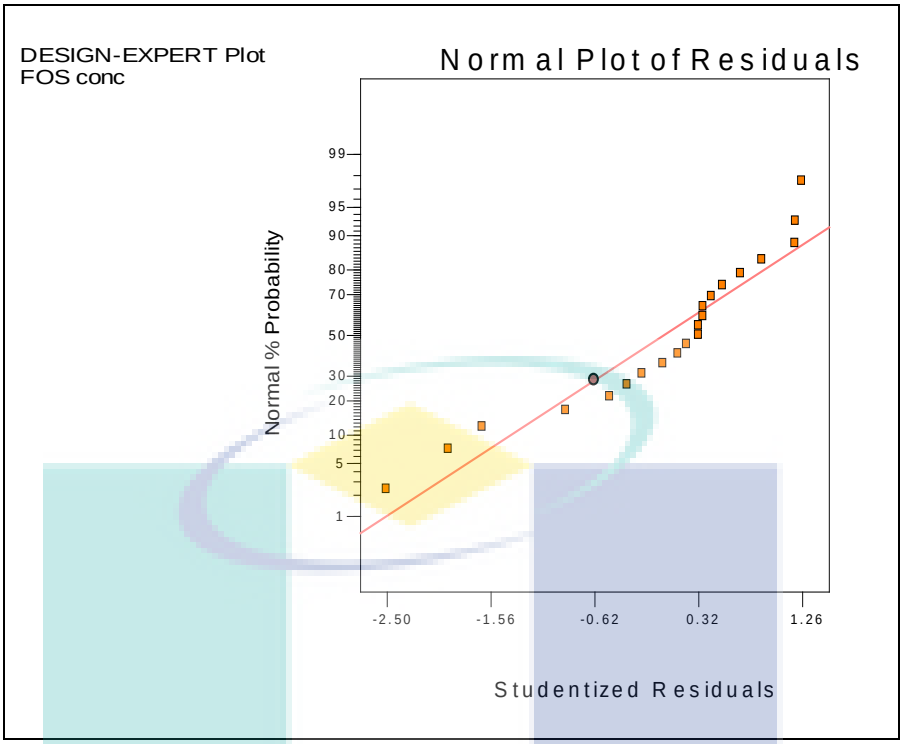


Figure 4.25: Normal Probability plot of residual for FOS production

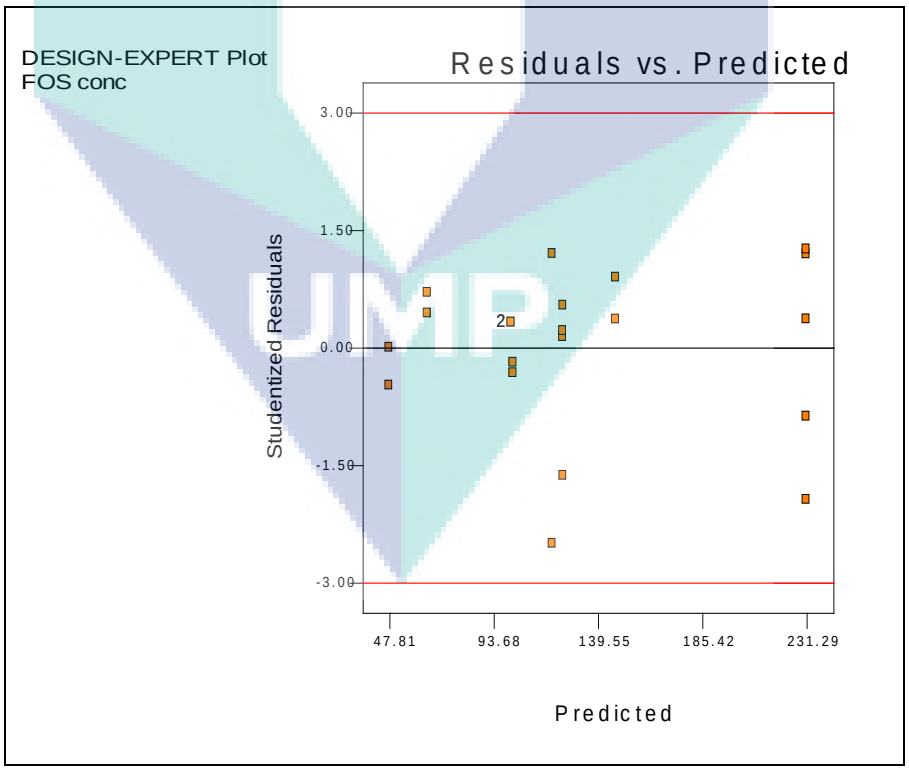


Figure 4.26: Plot of residual against predicted response for FOS production

The empirical model equations Eq. (4.7) form a mathematical correlation model that can be employed to predict and optimize the FOS concentration within the range of variable factors of this experiment.

The effect of the coconut sugar concentration and the temperature process variables on the FOS production was further analyzed using a simulated three-dimensional response surface and contour plots according to the backward quadratic model. The effect of the coconut sugar concentration and the temperature on the FOS production depicted in Figures 4.25 and 4.26 demonstrated that the FOS concentration increased when the coconut sugar concentration increased from 600 g/L to 800 g/L and as the temperature changed from 50 ^{o}C to 70 ^{o}C until it hits the optimum value (243 g/L) at 750 g/L and 55 ^{o}C, respectively. To put it concisely, temperature was the most significant factor affecting the FOS concentration (F value = 71.97) followed by the coconut sugar concentration (F value = 13.42). Figures 4.27 (a), (b), and (c) show the interaction between the significant models with the FOS production. The increase in the reaction temperature (50 ^{o}C to 70 ^{o}C) results in the increase of the FOS concentration. This is because the FTase in the reaction exhibited actively the increase of temperature. The FOS concentration reaches optimum when the coconut sugar increased as discussed previously, and this indicates the sucrose contents in the coconut sugar itself.

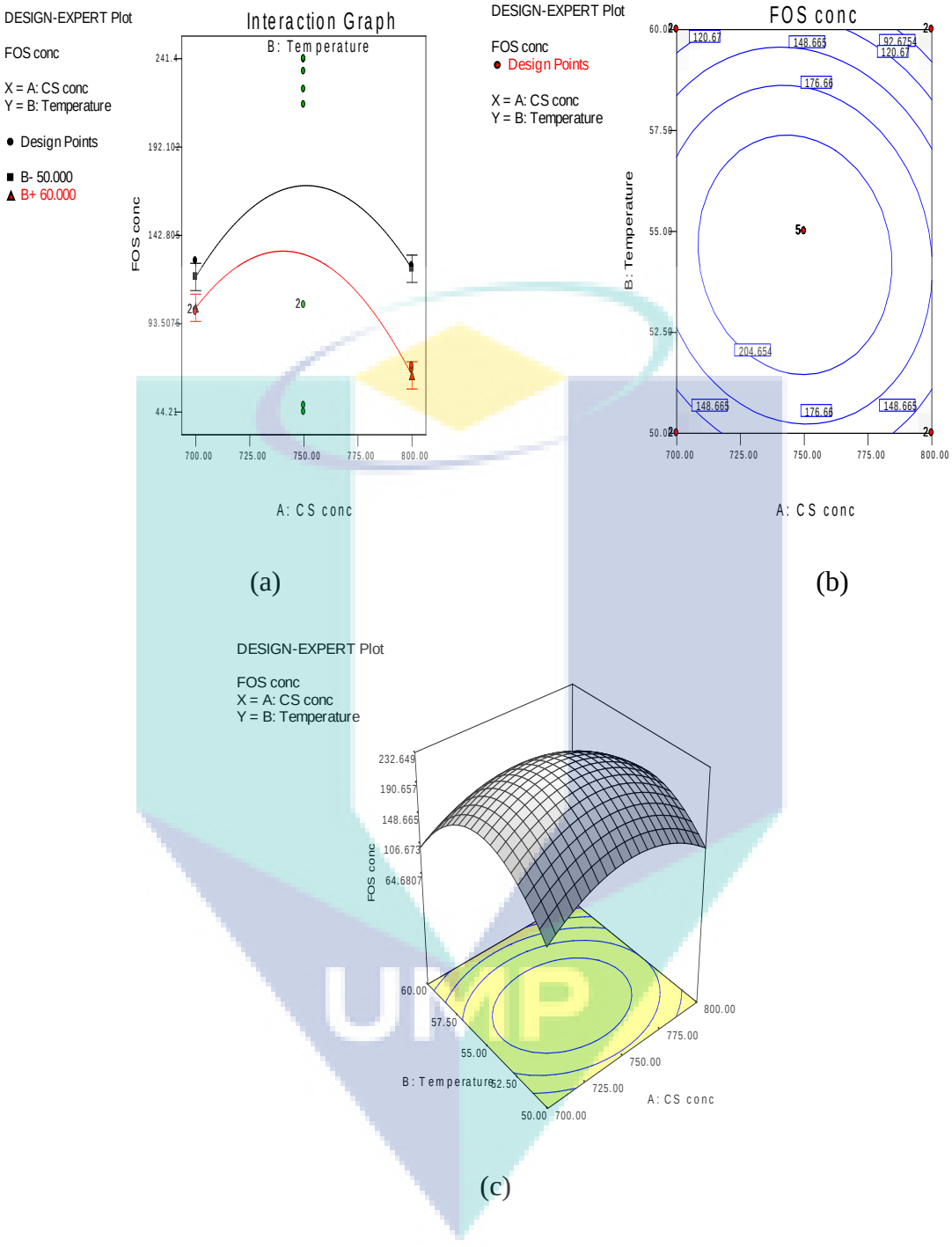


Figure 4.27: a) Interaction graph, (b) contour plot and (c) 3D surface of FOS production from the model equation: effect of coconut sugar concentration and temperature

4.5.2 Concluding Remarks of CCD Study

Table 4.8 depicted the summary of the optimization process that obtained regarding from the initial process of experimental work.

Table 4.8: Summary of reaction optimization using experimental design for FOS production from coconut sugar

	Before optimization	After optimization
Reaction parameter:		
Coconut sugar, g/L	700	750.73
FTase concentration, g/L	0.1	0.1
Temperature, ^{o}C	55	54.34
pH	5.5	5.5
Reaction time, hrs	5	5
Agitation speed	150	150
Response:		
FOS concentration, g/L	197.31	
a) Predicted		243.23
b) Actual		244.16
Conversion yield, %	28.14%	
a) Predicted		32.40
b) Actual		32.52

4.6 VALIDATION AND CONFIRMATION RUN OF EMPIRICAL MODEL ADEQUACY

Adequacy of the developed empirical model needs to be verified or validated in order to confirm the prediction accuracy, which is generated by the regression equation in predicting the FOS production at any particular coconut sugar concentration and temperature within the range of level defined previously. Experimental rechecking was performed using conditions that were previously used (Table 4.9). As suggested by the

software is only one condition for validation so it is directly selected as confirmation run of empirical model adequacy. The obtained actual values and its associated predicted values from the selected experiments were compared for further residual and percentage error analysis. The percentage error between actual and predicted value of both responses over a selected range of operating levels are calculated based on Eqs. (4.8) and (4.9). (Zularisam et al., 2009)

$$\text{Residual} = (\text{Actual value} - \text{Predicted value}) \tag{4.8}$$

$$\% \text{ Error} = \frac{\text{Residual}}{\text{Actual value}} \times 100\% \tag{4.9}$$

Results of Table 4.10 have shown that the percentage errors are ranging from 3.62% for FOS production respectively. Thus implied that the empirical model developed were considerably accurate for responding terms as the percentage error between the actual and predicted values were well within the value of 4%, suggesting that the model adequacy is reasonably within the 96% of prediction interval. By this means further analysis with regards to ideal operational process for optimal FOS production would be based on this developed model.

Table 4.9: The results of verification process

Run Factor		**FOS Concentration**			
Substrate Concentration	**Temperature**	**Actual**	**Predicted**	**Residual**	**Error**
746.33	54.43	241.09	232.37	8.72	3.62%
750.00	55.00	234.22	231.29	2.93	1.25%
680.00	55.00	240.90	231.29	9.61	3.99%

Table 4.10: The result of confirmation run

Run Factor		FOS Concentration			
Substrate Concentration	**Temperature**	**Actual**	**Predicted**	**Residual**	**Error**
746.33	54.43	241.09	232.37	8.72	3.62%

4.7 SCALE UP BY USING THE EMPIRICAL MODEL EQUATION

The implementation of the model equation has been further analyzed with the industrial-scale application, which is the reaction carried out using a self-fabricated batch reactor (10 L). To put it succinctly, the FOS production could be scaled up to the 10 L level following the conditions optimized at the bench-scale level. The results showed that FOS yields were maximum (30.13% w/w) after 5 hours of reaction with 753 g/L of the coconut sugar concentration at 54 ^{o}C, which is in agreement with the results obtained during optimization studies using RSM. Only 10% (v/v) FTase was required for the process. The minor deviation in the yields of the shake flask level (32.52%) and the scaled-up level (30.13%) can be attributed to the large volume-to-substrate ratio and to the mechanical agitation in the scaled-up process.

CHAPTER 5

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1 GENERAL CONCLUSIONS

This chapter presents a brief overview of the results and discussions, in order to summarize the work and to combine the detailed conclusion that has been presented previously in every chapter. The objective of this study is to produce the product interest that is Fructo-oligosaccharides (FOS). This expensive product is very important in human health which functions as prebiotics and alternative sweetener for diabetics. Mainly, this product has already been industrially scale manufactured by a few advanced countries like Japan and US of America. The production of FOS in this scale uses the conventional method which is two stages, that is the production of cultured enzyme (FTase) from microbes and the production of FOS from the reaction between the cultured enzyme and sucrose. In the current study, FOS has been produced with the introduction of coconut sugar as a new source of sucrose and the enzymatic reaction has been carried out for one stage only by using commercial enzyme substituting the cultured enzyme from microbes. In order to study the new invention, this research has two phases of framework. There are characterizations of materials and experimental work which delegates for three stages which are OFAT and screening process, optimization and finally validation of model equation and scale up.

The characterization had been done for all raw materials which were coconut sugar as the substrate, FTase and interest products FOS. All the materials had been characterized with FTIR for functional group determination. While there were another

five characterizations have been determined due to its sugar contents, moisture contents, viscosity and morphology for coconut sugar. Concluding from the characterization of coconut sugar beginning with total sugar contents in all the coconut sugar parts that was coconut sugar led to the highest sucrose contents (71%) followed by coconut milk (38%) and coconut water (9%). The sucrose contents in coconut sugar are very high which is 71% and followed by glucose (13%) and fructose (9%) content. While there is about 7.5% of water contents in coconut sugar that resulted high viscosity if the temperature increased otherwise. The best condition of the liquid movement that was indicated from the viscosity study that had been plotted by Arrhenius formula is 700 g/L and 55 ^{o}C of reaction substrate concentration and temperature respectively. Finally a few images could be found in the coconut sugar characterizations which were indicated for FTIR study and morphology study by microscope with camera. The enzyme activity determined after reducing 1 unit mol glucose after 1minute with the enzyme activity measured at 401 U/mL while the molecular weight of FTase is 145 KDa. FTase was stable at temperature ranged (50 ^{o}C to 60 ^{o}C). Below than 50 ^{o}C and more than 60 ^{o}C the enzyme was inactivated. While FTase was exhibited stable at pH ranged from (5.0 to 6.0). The image from FTIR study clearly showed that the highest peak was C-NH compound, followed by N-HNH compound and C-X compound. Finally, the characterization of FOS had been studied. As other sugar standard, FOS FTIR showed the highest peak for C-OH compound. There were no other strange compounds and it could be viewed by the lowest peak at C-X compound. After all the characterization parts had been done, the second stage of this study was the reaction process.

OFAT study concluded that reaction time in the significant range for FOS production was 4 to 6 hours. While for the concentration effects on the production of FOS is 500 g/L to 800 g/L and 0.01% to 0.12% of coconut sugar concentration and enzyme concentration respectively. While the significant range that had been observed was 50 ^{o}C to 70 ^{o}C and pH 4.0 to 6.5 of reaction temperature and pH respectively. While the significant limit for agitation speed was 150 rpm. In the screening process, concluded only two parameters (coconut sugar concentration and reaction temperature) were significant and another two (enzyme concentration and pH) were recommended as constant.

The empirical model from RSM developed was reasonably accurate for FOS concentration as all actual values for the confirmation runs were interval in the range in which, there was an expectation for any value to fall into 97% of the time. The maximum desirability value of 0.980 was achieved for FOS concentration was 243.23 g/L. The maximum yield of FOS finally obtained from the thorough study with two phases of framework which was the best range for the best parameter that was coconut sugar concentration with 750.73 g/L and reaction temperature with 55 ^{o}C producing over 30% yield of subsrate 243.23 g/L of FOS concentration.

As conclusion, this study has successfully produce FOS from a new approach of alternative sucrose which is coconut sugar with the reaction of commercial FTase with optimum conditions of 750.73 g/L of coconut sugar concentration and 10% g/L of FTase. The reaction had been carried out in the batch reactor at 55 ^{o}C, pH 5.5 acetate buffer and 150 rpm of reaction temperature, pH and agitation speed. The reaction mixture has been carried out for 5 hours to produce over 32.52% of FOS production yields. The production of FOS from coconut sugar was also capable to scale up to industrial level when the scale up study showed the positive response which was the maximum yield of FOS with the production yield of 30.13% of subsrate concentration.

5.2 RECOMMENDATION FOR FUTURE WORK

A number of recommendations are proposed to enhance the production of FOS from coconut sugar in order to increase the yield of the FOS concentration, fully understand and progress in a number of aspects of this work.

i) The production cost can be reduced by the utilization of batch continous reactor application to the future work as the FTase can be recycled and reused.

ii) Membrane technology (EMR) alternatively can be used in the batch continous reactor to filter the enzymes and the degradation of FTase can be studied for the recycling purposes.

iii) The production of FOS by commercial FTase has been applied in the batch reactor. Therefore, other types of FTase locally produced could be applied to the production of FOS from coconut sugar.

iv) Since coconut sugar used in this study was in liquid form, a few steps for the liquid preparation must be taken and if mistakes happened such as the existance of inhibitor while grinding the cubic coconut sugar and using the unsterilzed tools the reaction will be compromised and the results would not be accurate. It is highly recommended to make the reaction of FTase powder with the coconut sugar in cubes as well.

v) Immobolization of FTase can be implemented in the future ty in order to elucidate the study and achive higher yield of FOS production.

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APPENDIX A:

Experimental Flowchart, Correlation Chart, Standard Methods and Standard Curves

APPENDIX A1

OPERATIONAL FRAMEWORK

Characterization	**OFAT and Screening Process by using FFD**	**Optimization by using RSM**	**Validation of Empirical Model Adequacy and Scale Up**
- **Coconut sugar** Total Sugar contents -sucrose Determination of sucrose,fructose and glucose was detected by HPLC. Moisture content Determination of water contents in coconut sugar have been done with three steps: 1)weigh the coconut sugar that be minced (W1)	- **OFAT** The objective of running OFAT is to determine the significant parameters with the significant range. Six parameters have been selected which are -reaction time (1 to 10 hours) -coconut sugar concentration (300 g/L to 800 g/L) -enzyme concentration (0 g/L to 0.15 g/L) -temperature	The objective of optimization is to determine the optimum conditions and parameters for maximum production of FOS. The optimization has been done using Response Surface Method (RSM) In optimization only two factors will proceed which are i) coconut sugar concentration ii) temperature and the response will remain which is FOS production.	**-Validation of model equation** The objective of validation is to validate the equation that is to be obtained from the model design in RSM. The confirmation run has been obtained and the sample has been triplicated to get the average of FOS concentration. The maximum and minimum desirability as calculated

2)dry the coconut sugar in the oven with the heat at 100 ^{o}C 3)weigh the dried coconut sugar (W2) Then calculate W2/W1 x 100 Funtional group Determination of Functional group in coconut sugar can be directly analyzed by using FTIR Viscosity Determination of viscosity of coconut sugar solution can be analyzed by viscometer. **- Fructosyltransferase(FTase)** Quantitative (molecular weight) Determination of molecular	(20 ^{o}C to 80 ^{o}C) -pH (4.0 to 6.5) -agitation speed (50 rpm to 300 rpm) The samples of each experiment will be analyzed by using HPLC and all the experiments will be triplicate and the results of analysis will be calculated by average of each sample. The more specific range will be conducted on the screening process. **- Screening process** The objective of screening process is to determine the best range and the most significant	In RSM the interaction between the factors will be observed and the important part of this stage is the equation obtained from the data response through experimental works. The detail of the RSM will be discussed in the subtopic	by the software and more or less of the points will be residuals or errors of the validation equation. **-Scale up** The objective of scale up is to implement the limit and parameters into the industrial scale. The experiment has been done in the laboratory using 10 L self-fabricated enzymatic reactor. The reaction mixture that contains coconut sugar solution, FTase and buffer solution with total volume of 10 L will be filled in the reactor with the condition

weight of FTase is by using SDS Page methodology Qualitative (enzyme activity) Determination of enzyme activity is by using the reaction of reacted FTase with sucrose. The reaction mixture will be directly analyzed. Stability of FTase Determination of FTase stability due to its activity affected with temperature and pH. Functional group Determination of Functional group in coconut sugar can directly be analyzed by using Fourier Transform Infra Red	factors before it is implanted in optimization. The screening process has been done using Two Factorial Design (FFD) by Design Expert 6.0.8. These designs will help to screen many factors to discover the vital range and how they interact. four parameters were selected in this process and there are i)coconut sugar concentration ii)enzyme concentration iii)temperature iv)pH The design of the process is according to the response which is FOS production. The simulation of the filtration process will generate the response data.		that has already been optimized from the OFAT, FFD and RSM.

(FTIR). **-Fructooligosaccahrides (FOS)** Funtional group Determination of Functional group in coconut sugar can be directly analyzed by using Fourier Transform Infra Red (FTIR).	(The details of the utilization of Design expert software for FFD will be detailed in subtopic		

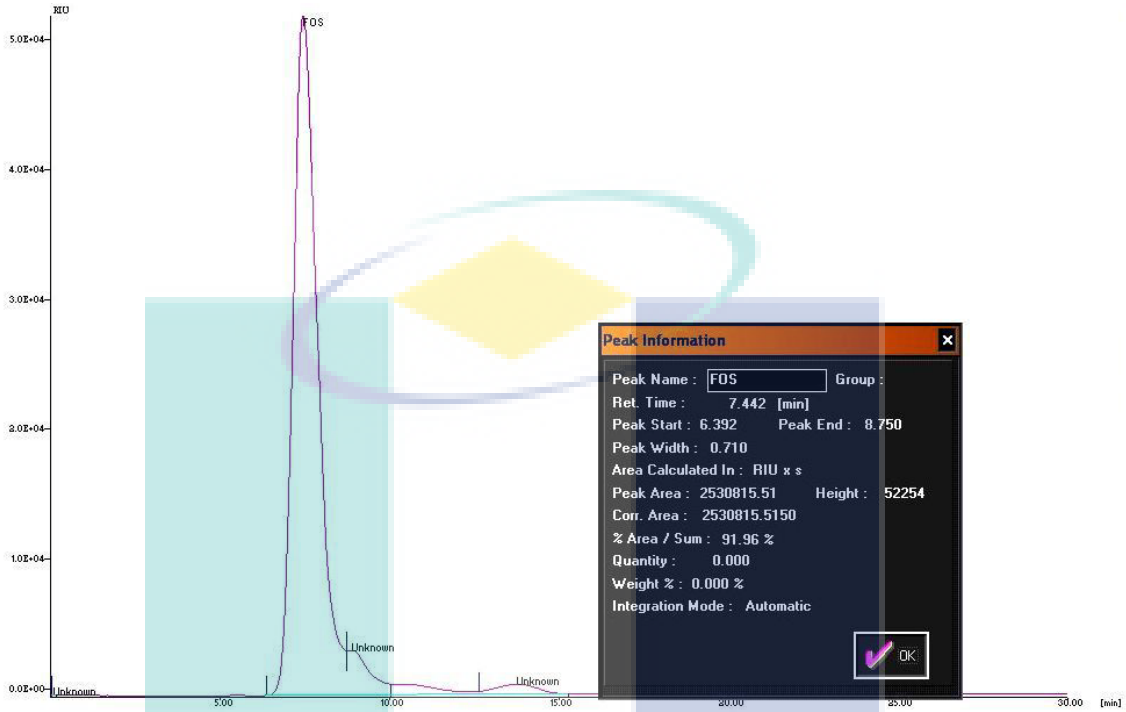
APPENDIX A2

FTIR CORRELATION CHART

Known Compounds	Groups	Wave number (cm-1)	Possible functional group	Range
Starch, glucose, fructose, sucrose, maltose, maltotriose, fructo-oligosaccharide	Polysaccharides	3400	Alcohol (1,2,3,Ar)	3100-3500
		2940	Alkane	2800-3000
		1480	Alkane	1420-1480
		1370	Alkane	1340-1400
		1170	Tertiary alcohol	1120-1220
		1120	Secondary alcohol	1050-1150
		1040	Aliphatic ether	1040-1170
		1000	Primary alcohol	1000-1080
		775	Ethyl	750-800
FTase	Proteins	3300	Alcohol (1,2,3,Ar)	3100-3500
			Amides	3200-3500
			Carboxylic acid	2900-3300
			1,2, or 3 amines	3200-3500
		1640	Alkene in aromatic	1630-1670
			Amides (mono and di substituted)	1640-1720
		1540	Mono substituted amide	1480-1580
		1100	Sec,tert amines	1480-1581
			Ether	1040-1170
			Esters	1080-1150
			Aldehydes	1020-1150
			Ketones	1070-1220

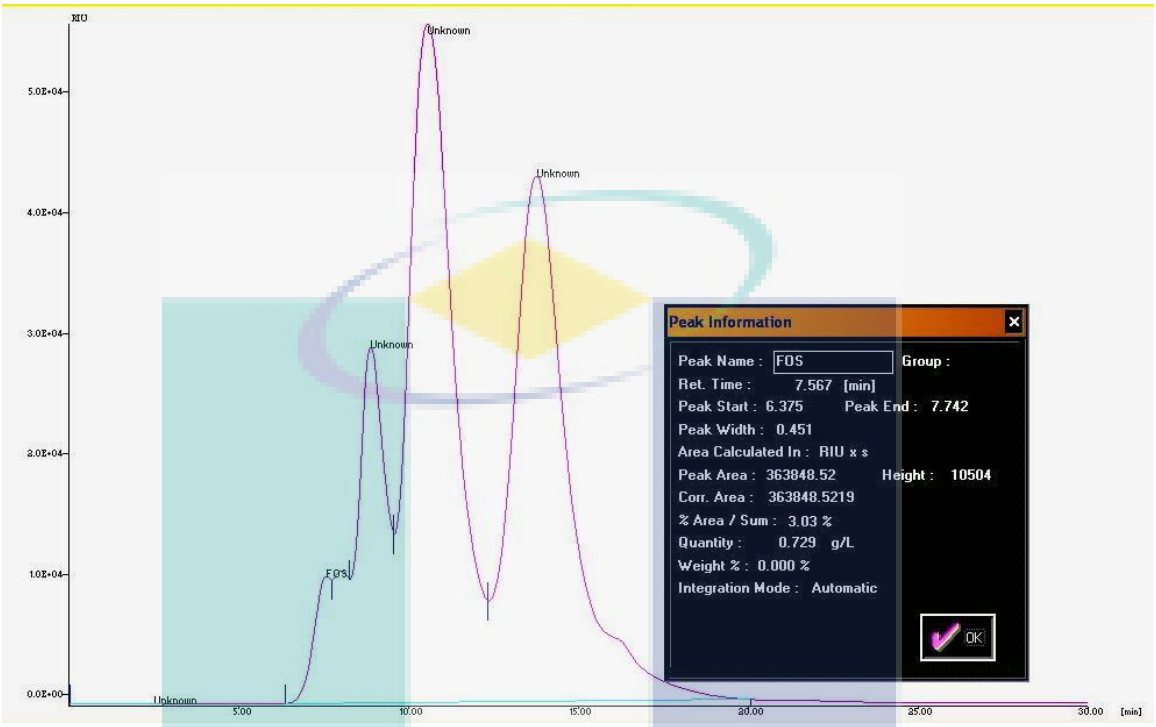
APPENDIX A3

STANDARD OF FOS (HPLC ANALYSIS)



APPENDIX A4

EXAMPLE OF HPLC RESULT ON FFD EXPERIMENTAL WORK

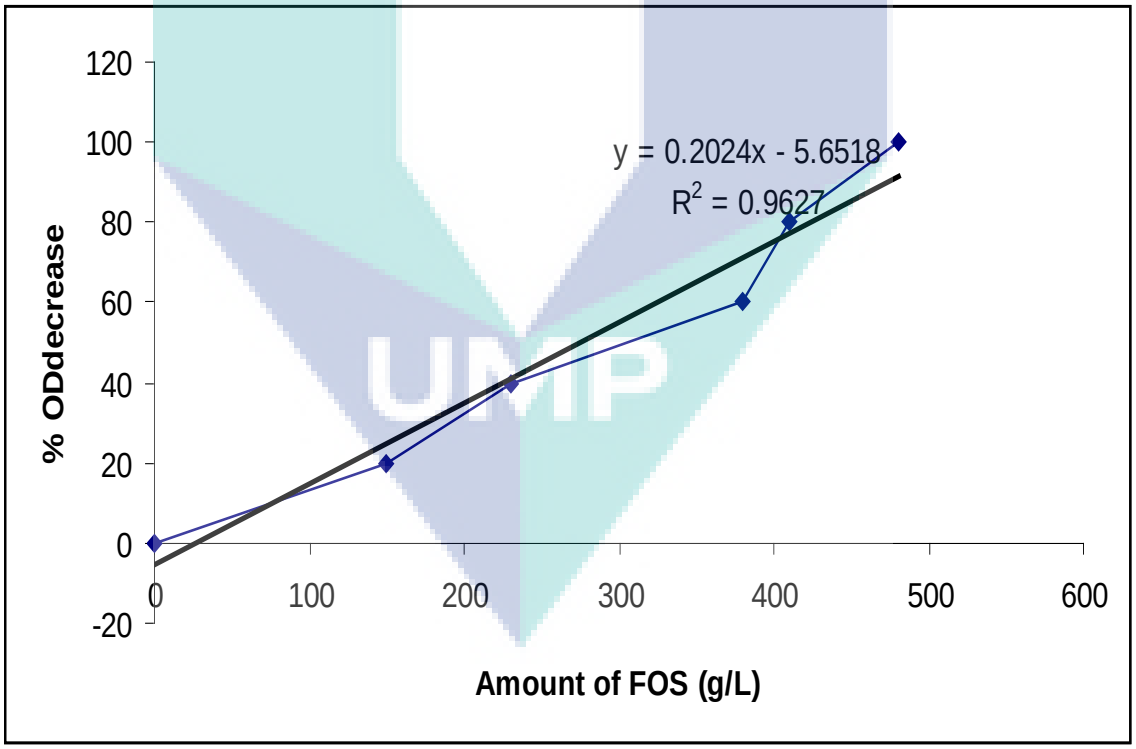


APPENDIX A5

CALCULATION OF FTASE ACTIVITY BY PHENOLPHTHALEIN METHOD

$$A = \frac{\%OD_{decrease} \times Y_p \times D_f \times 10^3}{MW \times t_i}$$

A	=	Enzyme activity (U/ml or µmol/ml) of unknown sample
% $OD_{decrease}$	=	$\frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100\%$
Y_p	=	mg of FOS equivalent to 100% $OD_{decrease}$ of the standard curve
D_f	=	dilution factor
t_i	=	incubation time
MW	=	molecular weight of FOS, 180.1559 g/mol



APPENDIX A6
WORKING SOLUTIONS FOR SDS-PAGE

1) Solution A (acrylamide stock solution), 100 ml
   30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide

2) Solution B (4x resolving gel buffer), 100 ml
   2 M Tris-HCl (pH 8.8), 75 ml
   Distilled water, 21 ml
   10% (w/v) SDS, 4 ml

3) Solution C (4x stacking gel buffer), 100 ml
   1 M Tris-HCl (pH 6.8), 50 ml
   Distilled water, 46 ml
   10% (w/v) SDS, 4 ml

4) Ammonium persulfate (10% w/v), 5 ml
   Distilled water, 5 ml
   0.5 g ammonium persulfate

5) Electrophoresis buffer, 1 L
   14.4 g glycine
   3 g Tris
   1 g SDS
   Use distilled water to make 1 L solution

6) 15% resolving gel preparation
   5 ml Solution A
   2.5 ml Solution B
   2.5 ml Distilled water
   50 µl 10% (w/v) ammonium persulfate
   10 µl TEMED

7) 5% stacking gel preparation

   0.67 ml Solution A

   1.0 ml Solution C

   2.3 ml distilled water

   30 µl 10% (w/v) ammonium persulfate

   10 µl TEMED

8) Staining solution, 1 L

   450 ml methanol

   450 ml distilled water

   100 ml glacial acetic acid

   1.0 g Coomassie Blue R-250

9) Destaining solution, 1 L

   800 ml distilled water

   100 ml methanol

   100 ml glacial acetic acid

APPENDIX A7

PREPARATION OF BUFFER SOLUTION

1) Sodium acetate buffer, 0.1 M

   Solution A: 11.55 ml glacial acetic acid per liter (0.2 M)

   Solution B: 16.4 g sodium acetate per liter (0.2 M)

   Referring to Table A-1 for desired pH, mix the indicated volumes of solution A and B, then diluted with distilled water to a total volume of 100 ml.

Table A-1: Preparation of 0.1 M Sodium Acetate Buffer

Desired pH	Solution A (ml)	Solution B (ml)
4.0	41.0	9.0
5.0	14.8	35.2

2) Potassium phosphate buffer, 0.1 M

   Solution A: 27.2 g KH_2PO_4 per liter (0.2 M)

   Solution B: 45.6 g K_2HPO_4 per liter (0.2 M)

   Referring to Table A-2 for desired pH, mix the indicated volumes of solution A and B, then diluted with distilled water to a total volume of 200 ml.

Table A-2: Preparation of 0.1 M Potassium Phosphate Buffer

Desired pH	Solution A (ml)	Solution B (ml)
5.0	87.7	12.3
6.0	39.0	61.0
7.0	5.3	94.7

3) Glycine-NaOH buffer, 0.1 M

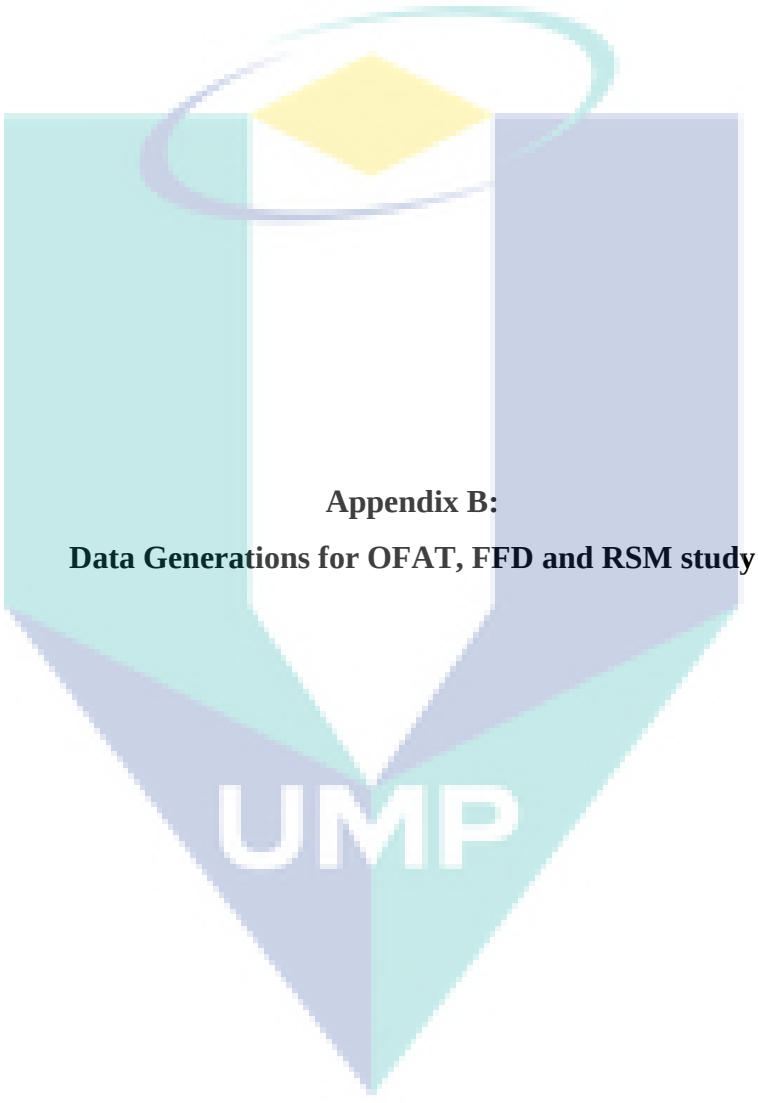
   Solution A: 15.01 g glycine per liter (0.2 M)

   Solution B: NaOH (0.2 M)

   Referring to Table A-3 for desired pH, mix the indicated volumes of solution A and B, then diluted with distilled water to a total volume of 200 ml.

Table A-3: Preparation of 0.1 M Glycine-NaOH Buffer

Desired pH	Solution A (ml)	Solution B (ml)
9.0	50.0	8.8
10.0	50.0	32.0



Appendix B:

Data Generations for OFAT, FFD and RSM study

DATA GENERATION (OFAT EXPERIMENT'S RESULTS)

Temperature Changes (^oC)	FOS Concentration			
	R1	R1	R2	Total
20	4.10	4.40	4.20	4.23
30	32.98	33.73	35.11	33.94
40	148.42	144.32	160.32	151.02
50	193.21	194.77	191.65	1913.21
60	178.92	179.45	180.13	179.50
70	92.21	91.62	90.37	91.64
80	43.76	40.98	38.86	41.20

pH Changes (pH)	FOS Concentration			
	R1	R1	R2	Total
4	56.37	54.91	56.45	55.91
4.5	95.12	97.24	96.18	96.18
5	130.16	141.77	121.55	131.16
5.5.	194.28	190.53	187.86	190.89
6	142.94	143.56	144.03	143.51
6.5	61.05	61.89	60.12	61.02

Coconut sugar concentration (g/L)	FOS Concentration			
	R1	R1	R2	Total
300	55.74	52.91	50.95	53.20
400	74.40	77.36	70.84	74.20
500	127.51	125.88	123.41	125.61
600	184.37	180.14	183.53	183.24
700	197.92	196.43	197.58	197.31
800	198.45	199.14	197.61	198.40

Enzyme concentration (g/L)	FOS Concentration			
	R1	R1	R2	Total
0	84.77	86.23	81.90	84.30
0.01	141.06	133.29	132.15	135.50
0.03	141.06	133.29	132.15	135.50
0.09	194.21	197.38	194.04	195.21
0.12	197.22	199.06	195.98	197.42
0.15	195.64	194.38	193.06	194.36

Reaction Time (h)	FOS Concentration			
	R1	R1	R2	Total
1	91.14	96.70	90.65	92.83
2	114.21	110.16	133.02	119.13
3	168.43	172.85	139.20	160.16
4	170.46	170.99	172.24	171.23
5	184.32	192.41	194.50	190.41
6	185.91	185.77	186.29	185.99
7	161.81	165.21	156.4	161.14
8	100.01	101.25	102.94	101.40
9	95.94	93.28	90.41	93.21
10	89.04	90.96	93.30	91.11

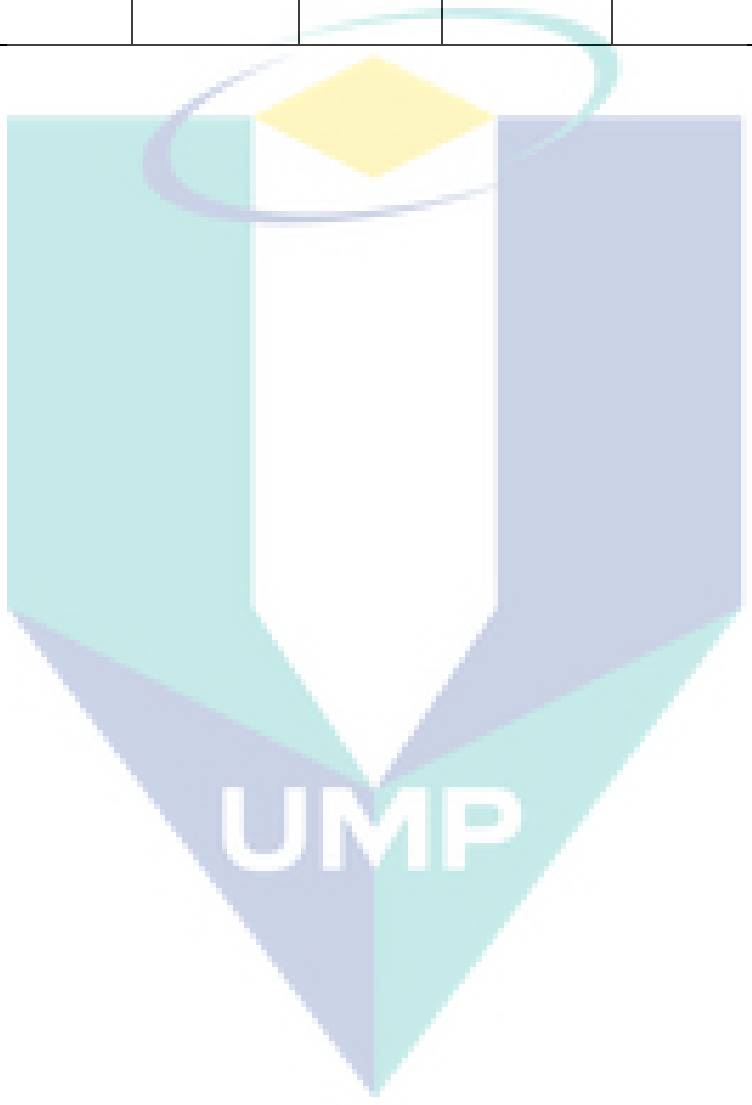
Agitation Speed	FOS Concentration			
	R1	R1	R2	Total
50	164.42	163.54	162.54	163.50
100	171.94	170.41	170.60	171.00
150	188.40	191.36	185.445	188.4
200	161.84	159.53	160.98	160.45
250	158.23	157.77	155.96	157.32
300	150.91	151.16	151.38	151.15

APPENDIX B2

DATA GENERATION FOR SCREENING PROCESS

Std	CS Conc (g/L)	Enzyme Conc (g/L)	Temp (^{o}C)	pH (pH)	FOS concentration (g/L)
1	500.00	0.08	50.00	5.00	45.30
2	500.00	0.08	50.00	5.00	35.30
3	800.00	0.08	50.00	5.00	77.00
4	800.00	0.08	50.00	5.00	67.30
5	500.00	0.12	50.00	5.00	37.60
6	500.00	0.12	50.00	5.00	56.90
7	800.00	0.12	50.00	5.00	70.20
8	800.00	0.12	50.00	5.00	64.30
9	500.00	0.08	70.00	5.00	58.50
10	500.00	0.08	70.00	5.00	56.75
11	800.00	0.08	70.00	5.00	56.4
12	800.00	0.08	70.00	5.00	77.67
13	500.00	0.12	70.00	5.00	118.43
14	500.00	0.12	70.00	5.00	41.82
15	800.00	0.12	70.00	5.00	60.64
16	800.00	0.12	70.00	5.00	45.43
17	500.00	0.08	50.00	6.00	131.74
18	500.00	0.08	50.00	6.00	71.56
19	800.00	0.08	50.00	6.00	161.13
20	800.00	0.08	50.00	6.00	87.35
21	500.00	0.12	50.00	6.00	57.30
22	500.00	0.12	50.00	6.00	67.71
23	800.00	0.12	50.00	6.00	118.43
24	800.00	0.12	50.00	6.00	182.68
25	500.00	0.08	70.00	6.00	21.18
26	500.00	0.08	70.00	6.00	22.60

27	800.00	0.08	70.00	6.00	25.17
28	800.00	0.08	70.00	6.00	32.52
29	500.00	0.12	70.00	6.00	12.51
30	500.00	0.12	70.00	6.00	21.43
31	800.00	0.12	70.00	6.00	33.04
32	800.00	0.12	70.00	6.00	58.12



APPENDIX B3

DATA GENERATION FOR OPTIMIZATION EXPERIMENTS RESULTS (RSM)

Std	CS Conc (g/L)	Temp (^{o}C)	FOS concentration (g/L)
1	700.00	50.00	128.54
2	700.00	50.00	100.99
3	800.00	50.00	125.21
4	800.00	50.00	125.79
5	700.00	60.00	100.99
6	700.00	60.00	99.99
7	800.00	60.00	67.95
8	800.00	60.00	69.91
9	680.00	55.00	154.21
10	680.00	55.00	150.23
11	820.00	55.00	128.36
12	820.00	55.00	112.11
13	750.00	48.00	103.93
14	750.00	48.00	103.93
15	750.00	62.00	47.81
16	750.00	62.00	44.21
17	750.00	55.00	224.22
18	750.00	55.00	234.22
19	750.00	55.00	215.68
20	750.00	55.00	240.90
21	750.00	55.00	241.4

APPENDIX C:

List of Publications

Journal/Paper

Noormazlinah, A. and Sakinah, A.M.M. 2010. Optimization of operating conditions using experimental design on synthesizing of Fructo-oligosaccharides from coconut sugar. *Journal of Bioresource Technology*.

Noormazlinah, A. and Sakinah, A.M.M. 2010. Effect of reaction parameters on synthesizing Fructooligosaccharides from coconut sugar. *Journal of Food Engineering.*

Noormazlinah, A. and Sakinah, A.M.M. 2010. Effect of reaction temperature and pH on Fructooligosaccharides production from natural coconut sugar. *Journal of Applied Science.*

Proceeding/Conference

Noormazlinah, A. and Sakinah, A.M.M. 2009. Effect of coconut sugar concentration on the Fructooligosaccharides from natural coconut. 8th UMT International Symposium on Sustainability Science and Management. May, 2-4. Hotel Primula Terengganu. Malaysia.

Noormazlinah, A. and Sakinah, A.M.M. 2009. Effect of reaction temperature and pH on Fructooligosaccharides production from natural coconut sugar. 23rd International Symposium Malaysian Chemical Engineers. August, 12-14. Hotel One Borneo Sabah, Malaysia.

Noormazlinah, A. and Sakinah, A.M.M. 2009. Maximization of Fructooligosaccharide production by using Response Surface Method (RSM). International Conference and Exhibition of Women Engineer. December, 26-28. Full paper accepted.

Exhibition

Gold Award for IENA, (Exhibition in Germany).