Optimization of *Aspergillus fijiensis* β-fructofuranosidase expression and production using *Pichia pastoris*, for the production of fructooligosaccharides from sucrose

by

Gerhardt Coetzee

Dissertation presented for the Degree

of

DOCTOR OF PHILOSOPHY
(CHEMICAL ENGINEERING)

in the Faculty of Engineering
at Stellenbosch University

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Supervisor
Prof Johann F. Görgens

April 2019

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes 3 unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Date: April 2019

Plagiarism Declaration

1. Plagiarism is the use of ideas, material and other intellectual property of another's work and to present it as my own.

2. I agree that plagiarism is a punishable offence because it constitutes theft.

3. I also understand that direct translations are plagiarism.

4. Accordingly, all quotations and contributions from any source whatsoever (including the internet) have been cited fully. I understand that the reproduction of text without quotation marks (even when the source is cited) is plagiarism.

5. I declare that the work contained in this assignment, except where otherwise stated, is my original work and that I have not previously (in its entirety or in part) submitted it for grading in this module/assignment or another module/assignment.

Initials and surname: G. Coetzee

Date: April 2019

Abstract

The South African sugar industry faces challenges affecting its profitability such as low international market price and the implementation of tax legislation by the South African National Treasury on sugar-sweetened beverages (SSBs). To alleviate these effects, the industry could produce alternative high-value, low-calorie products from sucrose such as short-chain fructooligosaccharides (scFOS). This product diversification may increase the industry's revenue while addressing the sugar tax legislation.

In this study, the β-fructofuranosidase from *Aspergillus fijiensis* ATCC 20611 was selected to produce scFOS from sucrose. Native (fopA) and protein-engineered (fopA_V1) versions of the enzymes were produced recombinantly in *Pichia pastoris*. Factors influencing heterologous protein production require empiric evaluation for each protein and thus the aim was to optimize the yeast expression system and cultivation processes to maximize β-fructofuranosidase production. On the genetic level, different yeast strains, promoters and gene codon-optimization techniques were compared. Dissolved oxygen controlled (DO-stat) and constant feeding strategies were compared in bioreactor cultivations to investigate the influence of yeast growth on volumetric enzyme titers. The application of the two enzymes to produce scFOS from industrial sugar streams were optimized using response surface methodology (RSM).

In shake flask experiments the *P. pastoris* DSMZ 70382 strain proved superior to X-33 when expressing Geneart® codon-optimized fopA under control of the *AOX1* and *GAP* promoters (12.1 U/ml and 3.2 U/ml for *AOX1* and 12.0 U/ml and 11.3 U/ml for *GAP*, respectively). Further bioreactor studies with *P. pastoris* DSMZ 70382 native fopA transformants showed that the *AOX1* promoter was superior to *GAP* while ATUM codon-optimization produced higher titers than Geneart® (13 702 U/ml and 2 718 U/ml for *AOX1* and 6 057 U/ml and 1 790 U/ml for *GAP*, respectively).

Constant feed cultivations produced higher growth rates for strains expressing the ATUM genes under the *GAP* promoter, but lower volumetric enzyme activities compared to DO-stat cultivations (2 129.25 and 1686.91 U/ml for GAPfopA and GAPfopA_V1, respectively, with DO-stat and 1413.36 and 1222.70 U/ml for GAPfopA and GAPfopA_V1, respectively, with constant feed). The GAPfopA strain produced higher enzyme activities than the GAPfopA_V1 for the constant feed and DO-stat method. Due to the shorter cultivation time, the constant feed method exhibited higher volumetric productivity for both strains (23.96 x 10^3 and 20.72 x 10^3 U/L/h for GAPfopA and GAPfopA_V1, respectively).

In scFOS production, the native and engineered enzymes were compared to evaluate whether the protein engineering afforded advantages in enzyme performance on non-ideal substrates (industrial sugar). RSM indicated optimum conditions to produce a target scFOS composition were 62 ^{0}C and 10 U/g sucrose using pure sugar. These conditions were applied to A-molasses and refinery molasses, however the desired scFOS composition was only successfully attained using A-molasses.

In conclusion, *P. pastoris* proved to be a suitable host for the high-level expression and production of functional codon-optimized native (GAPfopA) and engineered (GAPfopA_V1) versions of the fopA enzyme and that these enzymes can be applied for the batch production of scFOS from a selection of industrial sugar streams for the purpose of reducing process cost.

Opsomming

Die Suid-Afrikaanse suikerindustrie staar uitdagings in die gesig wat winsgewendheid affekteer, soos lae internasionale markprys en die implementasie van belastingwetgewing deur die Suid-Afrikaanse Nasionale Tesourie op suikerversoete drankies (SSBs). Om hierdie gevolge te verlig, kan die industrie alternatiewe hoë-waarde, lae-kalorie produkte vervaardig uit sukrose soos kort-ketting frukto-oligosakkariede (scFOS). Hierdie produkdiversifikasie kan die industrie se inkomste verhoog terwyl die suikerbelastingwetgewing aangespreek word.

In hierdie studie is die β-fruktofuranosidase van *Aspergillus fijiensis* ATCC 20611 gekies om scFOS uit sukrose te vervaardig. Natuurlike (fopA) en proteïen-gemanipuleerde (fopA_V1) weergawes van die ensiemes is rekombinant vervaardig in *Pichia pastoris*. Faktore wat die heteroloë proteïen vervaardiging beïnvloed, vereis empiriese evaluasie vir elke proteïen. Die doel was dus om die gis uitdrukkingsisteem en kultiveringsprosesse te optimeer om β-fruktofuranosidase te maksimeer. Op die genetiese vlak, is verskillende gislyne, promotors en geenkodonoptimeringstegnieke vergelyk. Opgeloste suurstof beheerde (DO-stat) strategië en konstante voerstrategië is vergelyk in bioreaktor kultiverings om die invloed van gisgroei op volumetriese ensieme titers te ondersoek. Die toepassing van die twee ensiemes om scFOS van industriële suikerstrome te vervaardig, is geoptimeer deur respons oppervlak metodologie (RSM) te gebruik.

In skudfles eksperimente was die *P. pastoris* DSMZ 70382-lyn superieur bo die X-33 as GeneArt® kodon-geoptimeerde fopA onder beheer van die *AOXI*- en *GAP*- promotors (12.1 U/ml en 3.2 U/ml vir *AOXI* en 12.0 U/ml en 11.3 U/ml vir *GAP*, onderskeidelik) uitgedruk is. Verdere bioreaktorstudies met *P. pastoris* DSMZ 70382 natuurlike fopA tranformante het gewys dat die *AOXI*-promotor superieur was bo *GAP* terwyl ATUM-

kodonoptimering hoër titers as GeneArt® gelewer het (13 702 U/ml en 2 718 U/ml vir *AOX1* en 6 057 U/ml en 1 790 U/ml vir *GAP*, onderskeidelik).

Konstante voerkwekings het hoër groeitempo's gegenereer vir lyne wat die ATUM-gene onder die *GAP*-promotor uitgedruk het, maar laer volumetriese ensiemaktiwiteite in vergelyking met DO-stat-kwekings (2 129.25 U/ml en 1686.91 U/ml vir GAPfopA en GAPfopA_V1, onderskeidelik, met DO-stat en 1413.36 en 1222.70 U/ml vir GAPfopA en GAPfopA_V1, onderskeidelik, met konstante voer). Die GAPfopA-lyn het hoër ensiemaktiwiteite gegenereer as die GAPfopA_V1 vir die konstante voer en DO-stat metode. As gevolg van die korter kwekingstyd, het die konstante voer metode hoër volumetriese produktiwiteit vir beide lyne vertoon (23.96 x 10^3 en 20.72 x 10^3 U/L/h vir GAPfopA en GAPfopA_V1, onderskeidelik).

In scFOS vervaardiging, is die natuurlike en gemanipuleerde ensiemes vergelyk om te evalueer of die proteïen-manipulering voordele in ensiemdoeltreffendheid op nie-ideale substrate (industriële suiker) oplewer. RSM het aangedui dat optimum kondisies om 'n doelwit scFOS-samestelling te vervaardig is 62 °C en 10 U/g sukrose wat suiwer suiker gebruik. Hierdie kondisies is toegepas op A-molasse en geraffineerde molasse, alhoewel die gewenste scFOS-samestelling slegs suksesvol verkry is deur A-molasse te gebruik.

Ter afsluiting, *P. pastoris* is bewys as 'n gepaste draer vir die hoë-vlak uitdrukking en produksie van funksionele kodon-geoptimeerde natuurlike (GAPfopA) en gemanipuleerde (GAPfopA-V1) weergawes van die fopA-ensiem en dat hierdie ensiemes toegepas kan word op die lotproduksie van scFOS van 'n deel van industriële suikerstrome met die doel om proseskostes te verminder.

Dedication

This thesis is dedicated to my family and friends for their continual interest and support throughout my studies.

Acknowledgements

Firstly, I would like to thank my supervisor Prof. Johann Görgens for providing me with the opportunity to work on this project and for his professional, technical and intellectual input that made this degree possible.

I would like to thank Dr. Eugéne van Rensburg for his input and advice, both professional and personal, and his encouragement throughout this endeavour.

I would also like to thank Dr. Heinrich Volschenk for allowing me to occupy a bench in his lab for such an extended period. I moved in and just didn't leave. I would also like to thank the other members of the Volschenk lab for their friendship and help, especially Dr. Kim Trollope, Dr. Riaan de Witt and Dr. María García Aparicio.

I would like to thank the National Research Foundation, Technology Innovation Agency and CSIR for their financial support that made this project possible.

Furthermore, I would like to thank everyone at the Department of Process Engineering who contributed to me reaching this goal. Thank you to the members of the Bioresource Group for sharing the experiences of being a postgraduate student, the frustrations and the successes.

Finally, but not least, I would like to thank all the technical staff that made sure that things ran smoothly or was fixed quickly when needed. Thank you to Mr. Arrie Arendse at the Department of Biochemistry for helping when equipment was misbehaving and a special thanks to Mr. Jos Weerdenburg and staff in the workshop at the Department of Process Engineering for helping to fix things when they broke and making them work better when not.

Table of contents

Declaration i

Plagiarism Declaration ii

Abstract iii

Opsomming v

Dedication vii

Acknowledgements viii

Table of contents ix

List of figures xiv

List of tables xvii

List of acronyms and abbreviations xix

Chapter 1 **Introduction** 1

1.1 Contextual background 1

1.2 Thesis outline 3

1.3 References 4

Chapter 2 **Literature review** 7

2.1 Fructooligosaccharides 7

2.1.1 Natural occurrence and structure 7

2.1.2 Health benefits of FOS 8

2.1.3 FOS production methods 8

2.1.4 Practical maxima for sucrose conversion to FOS 12

2.1.5 FOS production with recombinant enzymes 13

2.1.6 FOS market 14

2.2 *Pichia pastoris* for recombinant protein expression 15

2.2.1 Promoters 18

2.2.2 Codon optimisation 21

2.2.3 Fermentation media and operational conditions 22

2.3 Bioreactor fermentations 24

2.3.1 Batch and Fed-batch fermentations in a CSTR 26

2.4 Conclusion 29

2.4 References 31

Chapter 3 **Research objectives** 45

3.1 Objective one (Chapter 4): To produce a yeast expression system for the optimum production of β-fructofuranosidase 45

3.2 Objective two (Chapter 5): Optimize the production of the β-fructofuranosidase enzymes by using different fed-batch methods 46

3.3 Objective three (Chapter 6): Evaluate the difference between the native and an engineered β-fructofuranosidase enzyme to produce a specific scFOS composition 47

3.4 References 49

Chapter 4 **Heterologous expression of the codon-optimized *Aspergillus fijiensis* β-fructofuranosidase in *Pichia pastoris*** 51

Objective of dissertation in this chapter 51

Abstract 54

4.1 Introduction 55

4.2 Materials and Methods....56
4.2.1 Strains and media....56
4.2.2 Construction of expression cassettes....57
4.2.3 Yeast transformation....59
4.2.4 Protein production in shake flasks....59
4.2.5 Bioreactor cultivations....60
4.2.6 Enzyme activity assay....61
4.2.7 Quantifying specific enzyme activity....61
4.3 Results....62
4.3.1 Identifying the preferred host strain....62
4.3.2 Enzyme production in bioreactors....63
4.4 Discussion....66
4.5 References....70

***Chapter 5* Process development using different glycerol fed-batch methods for the production of a novel engineered β-fructofuranosidase enzyme in *Pichia pastoris*....75**

Objective of dissertation in this chapter....75
Abstract....78
5.1 Introduction....80
5.2 Materials and Methods....82
5.2.1 Microbial strains, media and plasmids....82
5.2.2 DNA cloning and yeast transformation....83
5.2.3 Transformant screening....83
5.2.4 Bioreactor cultivations....84
5.2.5 Sample analysis and calculations....86
5.2.6 Enzyme activity assay....87

5.2.7 Glycerol concentration 87

5.2.8 SDS-PAGE analysis 88

5.3 Results 88

5.3.1 Screening of *P. pastoris* transformants 88

5.3.2 Bioreactor cultivations 89

5.4 Discussion 92

5.5 References 99

***Chapter 6* Evaluation of the performance of an engineered β-fructofuranosidase from *Aspergillus fijiensis* to produce short-chain fructooligosaccharides from industrial sugar streams** 105

Objective of dissertation in this chapter 105

Abstract 108

6.1 Introduction 109

6.2 Methods 112

6.2.1 Materials 112

6.2.2 Bioreactor cultivations 112

6.2.3 Enzyme activity assay 114

6.2.4 Sugar analyses 114

6.2.5 Optimisation and validation of scFOS production 114

6.2.6 Central composite design 115

6.2.7 scFOS production from industrial substrates 115

6.2.8 Scale-up production of scFOS 117

6.3 Results and discussion 117

6.3.1 Characterization of processing parameters for scFOS production 117

6.3.2 Desirability analysis 124

6.3.3 Model validation 125

6.3.4 scFOS production using industrial substrates under optimised conditions 127

6.3.5 Scale-up production of scFOS 129

6.4 Conclusions 130

6.5 References 132

***Chapter 7* Conclusions and recommendations** 138

7.1 Conclusions 138

7.2 Recommendations 140

Appendices 141

Appendix A - Strain construction 141

Appendix B - Bioreactor cultivations 146

Appendix C – Mathematical models 148

List of figures

Figure 2-1. Structures of kestose (A), nystose (B) and fructofuranosyl nystose (C).....9

Figure 2-2. Two stage fructooligosaccharide (FOS) production process by submerged fermentation (SmF) (Adapted from Sangeetha et al., 2005).....11

Figure 2-3. Stirred-tank reactor (1) Inoculum connection (2) Ports for sensors (3) Motor (4) Sample valve (6) Sight glass (7) Air exhaust nozzle (8) Condenser (9) Pumps for acid, base and substrate addition (10) Off-gas analysers.....25

Figure 4-1. Comparison of the volumetric enzyme activity of *P. pastoris* X-33 (PX33) and *P. pastoris* DSMZ 70382 (P70382) containing the codon-optimized fopA gene (Geneart®) under transcriptional control of the alcohol oxidase (AG) and glyceraldehyde 3-phosphate dehydrogenase (GG) promoter, respectively. Cultivations were performed in shake flasks at 30 °C for 72 h at an agitation rate of 200 rpm. 63

Figure 4-2. Volumetric enzyme activity of positive *P. pastoris* transformants with the codon-optimized fopA gene from ATUM (grey bars) under control of the glyceraldehyde 3-phosphate dehydrogenase (*GAP*) promoter (A) and under control of the alcohol oxidase (*AOX1*) promoter (B) relative to the control containing the codon-optimized fopA gene from Geneart® (white bars) cultivated in shake flasks for 72 hrs at 30 °C at an agitation rate of 200 rpm. 64

Figure 4-3. Volumetric enzyme activity of *P. pastoris* AOX1fopA (Geneart®; P70382-AG), *P. pastoris* GAPfopA (Geneart®; P70382-GG), *P. pastoris* AOX1fopA (ATUM; P70382-AD2) and *P. pastoris* GAPfopA (ATUM; P70382-GD2) cultivated in 1.3 L fermenters in triplicate. Enzyme activity was determined at the point of maximum biomass. 65

Figure 5-1. Volumetric enzyme activity of *P. pastoris* transformants containing the *GAPfopA_V1* gene relative to the control with the *GAPfopA* gene in shake flasks. Cultivations were performed at 30 °C over 72 h at an agitation rate of 200 rpm. Error bars denote the standard error of triplicate experiments. 89

Figure 5-2. SDS-PAGE of the crude supernatant of the cultivations of two different feeding strategies: (A) constant feed with odd-numbered lanes representing GAPfopA_V1 and even-numbered lanes GAPfopA. Lanes 1 and 2 (33 h), lanes 3 and 4 (59 h) and lanes 5 and 6 (67 h). (B) DO-stat feed with odd-numbered lanes representing GAPfopA and even-numbered lanes GAPfopA_V1. Lanes 1 and 2 (107 h), lanes 3 and 4 (131 h) and lanes 5 and 6 (155 h). Lane M represents the molecular weight marker. 91

Figure 5-3. Dissolved oxygen (DO) level (blue) and O2 sparged (green) during the DO-stat and constant feed fermentations. The red line indicates the setpoint value at which the DO is controlled. 93

Figure 6-1. Response surface and contour plots at an 8 h reaction time for FOS percentage as a function of enzyme dosage and temperature for (A), (C) and (E) as percentage GF2, GF3 and GF4, respectively, produced by GAPfopA and (B), (D) and (F) produced by GAPfopA_V1. 122

Figure 6-2. Graph of desirability analyses performed to determine the minimum combination of enzyme dosage and temperature required for GAPfopA to produce a scFOS composition similar to Actilight® (± 3%) after an 8 h reaction. 125

Figure 6-3. Production of scFOS from 60% (w/v) sucrose as a function of time using GAPfopA (black) and GAPfopA_V1 (grey). The reaction was performed at 62.0 °C and pH 5.0 at an enzyme dosage of 10 U/g sucrose at an agitation rate of 120 rpm. The percentage GF2 (♦),

percentage GF3 (■) and percentage GF4 (▲) are shown where error bars denote the standard error of triplicate experiments.....126

Figure 6-4. Production of scFOS over a 12 h time period with GAPfopA (black) and GAPfopA_V1 (grey) in a 20 L bioreactor. The reaction was performed at 62.0 °C, pH 5.0 with an enzyme dosage of 10 U/g sucrose stirred at 200 rpm. The percentage GF2(♦), percentage GF3 (■) and percentage GF4 (▲) are shown. Error bars denote standard errors (n = 3).....130

Figure A-1. Gene sequence alignment of the two codon-optimized *fopA* genes by Geneart® and ATUM…….141

Figure B-1. Dry cell weight (DCW) concentrations (solid line) and volumetric enzyme activities (grey bars) over time for DO-stat cultivations for (A) GAPfopA and (B) GAPfopA_V1. Cultivations performed at 30 °C, pH 5 and dissolved oxygen controlled at 30% and enzyme activities determined at 107, 131 and 155 h. Error bars denote standard errors (n = 2)…….146

Figure B-2. Dry cell weight (DCW) concentrations (solid line) and volumetric enzyme activities (grey bars) over time for constant feed cultivations for (A) GAPfopA and (B) GAPfopA_V1. Cultivations performed at 30 °C, pH 5 and dissolved oxygen controlled at 30% and enzyme activities determined at 33, 59 and 67 h. Error bars denote standard errors (n = 2)……..147

List of tables

Table 2-1. Commercially available food-grade FOS 15

Table 2-2. Summary of heterologous proteins expressed in *P. pastoris* from fungi and bacteria since 2002. 17

Table 2-3. Elemental media composition of BSM, FM22 and d'Anjou. 23

Table 4-1. Microbial strains used in this study. 58

Table 4-2. Protein concentrations in the supernatant of 1.3 L bioreactors derived from the maximum activities of the cultivated strains after determining the specific activity of fopA with 2D Quant protein assay. 66

Table 5-1. Fermentation results for the two strains, GAPfopA and GAPfopA_V1, cultivated in 10 L bioreactors fed with two different substrate feeding strategies, DO-stat and constant feed. 90

Table 6-1. Central composite design of temperature (A) and enzyme dosage (B) for scFOS production for both GAPfopA and GAPfopA_V1. 116

Table 6-2. Observed responses after 8 h incubation with GAPfopA as percentages of total sugars and percentage of total scFOS. 118

Table 6-3. Observed responses after 8 h incubation with GAPfopA_V1 as percentages of total sugars and percentage of total scFOS. 119

Table 6-4. ANOVA of the model terms best fitting the relationship between the independent variables and responses. 123

Table 6-5. The reaction time required to produce the percentage scFOS that closely resembles Actilight® from industrial type substrates. All substrates diluted to 60% sucrose (w/v) and the reaction performed for 12 h at 62.0 °C with an enzyme dosage of 10 U/g sucrose and an agitation rate of 120 rpm. 128

List of acronyms and abbreviations

1-SST	sucrose:sucrose 1-fructosyltransferase
Adj. R^2	Adjusted R-squared
ANOVA	Analysis of variance
AOX1	Alcohol oxidase I promoter
ATCC	American type culture collection
BMGY	Buffered glycerol-complex media
BMMY	Buffered methanol-complex media
BSM	Basal salt medium
CCD	Central composite design
CSTR	Continuously stirred-tank (bio)reactor
CTAB	Cetyltrimethyl ammonium bromide
DCW	Dry cell weight
DHAS	Dihydroxyacetone synthase
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOT	Dissolved oxygen tension
DP	Degree of polymerization
DSF	Differential scanning fluorimetry
DSMZ	Deutsche Sammlung von Mikroorganism und Zellkulturen
FFase	β-fructofuranosidase

FLD1	Formaldehyde dehydrogenase
FOS	Fructooligosaccharides
FOSHU	Food for Specified Health Uses
FPLC	Fast protein liquid chromatography
ftf	Fructosyltransferase gene
GAP	Glyceraldehyde-3-phosphate dehydrogenase promoter
GB	Glycerol batch phase
GFB	Glycerol fed-batch phase
GF2	1-kestose
GF3	nystose
GF4	1^F-β-fructofuranosyl nystose
GFP	Green fluorescent protein
HPLC	High-performance liquid chromatography
ICL	Isocitrate lyase
ITD	Isothermal denaturation
lsdA	Levansucrase gene
NAD	nicotinamide adenine dinucleotide
PCR	Polymerase chain reaction
PEX	Peroxin
PGK	3-phosphoglycerate kinase
PID	Proportional-integral-derivative

Q_P	Volumetric productivity
Q_X	Biomass productivity
RI detector	Refractive index detector
RSM	Response surface methodology
scFOS	Short-chain fructooligosaccharides
SCP	Single-cell proteins
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SmF	Submerged fermentation
SSBs	Sugar-sweetened beverages
SSF	Solid-state fermentation
TEF	Translation elongation factor
vvm	Volume of air per liter of medium per minute
YPT	Yeast protein two
Y_{PS}	Product yield
Y_{XS}	Biomass yield

Chapter 1 Introduction

1.1 Contextual background

The South African sugar industry produces on average an estimated 2.2 million tons of sugar per season (annum). Of the total saleable sugar, about 60% is earmarked for the national market, whereas the excess is exported. Total sugar sales generate an estimated average direct income of R8 billion per annum. However, the international export profitability of sugar by the South African sugar industry is hampered by the low international market price (SASA, 2018). Furthermore, draft legislation has been tabled by the South African National Treasury for a tax on sugar-sweetened beverages (SSBs). These SSBs are defined as beverages with added caloric sweeteners such as sucrose, high-fructose corn syrup or fruit juice concentrates. Furthermore, SSBs containing less than 4 g/100ml sugar will not be taxed, but a sugar content above this threshold will be taxed with 2.1 cents per gram above the threshold (Economics Tax Analysis Chief Directorate, 2016; National Treasury, 2017). Beverages naturally rich in sugar (100% fruit juice, unsweetened milk and milk products) are tax exempt. Therefore, expanding the portfolio of the sugar industry with alternative products from sugar, such as short-chain fructooligosaccharides (scFOS), can increase the revenue of the sugar industry while addressing the sugar tax legislation.

Fructooligosaccharides (FOS) are high-value prebiotics used in functional foods (food containing health-giving additives) and can be produced by enzymatic modification of either inulin or sucrose (Crittenden and Playne, 1996). The revenue forecasts for the growth of FOS from 2005 to 2015 in Europe was from €27.2 million to €97.2 million with a compound growth rate of 10.7% from 2008 to 2015 (Frost & Sullivan, 2008) and a global market worth of $3.52 billion by 2024 (Grand View Research, 2016). FOS is not significantly digested by the human gastrointestinal tract and as a result is low-calorie (Roberfroid, 1999). Therefore, it will not

qualify as an added caloric sweetener as defined by the definition contained in the tax on SSBs (Dominguez et al., 2014), while converting sucrose to FOS could be a profitable alternative to exporting sugar.

FOS is produced by either the transfructolysation of sucrose by the enzymes fructosyltransferase (EC 2.4.1.9) or β-fructofuranosidase (FFase; EC 3.2.1.26) or the degradation of inulin by inulinases (endoinulinase; EC 3.2.1.7 and exoinulinase; EC 3.2.1.80). Commercially available FOS is currently produced by the enzymatic transfructosylation of sucrose in a two-stage process, where the enzyme is produced first followed by the production of FOS (Apolinário et al., 2014; Dominguez et al., 2014; Sangeetha et al., 2005). Various studies have been performed to optimise both of these two stages (Anane et al., 2016; Dominguez et al., 2014; Trollope et al., 2015). In this study both these stages were addressed; the first in chapters 4 and 5 and the second in chapter 6. Actilight®, a leading example of scFOS as a prebiotic food additive, consists of 37% kestose (GF2), 53% nystose (GF3) and 10% 1^F-β-fructofuranosyl nystose (GF4). Actilight® is produced by Tereos-Beghin Meiji through the transfructolysation of sucrose, and will serve as a technical benchmark for FOS-quality and chemical composition in this study (Lecerf et al., 2015).

There have been extensive studies in identifying, characterizing and expressing the fructosyltransferase or β-fructofuranosidase enzymes, but recombinant expression and genetic engineering have been limited (Fernandez et al., 2007; Singh and Singh, 2010). Heterologous expression of fructosyltransferases have been successful in *Pichia pastoris* (Trujillo et al., 2001), *Eschericia coli* (Van Hijum et al., 2002), *Saccharomyces cerevisiae* (Trollope et al., 2015) and homologous expression in *Aspergillus niger* (Zhang et al., 2017).

In this study, the β-fructofuranosidase enzyme from *A. fijiensis* ATCC 20611 was selected for sucrose conversion to scFOS, by heterologously producing the enzyme in recombinant *P.*

pastoris. The expression system and enzyme production will be optimised by evaluating different strains, promoters, codon-optimisation techniques and cultivation strategies. scFOS production will be optimised with the produced enzyme, in terms of temperature and enzyme dosage, and evaluated for various crude or refined types of industrial sucrose that may be used as feedstock for the conversion.

1.2 Thesis outline

This dissertation consists of 7 chapters. Chapter 2 discusses the growing global market and importance of FOS as a prebiotic for the use in functional foods for human health. The enzymes and methods of current industrial production are outlined in this chapter and the research in optimizing the production methods of FOS, both on a process and molecular level, is discussed. Finally, *P. pastoris* as a host for recombinant expression of these FOS producing enzymes is discussed. The molecular aspects of heterologous protein production in this yeast is presented here as well as the fermentation parameters. The aims and objectives derived from reviewing the literature and identifying existing gaps, to formulate the current work, is presented in Chapter 3. Chapter 4 presents the results for the determination of the best *P. pastoris* host strain, promoter and codon-optimization company to produce the β-fructofuranosidase enzyme. Chapter 5 discusses the results of scaled-up production of a codon-optimized native β-fructofuranosidase enzyme and an engineered version of this enzyme using different glycerol fed-batch methods. Chapter 6 describes the results of evaluating the differences in temperature and enzyme dosage between the enzymes from Chapter 5, using response surface methodology (RSM), to produce a specific target FOS composition similar to a commercial FOS product. The viability of using these enzymes to produce this FOS target composition with the conditions determined through RSM from industrial sugar streams is further assessed. Chapter 7 contains the main conclusions and recommendations for this study.

1.3 References

Anane, E., van Rensburg, E., Görgens, J.F., 2016. Comparison of constitutive and inducible β-fructofuranosidase production by recombinant *Pichia pastoris* in fed-batch culture using defined and semi-defined media. South African J. Chem. Eng. 22, 17–22.

Apolinário, A.C., De Lima Damasceno, B.P.G., De Macêdo Beltrão, N.E., Pessoa, A., Converti, A., Da Silva, J.A., 2014. Inulin-type fructans: A review on different aspects of biochemical and pharmaceutical technology. Carbohydr. Polym. 101, 368–378.

Crittenden, R.G., Playne, M.J., 1996. Production, properties and applications of food-grade oligosaccharides. Trends Food Sci. Technol. 7, 353–361.

Dominguez, A.L., Rodrigues, L.R., Lima, N.M., Teixeira, J.A., 2014. An Overview of the Recent Developments on Fructooligosaccharide Production and Applications. Food Bioprocess Technol. 7, 324–337.

Economics Tax Analysis Chief Directorate, 2016. Taxation of sugar sweetened beverages: Policy Paper - 8 July 2016 [WWW Document]. URL http://www.treasury.gov.za/publiccomments/Sugar sweetened beverages/POLICY PAPER AND PROPOSALS O%0AN THE TAXATION OF SUGAR SWEETENED BEVERAGES-8 JULY 2016.pdf

Fernandez, R.C., Ottoni, C. A., Da Silva, E.S., Matsubara, R.M.S., Carter, J.M., Magossi, L.R., Wada, M.A.A., De Andrade Rodrigues, M.F., Maresma, B.G., Maiorano, A.E., 2007. Screening of β-fructofuranosidase-producing microorganisms and effect of pH and temperature on enzymatic rate. Appl. Microbiol. Biotechnol. 75, 87–93.

Frost & Sullivan, 2008. Strategic Analysis of the European Human Food and Beverage Prebiotics Markets, Frost & Sullivan.

Grand View Research, 2016. Fructooligosaccharides (FOS) Market Worth $3.52 Billion By 2024 [WWW Document]. URL https://www.grandviewresearch.com/press-release/global-fructooligosaccharides-fos-market

Lecerf, J.M., Clerc, E., Jaruga, A., Wagner, A., Respondek, F., 2015. Postprandial glycaemic and insulinaemic responses in adults after consumption of dairy desserts and pound cakes containing short-chain fructo-oligosaccharides used to replace sugars. J Nutr Sci 4, 1–7.

National Treasury, 2017. Tax on Sugary Beverages [WWW Document]. URL http://www.treasury.gov.za/public comments/Sugar sweetened beverages/2017022701 - QandA Tax on Sugary Beverages.pdf

Roberfroid, M.B., 1999. Caloric value of inulin and oligofructose. J. Nutr. 129, 1436S–1437S.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2005. Recent trends in the microbial production, analysis and application of fructooligosaccharides. Trends Food Sci. Technol. 16, 442–457.

SASA, 2018. South African Sugar Association [WWW Document]. URL www.sasa.org.za (accessed 8.18.18).

Singh, R.S., Singh, R.P., 2010. Production of fructooligosaccharides from inulin by endoinulinases and their prebiotic potential. Food Technol. Biotechnol. 48, 435–450.

Trollope, K.M., Görgens, J.F., Volschenk, H., 2015. Semirational directed evolution of loop regions in *Aspergillus japonicus* β-fructofuranosidase for improved fructooligosaccharide production. Appl. Environ. Microbiol. 81, 7319–7329.

Trujillo, L.E., Arrieta, J.G., Dafhnis, F., García, J., Valdés, J., Tambara, Y., Pérez, M., Hernández, L., 2001. Fructo-oligosaccharides production by the *Gluconacetobacter*

diazotrophicus levansucrase expressed in the methylotrophic yeast *Pichia pastoris*. Enzyme Microb. Technol. 28, 139–144.

Van Hijum, S.A.F.T., Van Geel-Schutten, G.H., Rahaoui, H., Van der Maarel, M.J.E.C., Dijkhuizen, L., 2002. Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. Appl. Environ. Microbiol. 68, 4390–4398.

Zhang, J., Liu, C., Xie, Y., Li, N., Ning, Z., Du, N., Huang, X., Zhong, Y., 2017. Enhancing fructooligosaccharides production by genetic improvement of the industrial fungus *Aspergillus niger* ATCC 20611. J. Biotechnol. 249, 25–33.

Chapter 2 Literature review

2.1 Fructooligosaccharides

2.1.1 Natural occurrence and structure

FOS occurs naturally in microorganisms, many plants and vegetables such as leek, garlic and barley. Their functions vary from energy reserves in both plants and bacteria, contributing to biofilm formation in bacteria, protecting plants against drought and freezing and petal expansion in *Hemerocallis* (Banguela and Hernández, 2006; Bieleski, 1993; Kiska and Macrina, 1994). Generally, the concentrations of FOS range from 0.3% to 6% of fresh weight but for some resources, such as Jerusalem artichoke, it can be as high as 20% (Mussatto and Mancilha, 2007).

Fructans are fructose polymers derived from sucrose and their structures vary depending on their source. They usually consist of a common glucose residue with several fructose units attached by a β(2→1) or β(2→6) bond. The fructans with β(2→1) bonds are known as inulin and those with β(2→6) as levans. Their structures can be highly diverse and vary according to the degree of polymerisation (DP), the presence of branches, the type of linkages between fructose units and the position of the glucose residue (Apolinário et al., 2014; Ritsema and Smeekens, 2003). Both inulin and levans occur in microorganisms and plants. However, they differ in their DP where microbial fructans will generally have a higher DP than their plant counterpart (Banguela and Hernández, 2006; Velázquez-Hernández et al., 2009). Several fungal species produce fructans of the inulin type with a DP ranging from 3 – 10 and these are known as fructooligosaccharides (FOS) or short-chain fructooligosaccharides (scFOS) (Hidaka et al., 1988; Lorenzoni et al., 2014; Mussatto et al., 2012, 2009).

2.1.2 Health benefits of FOS

scFOS is an important ingredient in functional foods and serve as a source of prebiotics. These consist of the low DP oligosaccharides derived from sucrose (Fig. 2-1), predominantly present in the form of 1-kestose, nystose and 1^F-β-fructofuranosyl nystose (Nobre et al., 2015).

Functional foods were first established in Japan in 1984. Shortly thereafter, specific health-related foods called FOSHU (food for specified health uses) was established by the Ministry of Health and legislation introduced for their regulation. In 1996 the FOSHU list of foods comprised 58 approved foods of which 34 incorporated oligosaccharides as a functional ingredient (Crittenden and Playne, 1996; Menrad, 2003).

FOS has various functional and health properties. They only have about a third of the sweetness of a 10% sucrose solution (Yun, 1996) and therefore serve as a non-cariogenic sweetener with low caloric value (Roberfroid, 1999). FOS also promotes the growth of bifidobacteria in the colon and has been reported to protect against colon cancer, improve mineral absorption, enhance immunity and reduce cholesterol, phospholipids and triglycerides in the blood (Dominguez et al., 2014; Fernandez et al., 2007).

2.1.3 FOS production methods

The ability to produce FOS is spread over a wide variety of microorganisms including fungi, bacteria and yeasts. FOS can be produced by either the transfructolysation of sucrose by the enzymes fructosyltransferase (EC 2.4.1.9) or β-fructofuranosidase (FFase; EC 3.2.1.26) or the degradation of inulin by endoinulinase (EC 3.2.1.7). The fructosyltransferase and β-fructofuranosidase enzymes, which are the focus of this study, has a double-displacement reaction mechanism involving two steps, namely hydrolysis and transfer of a sugar monomer (Chuankhayan et al., 2010). The hydrolysis of sucrose produces a free glucose, while the

fructose is retained by the enzyme to form a fructosyl-enzyme intermediate. If the next acceptor is water the reaction results in the hydrolysis products of fructose and glucose. However, if the next acceptor is a fructan (sucrose, GF2 or GF3), the fructose moiety is transferred and results in FOS of increasing DP. The transfructosylating reaction predominantly occurs at sucrose concentrations above 200 g/L (Kim et al., 1996), whereas the same enzyme has a hydrolytic action at sucrose concentrations below 5 g/L.

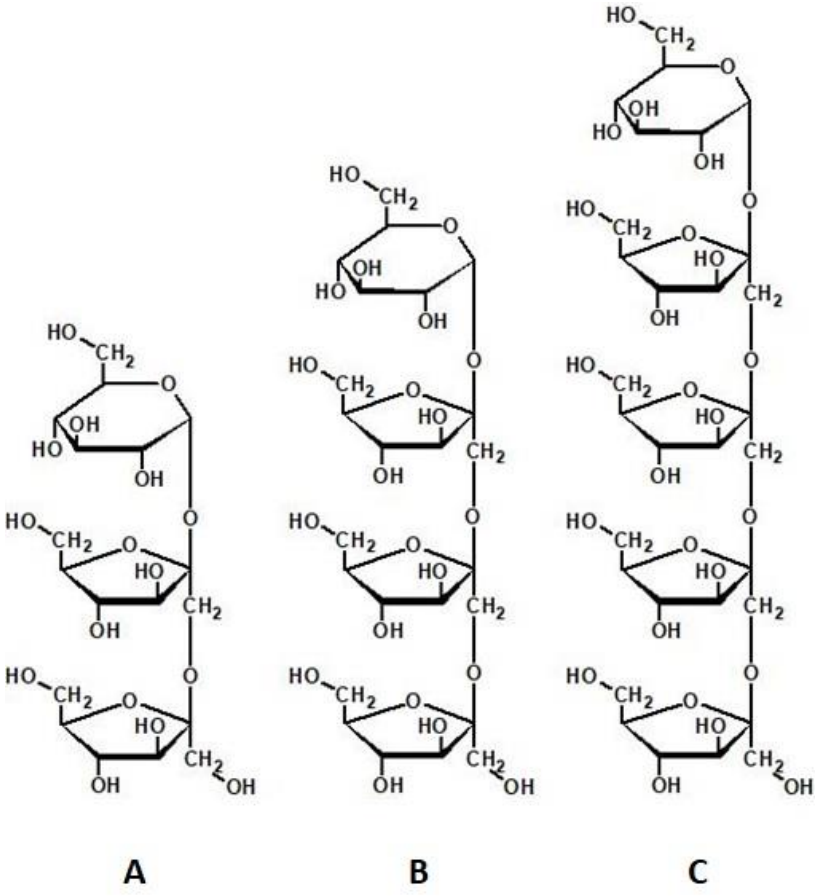


Figure 2-1. Structures of kestose (A), nystose (B) and fructofuranosyl nystose (C)

In an industrial process, FOS can be produced by using the whole cells of an enzyme-producing organism, which can be either suspended or immobilized, or the extracted enzyme in an immobilized or free form (Cruz et al., 1998; Nguyen et al., 1999; Sangeetha et al., 2005, 2004). However, the FOS produced from inulin degradation typically have a higher DP than that from

the transfructolysation of sucrose (DP 2-9 and DP 2-4, respectively) and only some will contain a terminal glucose moiety (Singh and Singh, 2010). Oligosaccharides can also be synthesized through chemical routes, but this is an intensive and time-consuming process that requires hazardous and expensive chemicals and produces low yields (Palcic, 1999; Prapulla et al., 2000). Thus, the enzymatic synthesis of FOS from sucrose is the preferred method for producing this product.

There are two fermentative methods that have been studied for producing FFase – Submerged Fermentation (SmF) and Solid-State Fermentation (SSF). There are many advantages to using SSF such as simplicity of use, decreased likelihood of contaminant growth, product concentration, high productivity, lower capital cost and energy consumption and the ability to use low cost agricultural and agro-industrial substrates. Also, the reduced water consumption of SSF results in smaller fermenters, requiring less downstream processing, reduced stirring and reduced sterilization costs, which makes the process more economically viable (Mussatto and Teixeira, 2010; Sangeetha et al., 2005). However, SSF also has several disadvantages that have hampered its adoption as an industrial technique for enzyme production such as difficulty to control process parameters (pH, temperature, moisture, aeration and oxygen transfer), difficulty in scale-up and heat build-up (Couto and Sanromán, 2006). There are numerous studies in shake flasks on the optimisation of FFase production in SmF and the production of FOS, with the focus on optimising the medium, aeration, cultivation time and agitation (Maiorano et al., 2008), while those for SSF are rare.

Therefore, commercially scFOS is currently being produced in a two-step batch process with the production of the enzyme first followed by the production of FOS second. (Fig. 2-2). Hidaka et al. (1988) investigated 11 FOS producing microorganisms and found *A. niger* ATCC 20611 to be a high enzyme producing strain and have high transfructosylating activity. The β-

fructofuranosidase (fopA) from *A. niger* ATCC 20611 was purified and characterised by Hirayama et al. (1989) and cloned and expressed in *S. cerevisiae* by Yanai et al. (2001). Since its discovery, the enzyme produced by this filamentous fungus has been one of the most effective for the commercial production of FOS from sucrose (Zhang et al., 2017).

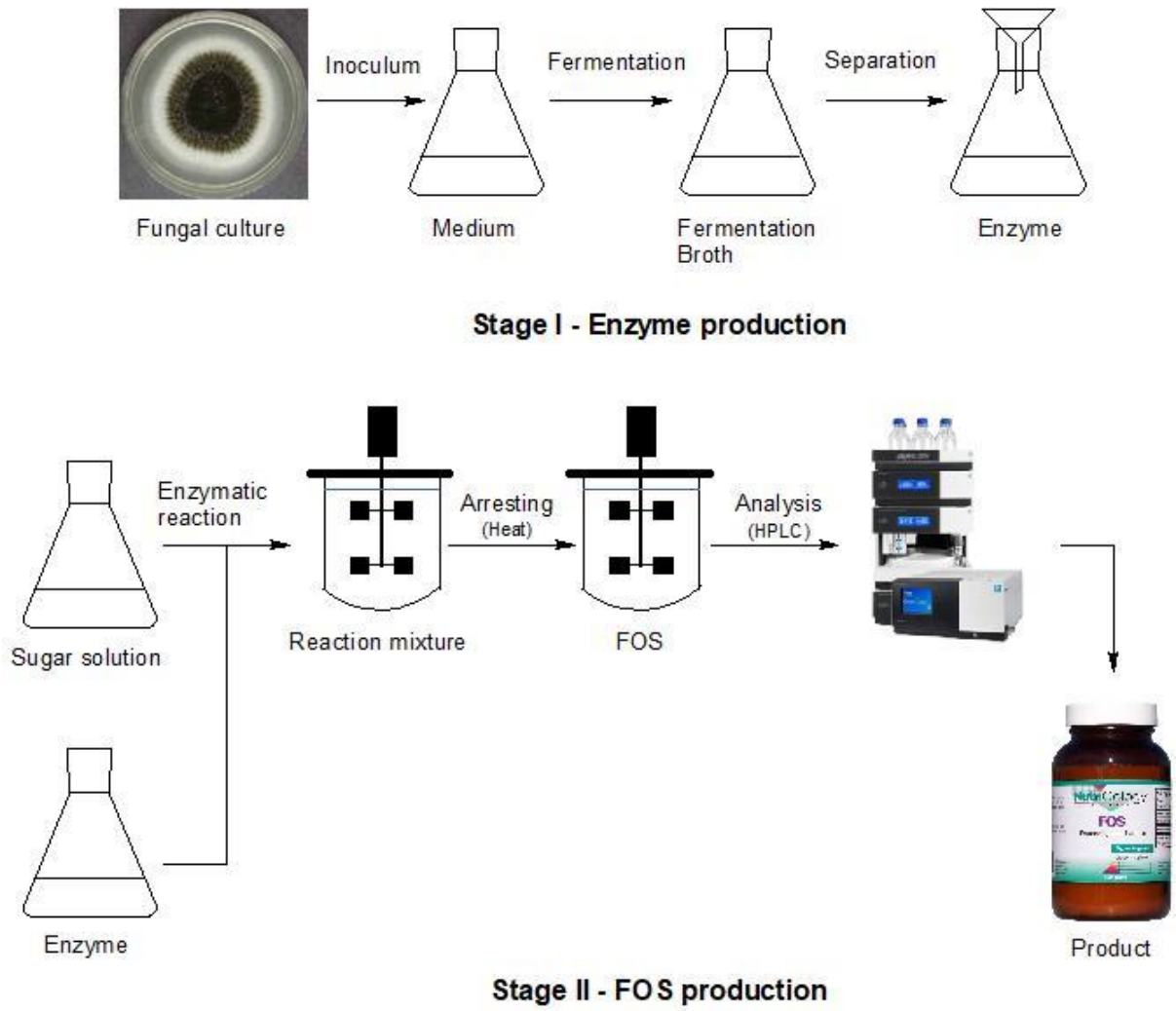


Figure 2-2. Two stage fructooligosaccharide (FOS) production process by submerged fermentation (SmF) (Adapted from Sangeetha et al., 2005).

Response surface methodology has been shown to be effective in optimising the production of fructooligosaccharides from sucrose. Nemukula et al. (2009) isolated a fructosyltransferase from *A. aculeatus* and determined the influence of pH, temperature, reaction time, enzyme and

sucrose concentrations for sucrose conversion to FOS, while Vega and Zúniga-Hansen (2011) evaluated the temperature and the concentrations of sucrose and enzyme to preferentially produce high concentrations of 1-kestose. Both these studies showed that you can tailor the FOS composition to your requirements by varying the above-mentioned process conditions. In Chapter 6 we will use RSM to both determine the influence of enzyme dosage and temperature on the formation of scFOS for the native fopA and protein-engineered fopA_V1 enzymes as well as the conditions needed to target a specific scFOS composition similar to Actilight®.

2.1.4 Practical maxima for sucrose conversion to FOS

Industrial FOS production by sucrose conversion with FFase enzymes generates a maximum yield of FOS of 55-60%, based on the initial sucrose concentration (Sangeetha et al., 2005). This is the maximum value of FOS attainable because the glucose liberated during the enzymatic process competitively inhibits the enzyme (Yun, 1996). Various studies performed attempted to alleviate this problem by removing the glucose. Yun et al. (1994) investigated a mixed enzyme reaction with the β-fructofuranosidase from *Aureobasidium pullulans* and the glucose oxidase from *A. niger*. The unreacted sucrose and released glucose were completely consumed and a FOS content of 98% was obtained based on a dry substance basis. The β-fructofuranosidase from *A. pullulans* was also used in conjunction with a commercial enzyme preparation of glucose isomerase from a *Streptomyces* sp. and resulted in an increased FOS yield based on initial sucrose concentration of 69% compared to 62% using only the β-fructofuranosidase (Yoshikawa et al., 2008). In another study, Nishizawa et al. (2001) were able to use a membrane reactor system to selectively remove glucose while leaving the sucrose and FOS behind and was able to achieve 93% FOS based on a final saccharide weight composition.

2.1.5 FOS production with recombinant enzymes

Several studies tried to improve the enzymatic production of FOS from sucrose when using recombinant enzymes as biocatalysts. In some instances, research focused on either screening for novel enzymes with high transfructosylating activity (Chen and Liu, 1996; Ghazi et al., 2007), optimising the production of the native enzymes with known transfructosylating capability (Balasubramaniem et al., 2001; Vandáková et al., 2004; Yun et al., 1997) or heterologous recombinant expression of these enzymes for the production of FOS (Spohner and Czermak, 2016; Trujillo et al., 2001). However, screening for transfructolysating enzymes is a laborious process and only a small number of these enzymes have sufficient transfructosylating activity to be of industrial relevance (Vega-Paulino and Zúniga-Hansen, 2012). There is currently no commercial enzyme preparation available with fructosyltransferase (β-fructofuranosidase, EC 3.2.1.26 or β-D-fructosyltransferase, EC 2.4.1.9) as the main activity for the sole purpose of producing scFOS. However, there are various food-grade commercial enzyme preparations, which contain these enzymes as side activities. Vega-Paulino and Zúniga-Hansen (2012) screened 25 commercial enzyme preparations for transfructolysation activity and found three with high activity (Viscozyme L, Pectinex Smash and Rohacept CM) that could possibly be used to produce FOS.

Although a number of other promising sources of FFases for FOS production have been reported (Chávez et al., 1997; Park et al., 2001; Yoshikawa et al., 2007) only a few reports of recombinant FFases are available. Rehm et al. (1998) expressed the *sst* gene of *Aspergillus foetidus* in *S. cerevisiae* producing 1-kestose from sucrose with the purified enzyme, whereas 1-kestose and 1-nystose was produced by expressing the *Gluconacetobacter diazotrophicus* levansucrase gene and the *ftf* gene from *Lactobacillus reuteri* in *Pichia pastoris* and *Escherichia coli*, respectively, and incubating the purified enzyme with sucrose (Trujillo et al., 2001; Van Hijum et al., 2002). More recently

Trollope et al. (2015) expressed an engineered fopA enzyme from *A. niger* ATCC 20611 in *S. cerevisiae*, while Zhang et al. (2017) overexpressed an almost identical version of this enzyme in its native host, *A. niger* ATCC 20611. Both these enzymes were capable of producing GF2, GF3 and GF4.

2.1.6 FOS market

The markets for functional foods vary considerably depending on the definition of what a functional food is. However, the global market can be estimated to be at least 33 billion US$ with the market for Europe exceeding 2 billion US$. In Germany alone, the functional dairy market has grown from 5 million US$ in 1995 to 419 million US$ in 2000 (Menrad, 2003).

The first company to produce FOS commercially was Meiji Seika Kaisha, Ltd. in Japan in 1984. Since then the number of companies producing FOS has grown considerably, and produce FOS and inulin to varying purities (Table 2-1). However, the only companies supplying individual FOS molecules for analytical purposes are Sigma Aldrich, Megazyme and Wako Chemicals GmbH (Nobre et al., 2015). Actilight® is the leading FOS product and has several health benefits proven in clinical trials and has been accepted by the European Food Safety Authority to regulate blood glucose (Lecerf et al., 2015; Paineau et al., 2014). The FOS market in Europe was projected to grow from €27.2 million in 2005 to €97.2 million in 2015 with FOS units shipped to increase from 9 080 tonnes to 25 580 tonnes in the same time (Frost & Sullivan, 2008).

Table 2-1. Commercially available food-grade FOS

Substrate	Company	Country	Product name	Type of fructan
Sucrose	Meiji Seika Kaisha	Tokyo, Japan	Meioligo	FOS
	GTC Nutrition	Golden, Colorado, US	NutraFlora®	FOS
	Cheil Foods and Chemicals	Seoul, Korea	Oligo-Sugar	FOS
	Victory Biology Engineering	Shanghai, China	Beneshine™ P-type	FOS
	Beghin-Meiji Industries	Paris, France	Actilight® Profeed®	FOS
Inulin	BENEO-Orafti	Brussels, Belgium	Orafti®	Inulin and oligofructose
	Cosucra	Warcoing, Belgium	Fibruline® Fibrulose®	Inulin and oligofructose
	Sensus	Roosendaal, Netherlands	Frutafit® inulin Frutalose®	Inulin and oligofructose
	Nutriagaves de Mexico S.A. de C.V.	Ayotlan, Jalisco, Mexico	OLIFRUCTINE-SP®	Inulin and oligofructose

Adapted from Dominguez et al. (2014) and Nobre et al. (2015).

2.2 *Pichia pastoris* for recombinant protein expression

It has been shown that the improvement of the production strain can reduce the cost of a bioprocess markedly without significant capital outlay (Chiang, 2004). Additionally, a superior production strain will invariably result in a superior production process, which makes the optimisation of the strain worthwhile. Various molecular techniques can be applied to improve

the expression of recombinant proteins from *P. pastoris* but due to the typical "biological" variation in results from these techniques, each one has to be evaluated empirically, on a case by case basis.

P. pastoris has become a very important host for recombinant protein expression. Phillips Petroleum used it for the commercial production of single-cell proteins (SCP) almost 40 years ago. However, the increase in the price of methanol as a result of the oil crisis made the process too costly. This host was patented by Phillips Petroleum and made available for research purposes and was developed for recombinant protein expression in the 1980s (Cregg et al., 2000, 1993). Since then the genome sequences for the original SCP production strain CBS 7435, strain GS115 and the related strain DSMZ 70382 have been published with the latter free to use for commercial enzyme production (De Schutter et al., 2009; Küberl et al., 2011; Mattanovich et al., 2009). Due to the extensive research into this platform over 400 proteins have been expressed (Table 2-2) ranging from human to invertebrate (Cereghino and Cregg, 2000).

The advantages of yeast hosts for recombinant protein expression are their protein processing capabilities, which is similar to that of other eukaryotes, their amenability to genetic manipulation and the absence of endotoxins. The most well-known yeast host for recombinant protein expression was *S. cerevisiae* and this was primarily due to the extensive knowledge available of its genetics, physiology, biochemistry and fermentation technologies. However, due to some inherent disadvantages of *S. cerevisiae* (Crabtree positive, poor plasmid stability and low protein yields), the search for yeast hosts was expanded. Of these new yeast hosts, the methylotrophic yeasts proved to very successful with *Hansenula polymorpha* and *P. pastoris* being some of the most prominent (Buckholz and Gleeson, 1991; Porro et al., 2005). Yang et al. (2016) could express a fructosyltransferase from *A. niger* YZ59 in *P. pastoris* GS115 and

Table 2-2. Summary of heterologous proteins expressed in *Pichia pastoris* from fungi and bacteria since 2002.

Protein expressed	Promoter	Function	Reference
Bacteria			
Escherichia coli AppA	*AOX1*	Use in the animal feed industry to release inorganic phosphate	(Stahl et al., 2003)
Gluconoacetobacer diazotrophicus exo-levanase	*AOX1/GAP*	Fructose-releasing potential for high-fructose syrup production	(Menéndez et al., 2004)
Thermus aquaticus YT-1 aqualysin I	*GAP*	Heat-stable subtilisin-type serine protease	(Olędzka et al., 2003)
Fungi			
Aspergillus oryzae tannase	*AOX1*	Hydrolyses the ester and depside bonds of gallotannins and gallic acid esters	(Zhong et al., 2004)
Candida antartica CBM–CALB fusion protein	*AOX1*	Hydrolyzes triglycerides	(Jahic et al., 2003)
Candida parapsilosis lipase/acyltransferase	*AOX1*	Catalyses alcoholysis of esters	(Brunel et al., 2004)
Rhizopus oryzae lipase	*FLD1/AOX1*	Catalyses breakdown of triglycerides	(Resina et al., 2004)
Trametes versicolor cellobiose dehydrogenase (CDH)	*AOX1*	Oxidation of reducing-end groups of cellobiose	(Stapleton et al., 2004)
Trametes versicolor laccase (lcc1)	*AOX1*	Oxidation of phenolic substrates	(O'Callaghan et al., 2002)
Aspergillus niger fructosyltransferase	*AOX1*	Synthesize FOS	(Yang et al., 2016)

Adapted from Macauley-Patrick et al. (2005)

obtained a volumetric activity of 1020 U/ml in a 5L bioreactor. Therefore, for this study, *P. pastoris* was selected as the host for the recombinant expression of fopA. There are several *P. pastoris* host strains available such as CBS 704, CBS 2612, CBS 7435, CBS 9173-9189 (Westerdijk Institute) and DSMZ 70877 (German Collection of Microorganisms and Cell Cultures) as well as X-33, GS115, KM71 and SMD1168 strains from Invitrogen (Ahmad et al., 2014). However, expression of recombinant proteins may vary between the different strains and the use of some are restricted by patents. Ang et al. (2016) showed that KM71H strain had higher expression of the human DNA topoisomerase I (2.26 ng/ml) compared to that of the X-33 strain (0.75 ng/ml), while Blanchard et al. (2008) found that strain GS115 could expressbiologically active N-glycosylated ^{15}N-labeled human chorionic gonadotropin (phCG) in contrast to X-33. In this study, the strains DSMZ 70382 (CBS 704) and X-33 were evaluated for the expression and production of the fopA enzyme (Chapters 5 and 6).

2.2.1 Promoters

The predominant promoters used in *P. pastoris* for recombinant protein expression are alcohol oxidase (*AOX1)* and glyceraldehyde-3-phosphate dehydrogenase (*GAP)*. *P. pastoris* possesses two alcohol oxidase genes, *AOX1* and *AOX2*, which encode for the enzyme. Of the two enzymes, AOX1 is produced in greater quantities in the native organism application, where it is responsible for up to 95% of the total expressed alcohol oxidase, which is essential for growth on methanol. The *AOX1* promoter is therefore used the most for foreign protein expression and is regulated by a repression\derepression mechanism as well as an induction mechanism. There are several advantages to using the *AOX1* promoter, such as the tight regulation of the transcription by the previously described mechanism, high levels of foreign proteins can be expressed, high cell biomass can be achieved before the gene product is induced and induction of transcription is simple to do. However, methanol is used for induction and this can be a fire

hazard when stored in large quantities. Monitoring the levels of methanol in the bioreactor medium is also difficult, as the organism is highly sensitive to methanol under- or over-feeding, while methanol is produced from petrochemical sources, making it unusable for the production of food products such as FOS (Macauley-Patrick et al., 2005).

The glyceraldehyde-3-phosphate dehydrogenase (*GAP)* promoter has emerged as a promising alternative to the *AOX1* promoter for recombinant protein production in *P. pastoris*. Glyceraldehyde-3-phosphate dehydrogenase is a NAD-binding enzyme, which plays an important role in the glycolysis and gluconeogenesis pathways of the methylotrophic yeasts (Çalık et al., 2015). The host can use either glucose or glycerol as a substrate for expression of recombinant proteins from the cells. Therefore, the use of volatile, flammable methanol can be avoided, and bio-based feedstocks can be used for food grade products such as FOS. In the *AOX1* system, protein production is limited by the availability of methanol (inducible promoter), while the *GAP* system biomass and protein production occur simultaneously (constitutive promoter). Other advantages are that the *GAP* system in fed-batch culture requires minimal control of the feeding strategy, can have longer protein production periods and genes are expressed when cells are grown on glucose, glycerol or methanol (Potvin et al., 2012).

The literature on whether the *GAP* or *AOX1* promoter is more efficient in expressing heterologous proteins is varied. When functional mammalian transport proteins were expressed with both these promoters, the *GAP* promoter had levels 5 times higher than that of *AOX1* (Döring et al., 1998), while Delroisse et al. (2005) showed *GAP* produced protein levels twice that of *AOX1* in shake flasks. In contrast, Boer et al. (2000) showed increased yield from *AOX1*

expressing a cellobiohydrolase and Vassileva et al. (2001) also had higher levels of hepatitis B surface antigen from a single copy *AOX1* integrant compared to the single copy *GAP* integrant.

There are, however, several other inducible and constitutive promoters used with varying success. One promising inducible promoter as an alternative to *AOX1* is the glutathione-dependent enzyme formaldehyde dehydrogenase (*FLD1*) promoter. The advantage to this promoter is that it can be induced by either methanol or methylamine. Cos et al. (2005) were able to produce *Rhizopus oryzae* lipase controlled by the *FLD1* promoter in a methanol-free fed-batch system with production values similar to those of the gene controlled by *AOX1*. Furthermore, Duan et al. (2009) were able to co-express two different proteins controlled by either the *AOX1* or *FLD1* promoters in *P. pastoris*. A green fluorescent protein (GFP) was placed under the control of the *FLD1* promoter while a portion of a gelatin gene was paced under control of the *AOX1* promoter. When fed with methanol both these proteins were expressed and when fed with methylamine only the GFP was expressed. Two more promoters with an alternative inducible substrate are *PEX8* and *ICL1*. *PEX8* can be induced by either methanol or oleic acid and *ICL1* with ethanol (Liu et al., 1995; Menendez et al., 2003). Dihydroxyacetone synthase (*DHAS*) is another strong methanol-induced promoter with expression levels up to 20% of total cell proteins (Gellissen, 2000).

Constitutive promoters as alternatives to *GAP* are *YPT1*, 3-phosphoglycerate kinase (*PGK1*) and translation elongation factor EF-1 (*TEF1*). *YPT1* is a weak promoter while *TEF1* has shown to be comparable to *GAP* in expression and a strong alternative. *PGK1* requires either glucose,

methanol or glycerol for expression with glucose producing the highest yields (Ahn et al., 2007; de Almeida et al., 2005; Sears et al., 1998).

2.2.2 Codon optimisation

Since the deciphering of the genetic code, it was discovered that 61 codons (triplets of nucleotide base's) encode for 20 amino acids, with the remaining three codons terminating translation. Therefore, multiple codons can code for the same amino acid and is why the genetic code is referred to as being degenerate. This degeneracy of the genetic code means that different nucleotide sequences can code for the same amino acid sequence and hence the same protein (Quax et al., 2015). However, the frequency with which different organisms use various codons differs substantially and can have a significant effect on the expression of a heterologous protein in a host organism. It is generally accepted that the more rare codons a gene possesses with regards to the preferences of the host in which it is expressed, the lower the expression of the heterologous protein will be. This codon bias can also affect protein folding and differential regulation of protein expression (Quax et al., 2015).

Codon-optimisation can be used to alter the nucleotide sequence in the target gene to replicate the preferred codon usage of the host and this can either be achieved through site-directed mutagenesis or by resynthesising the entire gene (Gustafsson et al., 2004; Quax et al., 2015). Codon-optimisation has been successfully used for the expression of heterologous proteins in *P. pastoris* with increased expression of human cystatin C (Li et al., 2014), lipase from

Yarrowia lipolytica (Zhou et al., 2015), endoinulinase from *A. niger* (He et al., 2014) and a β-fructosidase from *Thermotoga maritima* (Menéndez et al., 2013).

2.2.3 Fermentation media and operational conditions

Standard conditions for fed-batch processes with *P. pastoris* have been published, i.e. medium, pH, feeding strategy, etc. However, different promoters and product requirements necessitate individual optimisation tailored to specific strains. The most commonly used medium for high cell density fermentations with *P. pastoris* is the basal salt medium (BSM) introduced by Invitrogen Co (Cos et al., 2006). This medium consists of a basal salt component, trace salts solution (PTM_1) and ammonium hydroxide as the nitrogen source. The carbon sources are glycerol, methanol or a combination thereof (Çelik and Çalık, 2012). The BSM medium is not the optimum media for all situations and two other media have been developed, i.e. one by D'Anjou and Daugulis (2000) and the FM22 medium by Stratton et al. (1998). The composition of the BSM and FM22 media are similar but some elements, such as potassium, magnesium and phosphor, in the medium described by D'Anjou and Daugulis (2000) are significantly lower than the other two media (Table 2-3). The nitrogen source for both the BSM and FM22 media is ammonium hydroxide and is added to the fermentation to control the pH, while the nitrogen is only added to the D'Anjou medium at the beginning of the fermentation. Anane et al. (2016) further optimised the BSM medium by evaluating the effect of the PTM_1 solution on the expression of a β-fructofuranosidase in *P. pastoris* under the control of the *GAP* and *AOX1* promoters.

Temperature is another factor of major importance in the expression of recombinant proteins by yeasts. The *Pichia* Fermentation Protocol (Invitrogen) recommends a cultivation temperature of 30 ºC, while temperatures above 32 ºC have been associated with the cessation of protein expression (Inan et al., 1999). Furthermore, lowering the temperature has shown

some positive effects on protein expression. Li et al. (2001) performed a study at 23 ºC and was able to increase the expression of herring antifreeze protein 3-fold. They speculated that this may be due to enhanced protein folding pathway and increased cell viability at lower temperatures, which reduces lysis of the cells and the subsequent release of proteases.

Table 2-3. Elemental media composition of basal salt medium (BSM), FM22 and d'Anjou.

Element	BSM (g/l)	FM22 (g/l)	D'Anjou (g/l)
N	NH_4OH (pH control)	1.06 + NH_4OH (pH control)	4.24
P	12.27	9.76	2.73
K	11.05	18.74	3.45
Mg	1.47	1.15	0.46
Ca	0.27	0.12	0.10
S	5.51	5.46	5.47
Cl	-	-	0.17

Adapted from Cos et al. (2006)

The pH of the fermentation culture can have a significant effect on the protease activity and the stability of expressed proteins. The pH range for fermentations is usually between pH 5 and 6, but *P. pastoris* is capable of growing in a range of pH 3 to 7 (Cregg et al., 1993). Numerous studies have reported on the optimal pH range for protein expression. The pH range from some of these studies was shown to be between 5 and 8. In this range, protease activity was minimised, and protein stability maintained. An increase in pH results in the loss of cell viability and decreases the activity or stability of the recombinant protein lower recombinant product stability or activity (Idiris et al., 2010; Kobayashi et al., 2000; Ohya et al., 2002).

The *Pichia* Fermentation Protocol (Invitrogen) recommends a standard process to maintain sufficient dissolved oxygen (DO) in the culture media. A cascade system is used where

compressed air is fed at a rate of 0.1 to 1 vvm (litres of air per litres of initial fermentation volume per minute), agitation varied from 100 to 1500 rpm and oxygen-enriched air fed when needed to maintain a DO of >20%. Maintaining the DO at 20 – 30% is the preferred level for fermentation of *P. pastoris.* However, there have been studies that used hypoxic conditions for protein expression. Hu et al. (2008) produced high cell concentrations and high protein expression under oxygen-limiting conditions. This again emphasizes that there is not one set of parameters for optimum production of a particular protein/enzyme of interest, but that empirical optimisation is required for the expression of each new product.

2.3 Bioreactor fermentations

As was previously mentioned, the enzymes used for producing FOS have either been produced in a submerged fermentation system (SmF) or a solid-state fermentation system (SSF), and that the SmF is the one used in the commercial production of FOS. Since this project will make use of SmF in a stirred-tank fermenter, it will be discussed in more detail here.

There are five major types of fermenters used in SmF and they are stirred-tank fermenter, bubble column fermenter, airlift fermenter, fluidised-bed fermenter and trickle-bed fermenter. The continuously stirred-tank (bio)reactor (CSTR) is typically a cylindrical vessel with a working height to diameter ratio of 3 to 4. The CSTR has a central shaft with a number of impellers attached to it at intervals of about 1 impeller diameter apart. The impellers can direct the flow radially or axially. The reactor is equipped with baffles (which improves mixing and

oxygen transfer) that extend from the wall into the vessel and are usually 8 – 10% of the vessel diameter (Christi, 1999).

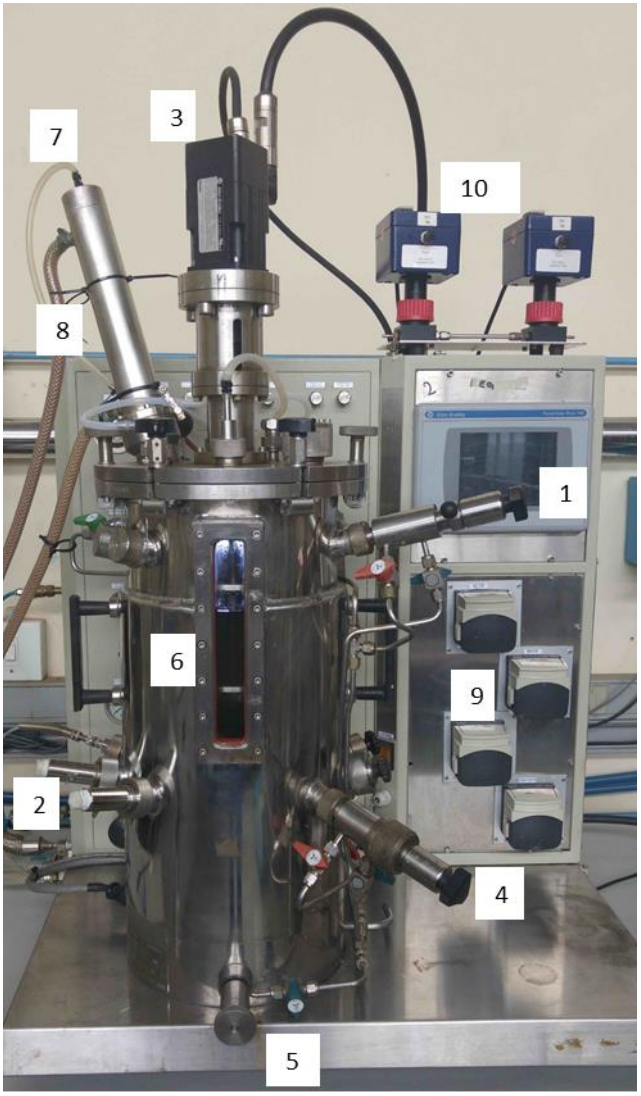


Figure 2-3. Stirred-tank reactor (1) Inoculum connection (2) Ports for sensors (3) Motor (4) Sample valve (6) Sight glass (7) Air exhaust nozzle (8) Condenser (9) Pumps for acid, base and substrate addition (10) Off-gas analysers.

The reactor is a pressure vessel that is designed to be sterilised with steam and to tolerate high pressures, high temperatures and also full vacuum. Modern commercial fermenters are usually made of stainless steel of the type 316L but the type 304L can be used as an alternative in less corrosive conditions. General features are ports for several sensors (pH, DO, foam and temperature) as well as several steam sterilisable connections for sampling and the addition of

compounds which is situated above the culture level (Fig. 2-3). A steam sterilisable harvest valve is located at the bottom of the vessel. The agitator shaft can either enter the vessel from the bottom or top and have attached impellers which create flow inside the vessel which is broken by the baffles for efficient mixing. Filter sterilised gas enters through the sparger and the exhaust gas exits through a heat exchanger which condenses the water in the gas and returns it to the vessel.

2.3.1 Batch and Fed-batch fermentations in a CSTR

Batch fermentation in a CSTR is the simplest example of fermentation and is generally used for the generation of cell biomass or a product. The batch fermentation is a closed system and it contains all the nutrients for the growth of the organism from the start of the process. It can either be performed in a shake flask or if better control is required, in a bioreactor (fermenter). The fermentation for a batch system ends when the growth of the microorganism stops, the desired time has elapsed, or the amount of product needed has been produced. The advantages of a batch system are its ease of use, low chance of contamination and the production of secondary metabolites that are not growth related. The disadvantages are the accumulation of (toxic) products, which inhibit cell growth, substrate concentrations that can have inhibitory effects on cell growth have to be kept low and can reduce the amount of product and can lead to increased downtime in industrial processes for cleaning etc. (Macauley-Patrick and Finn, 2008).

Fed-batch cultures start out as batch cultures and can then either be, depending on the feeding strategy, a variable volume or a fixed volume culture. A fixed volume fermentation is when at a certain point of the fermentation a certain volume is removed from the fermentation and replaced with an equal volume of fresh nutrients. With the variable volume fermentation, nothing is removed from the bioreactor during the fermentation and the volume increase is due

to the adding of fresh medium and nutrients during this period. This strategy can result in high cell densities and product concentrations and allows the operator to control the growth of the organism at a chosen specific growth rate (Macauley-Patrick and Finn, 2008; Stanbury et al., 2003).

The fed-batch strategy based on maintenance of a constant dissolved-oxygen (DO) concentration is referred to as DO-stat. In this strategy, new feed addition to the fed-batch is controlled by the online DO-measurement, which indicates whether the feed of carbon source should be increased (DO too high) or decreased (DO too low), due to the consumption of oxygen during carbon source conversion (Potvin et al., 2012). The DO-stat strategy strives to maintain a suitable level of dissolved oxygen in the medium for two reasons: Firstly, a sufficient concentration of dissolved oxygen is required for the production of high-density biomass and to minimise the build-up of partially oxidised substrates, while secondly, dissolved oxygen is also required to maintain an active healthy culture for extended periods of time after induction. The DO-stat strategy has also been implemented to avoid a scenario where the demand for oxygen exceeds the system's ability to supply, thus leading to hypoxic conditions that affect cell viability and product formation (Chung, 2000).

Although DO-stat has been effectively implemented in several studies for control of the feed-rate to *P. pastoris* fed-batch cultures (Ferreira et al., 2012; Hu et al., 2008; Lee et al., 2003), it is not completely dependable. During highly active cell growth the residual concentrations of methanol and DO in the culture will be low, and the methanol levels may become limiting if DO-stat system does not respond quickly enough to address the deficiency resulting in temporarily reduced productivity until feed commences. Conversely, if a culture experiences higher-than-expected methanol concentration due to overfeeding by the DO-stat system the cell growth will become inhibited, which slows the rate of methanol and DO consumption. The

DO-stat will respond to the increased DO concentrations by increasing methanol feed, which will worsen the situation and lead to the shutdown of the microbial growth, metabolism and enzyme production (Yamawaki et al., 2007).

The other feeding strategy is µ-stat, where a constant specific growth rate is maintained by regulating the substrate feed via mass balance equations. Numerous studies have proven the effectiveness of this strategy with Trinh et al. (2003) reported on the differences in the production of mouse endostatin when the feeding strategies DO-stat, µ-stat and one based on methanol consumption were compared. The production of total endostatin was comparable for all the strategies tested. However, the µ-stat feeding strategy resulted in a 2x increase in specific production per biomass and per methanol. Despite the advantages of µ-stat, changes in initial conditions or culture growth characteristics could lead to the accumulation of methanol and the inhibition of protein production. To compensate for this, the specific growth rate is usually kept lower than μ_{max}, which reduces the productivity of the process (Potvin et al., 2012; Zhang et al., 2000).

The cultivation process for recombinant enzyme production by *P. pastoris* under control of the *AOX1* promotor usually consists of three phases. Phase I being the glycerol batch phase for initial cell growth, while Phase II is the glycerol fed-batch phase for the derepression of the *AOX1* promoter and further accumulation of biomass, and Phase III the induction of the *AOX1* promoter with methanol for the expression of the recombinant protein. For fermentations with the *GAP* promoter, the glycerol feed during Phase II is simply extended for the duration of the fermentation, as it coincides with recombinant enzyme production. The DO-stat feeding strategy has been used successfully for recombinant protein expression in *P. pastoris* for both the feed of methanol during Phase III (Lim et al., 2003; Woo et al., 2005) and for the feed of glycerol for a prolonged Phase II (Anane et al., 2016; Ferreira et al., 2012). A

fructosyltransferase from *A. niger* (Yang et al., 2016) and the fopA enzyme in this study (Anane et al., 2016) have both been expressed in *P. pastoris* using DO-stat feeding strategy, but the protein-engineered version fopA_V1 has only been expressed in *S. cerevisiae* in 96-deep-well microplates (Trollope et al., 2015).

2.4 Conclusion

To conclude, fructooligosaccharides are a high-value, prebiotic product derived from sucrose that offers a technically viable means to divert sucrose into healthier products that will also avoid the imminent sugar tax. There have been many studies attempting to optimise the production of FOS from sucrose, by either screening for new FOS-producing enzymes or optimising the process of producing FOS. More recently, molecular techniques have been successfully applied to increase the production of FOS by either the recombinant expression of various FOS producing enzymes in different host organisms (*P. pastoris*, *S. cerevisiae* etc.) or the molecular engineering of the enzymes itself.

The native codon-optimized fopA enzyme has been expressed in *P. pastoris* under control of the *GAP* and *AOX1* promoters using DO-stat fed cultivation while the protein engineered variant, fopA_V1, has only been expressed in *S. cerevisiae* in shake flasks. (Anane et al., 2016; Trollope et al., 2015). However, the comparison of different *P. pastoris* strains, promoters, codon-optimization methods and fed-batch techniques to improve enzyme production has not been attempted for these enzymes. While increased thermostability for fopA_V1 has been demonstrated by Trollope et al. (2015) using isothermal denaturation (ITD) and differential scanning fluorimetry (DSF) the results of these techniques are more theoretical and do not necessarily reflect what would occur in an actual scFOS production process. Thus, optimum conditions for scFOS production for these enzymes have also not been determined. Finally, pure sucrose has been used to produce scFOS for the fopA and fopA_V1 enzymes (Hirayama

et al., 1989; Nishizawa et al., 2001; Trollope et al., 2015; Yanai et al., 2001) while the use of molasses has not been reported. Dorta et al. (2006) used the intracellular extract from *A. niger* ATCC 20611 for production of FOS from B-cane molasses but this would mainly consist of a sucB invertase and not fopA, which is an extracellular enzyme (Goosen et al., 2007).

Therefore, this study will consider process optimisation and strain development for improved production of FFase, more specifically the fopA protein-engineered variant (increased thermostability, reduced glucose inhibition and increased specific activity), and the application of the resulting enzyme for scFOS production. A unique aspect of this optimisation is the comparison of the previously-known version of the fopA-enzyme with the newer protein-engineered fopA_V1 enzyme, both proposed in *P. pastoris*, with consideration of the impact of the unique properties of the engineered enzyme on both its production and application to FOS synthesis from various industrially-relevant sucrose sources.

2.4 References

Ahmad, M., Hirz, M., Pichler, H., Schwab, H., 2014. Protein expression in *Pichia pastoris*: Recent achievements and perspectives for heterologous protein production. Appl. Microbiol. Biotechnol. 98, 5301–5317.

Ahn, J., Hong, J., Lee, H., Park, M., Lee, E., Kim, C., Choi, E., Jung, J., Lee, H., 2007. Translation elongation factor 1- α gene from *Pichia pastoris*: molecular cloning, sequence, and use of its promoter. Appl. Microbiol. Biotechnol. 74, 601–608.

Anane, E., van Rensburg, E., Görgens, J.F., 2016. Comparison of constitutive and inducible β-fructofuranosidase production by recombinant *Pichia pastoris* in fed-batch culture using defined and semi-defined media. South African J. Chem. Eng. 22, 17–22.

Ang, R.P., Teoh, L.S., Chan, M.K., Miswan, N., Khoo, B.Y., 2016. Comparing the expression of human DNA topoisomerase I in KM71H and X33 strains of *Pichia pastoris*. Electron. J. Biotechnol. 21, 9–17.

Apolinário, A.C., De Lima Damasceno, B.P.G., De Macêdo Beltrão, N.E., Pessoa, A., Converti, A., Da Silva, J.A., 2014. Inulin-type fructans: A review on different aspects of biochemical and pharmaceutical technology. Carbohydr. Polym. 101, 368–378.

Balasubramaniem, A.K., Nagarajan, K.V., Paramasamy, G., 2001. Optimization of media for β-fructofuranosidase production by *Aspergillus niger* in submerged and solid state fermentation. Process Biochem. 36, 1241–1247.

Banguela, A., Hernández, L., 2006. Fructans: From natural sources to transgenic plants. Biotecnol. Apl. 23, 202–210.

Bieleski, R.L., 1993. Fructan Hydrolysis Drives Petal Expansion in the Ephemeral Daylily Flower. Plant Physiol. 103, 213–219.

Blanchard, V., Gadkari, R.A., George, A.V.E., Roy, S., Gerwig, G.J., Leeflang, B.R., Dighe, R.R., Boelens, R., Kamerling, J.P., 2008. High-level expression of biologically active

glycoprotein hormones in *Pichia pastoris* strains - Selection of strain GS115, and not X-33, for the production of biologically active N-glycosylated 15N-labeled phCG. Glycoconj. J. 25, 245–257.

Boer, H., Teeri, T.T., Koivula, A., 2000. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7a secreted from *Pichia pastoris* using two different promoters. Biotechnol. Bioeng. 69, 486–494.

Brunel, L., Neugnot, V., Landucci, L., Boze, H., Moulin, G., Bigey, F., Dubreucq, E., 2004. High-level expression of *Candida parapsilosis* lipase/acyltransferase in *Pichia pastoris*. J. Biotechnol. 111, 41–50.

Buckholz, R.G., Gleeson, M.A., 1991. Yeast systems for the commercial production of heterologous proteins. Nat. Biotechnol. 9, 1067–1072.

Çalık, P., Ata, Ö., Güneş, H., Massahi, A., Boy, E., Keskin, A., Öztürk, S., Zerze, G.H., Özdamar, T.H., 2015. Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters. Biochem. Eng. J. 95, 20–36.

Çelik, E., Çalık, P., 2012. Production of recombinant proteins by yeast cells. Biotechnol. Adv. 30, 1108–1118.

Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris* 24, 45–66.

Chávez, F.P., Rodriguez, L., Díaz, J., Delgado, J.M., Cremata, J. a., 1997. Purification and characterization of an invertase from *Candida utilis*: Comparison with natural and recombinant yeast invertases. J. Biotechnol. 53, 67–74.

Chen, W.C., Liu, C.H., 1996. Production of β-fructofuranosidase by *Aspergillus japonicus*. Enzyme Microb. Technol. 18, 153–160.

Chiang, S.J., 2004. Strain improvement for fermentation and biocatalysis processes by genetic

engineering technology. J. Ind. Microbiol. Biotechnol. 31, 99–108.

Christi, Y., 1999. Fermentation (Industrial): Basic considerations, in: Robinson, R., Batt, C., Patel, P. (Eds.), Encyclopedia of Food Microbiology. Academic Press, London, pp. 663–674.

Chung, J.D., 2000. Design of metabolic feed controllers: Application to high-density fermentations of *Pichia pastoris*. Biotechnol. Bioeng. 68, 298–307.

Cos, O., Ramón, R., Montesinos, J.L., Valero, F., 2006. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. Microb. Cell Fact. 5, 17.

Cos, O., Resina, D., Ferrer, P., Montesinos, J.L., Valero, F., 2005. Heterologous production of *Rhizopus oryzae* lipase in *Pichia pastoris* using the alcohol oxidase and formaldehyde dehydrogenase promoters in batch and fed-batch cultures. Biochem. Eng. J. 26, 86–94.

Couto, S.R., Sanromán, M.Á., 2006. Application of solid-state fermentation to food industry-A review. J. Food Eng. 76, 291–302.

Cregg, J.M., Cereghino, J.L., Shi, J., Higgins, D.R., 2000. Recombinant protein expression in *Pichia pastoris*. Mol. Biotechnol. 16, 23–52.

Cregg, J.M., Vedvick, T.S., Raschke, W.C., 1993. Recent advances in the expression of foreign genes in *Pichia pastoris*. Nat. Biotechnol. 11, 905–910.

Crittenden, R.G., Playne, M.J., 1996. Production, properties and applications of food-grade oligosaccharides. Trends Food Sci. Technol. 7, 353–361.

Cruz, R., Cruz, V.D., Belini, M.Z., Belote, J.G., Vieira, C.R., 1998. Production of fructooligosaccharides by the mycelia of *Aspergillus japonicus* immobilized in calcium alginate. Bioresour. Technol. 65, 139–143.

D'Anjou, M.C., Daugulis, A.J., 2000. Mixed-feed exponential feeding for fed-batch culture of recombinant methylotrophic yeast. Biotechnol. Lett. 22, 341–346.

de Almeida, J.R.M., de Moraes, L.M.P., Torres, F.A.G., 2005. Molecular characterization of the 3-phosphoglycerate kinase gene (*PGK1*) from the methylotrophic yeast *Pichia pastoris*. Yeast 22, 725–737.

De Schutter, K., Lin, Y.C., Tiels, P., Van Hecke, A., Glinka, S., Weber-Lehmann, J., Rouzé, P., Van De Peer, Y., Callewaert, N., 2009. Genome sequence of the recombinant protein production host *Pichia pastoris*. Nat. Biotechnol. 27, 561–566.

Delroisse, J.M., Dannau, M., Gilsoul, J.J., El Mejdoub, T., Destain, J., Portetelle, D., Thonart, P., Haubruge, E., Vandenbol, M., 2005. Expression of a synthetic gene encoding a *Tribolium castaneum* carboxylesterase in *Pichia pastoris*. Protein Expr. Purif. 42, 286–294.

Dominguez, A.L., Rodrigues, L.R., Lima, N.M., Teixeira, J.A., 2014. An Overview of the Recent Developments on Fructooligosaccharide Production and Applications. Food Bioprocess Technol. 7, 324–337.

Döring, F., Klapper, M., Theis, S., Daniel, H., 1998. Use of the glyceraldehyde-3-phosphate dehydrogenase promoter for production of functional mammalian membrane transport proteins in the yeast *Pichia pastoris*. Biochem Biophys Res Commun 250, 531–535.

Dorta, C., Cruz, R., de Oliva-Neto, P., Moura, D.J.C., 2006. Sugarcane molasses and yeast powder used in the fructooligosaccharides production by *Aspergillus japonicus*-FCL 119T and *Aspergillus niger* ATCC 20611. J. Ind. Microbiol. Biotechnol. 33, 1003–1009.

Duan, H., Umar, S., Hu, Y., Chen, J., 2009. Both the *AOX1* promoter and the *FLD1* promoter work together in a *Pichia pastoris* expression vector. World J. Microbiol. Biotechnol. 25, 1779–1783.

Fernandez, R.C., Ottoni, C. A., Da Silva, E.S., Matsubara, R.M.S., Carter, J.M., Magossi, L.R., Wada, M. a a, De Andrade Rodrigues, M.F., Maresma, B.G., Maiorano, A.E., 2007. Screening of β-fructofuranosidase-producing microorganisms and effect of pH and

temperature on enzymatic rate. Appl. Microbiol. Biotechnol. 75, 87–93.

Ferreira, A.R., Ataíde, F., Von Stosch, M., Dias, J.M.L., Clemente, J.J., Cunha, A.E., Oliveira, R., 2012. Application of adaptive DO-stat feeding control to *Pichia pastoris* X33 cultures expressing a single chain antibody fragment (scFv). Bioprocess Biosyst. Eng. 35, 1603–1614.

Frost & Sullivan, 2008. Strategic Analysis of the European Human Food and Beverage Prebiotics Markets, Frost & Sullivan.

Gellissen, G., 2000. Heterologous protein production in methylotrophic yeasts. Appl. Microbiol. Biotechnol. 54, 741–751.

Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Ferrer, M., Ballesteros, A., Plou, F.J., 2007. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus*. J. Biotechnol. 128, 204–211.

Goosen, C., Yuan, X., Munster, J.M. Van, Ram, A.F.J., van der Maarel, M.J.E.C., Dijkhuizen, L., 2007. Molecular and biochemical characterization of a novel intracellular invertase from *Aspergillus niger* with transfructosylating activity. Eukaryot. Cell 6, 674–681.

Gustafsson, C., Govindarajan, S., Minshull, J., 2004. Codon bias and heterologous protein expression. Trends Biotechnol. 22, 346–353.

He, M., Wu, D., Wu, J., Chen, J., 2014. Enhanced expression of endoinulinase from *Aspergillus niger* by codon optimization in *Pichia pastoris* and its application in inulooligosaccharide production. J. Ind. Microbiol. Biotechnol. 41, 105–114.

Hidaka, H., Hirayama, M., Sumi, N., 1988. A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 52, 1181–1187.

Hirayama, M., Sumi, N., Hidaka, H., 1989. Purification and properties of a fructooligosaccharide-producing β-fructofuranosidase from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 53, 667–673.

Hu, X.Q., Chu, J., Zhang, Z., Zhang, S.L., Zhuang, Y.P., Wang, Y.H., Guo, M.J., Chen, H.X., Yuan, Z.Y., 2008. Effects of different glycerol feeding strategies on S-adenosyl-l-methionine biosynthesis by PGAP-driven *Pichia pastoris* overexpressing methionine adenosyltransferase. J. Biotechnol. 137, 44–49.

Idiris, A., Tohda, H., Kumagai, H., Takegawa, K., 2010. Engineering of protein secretion in yeast: strategies and impact on protein production. Appl. Microbiol. Biotechnol. 86, 403–417.

Inan, M., Chiruvolu, V., Eskridge, K.M., Vlasuk, G.P., Dickerson, K., Brown, S., Meagher, M.M., 1999. Optimization of temperature-glycerol-pH conditions for a fed-batch fermentation process for recombinant hookworm (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) production by *Pichia pastoris*. Enzyme Microb. Technol. 24, 438–445.

Jahic, M., Gustavsson, M., Jansen, A.K., Martinelle, M., Enfors, S.O., 2003. Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes. J. Biotechnol. 102, 45–53.

Kiska, D.L., Macrina, F.L., 1994. Genetic analysis of fructan-hyperproducing strains of *Streptococcus mutans*. Infect Immun 62, 2679–2686.

Kobayashi, K., Kuwae, S., Ohya, T., Ohda, T., Ohyama, M., Ohi, H., Tomomitsu, K., Ohmura, T., 2000. High-level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation. J. Biosci. Bioeng. 89, 55–61.

Küberl, A., Schneider, J., Thallinger, G.G., Anderl, I., Wibberg, D., Hajek, T., Jaenicke, S., Brinkrolf, K., Goesmann, A., Szczepanowski, R., Pühler, A., Schwab, H., Glieder, A., Pichler, H., 2011. High-quality genome sequence of *Pichia pastoris* CBS7435. J. Biotechnol. 154, 312–320.

Lecerf, J.M., Clerc, E., Jaruga, A., Wagner, A., Respondek, F., 2015. Postprandial glycaemic

and insulinaemic responses in adults after consumption of dairy desserts and pound cakes containing short-chain fructo-oligosaccharides used to replace sugars. J Nutr Sci 4, 1–7.

Lee, C.Y., Nakano, A., Shiomi, N., Lee, E.K., Katoh, S., 2003. Effects of substrate feed rates on heterologous protein expression by *Pichia pastoris* in DO-stat fed-batch fermentation. Enzyme Microb. Technol. 33, 358–365.

Li, Y.M., Li, D.J., Xu, X.J., Cui, M., Zhen, H.H., Wang, Q., 2014. Effect of codon optimization on expression levels of human cystatin C in *Pichia pastoris*. Genet. Mol. Res. 13, 4990–5000.

Li, Z., Xiong, F., Lin, Q., D'Anjou, M., Daugulis, A.J., Yang, D.S.C., Hew, C.L., 2001. Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. Protein Expr. Purif. 21, 438–445.

Lim, H.K., Choi, S.J., Kim, K.Y., Jung, K.H., 2003. Dissolved-oxygen-stat controlling two variables for methanol induction of rGuamerin in *Pichia pastoris* and its application to repeated fed-batch. Appl. Microbiol. Biotechnol. 62, 342–348.

Liu, H., Tan, X., Russel, K.A., Veenhuis, M., Cregg, J.M., 1995. *PER3*, a gene required for peroxisome biogenesis in *Pichia pastoris*, encodes a peroxisomal membrane protein involved in protein import. J. Biol. Chem. 270, 10940–10951.

Lorenzoni, A.S.G., Aydos, L.F., Klein, M.P., Rodrigues, R.C., Hertz, P.F., 2014. Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from *Aspergillus aculeatus*. Carbohydr. Polym. 103, 193–197.

Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M., 2005. Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22, 249–70.

Macauley-Patrick, S., Finn, B., 2008. Modes of fermenter operation, in: McNeil, B., Harvey, L.M. (Eds.), Practical Fermentation Technology. John Wiley & Sons, Ltd, pp. 69–95.

Maiorano, A.E., Piccoli, R.M., Da Silva, E.S., De Andrade Rodrigues, M.F., 2008. Microbial

production of fructosyltransferases for synthesis of pre-biotics. Biotechnol. Lett. 30, 1867–1877.

Mattanovich, D., Graf, A., Stadlmann, J., Dragosits, M., Redl, A., Maurer, M., Kleinheinz, M., Sauer, M., Altmann, F., Gasser, B., 2009. Genome, secretome and glucose transport highlight unique features of the protein production host *Pichia pastoris*. Microb. Cell Fact. 8, 29–41.

Menéndez, C., Hernández, L., Banguela, A., País, J., 2004. Functional production and secretion of the *Gluconacetobacter diazotrophicus* fructose-releasing exo-levanase (LsdB) in *Pichia pastoris*. Enzyme Microb. Technol. 34, 446–452.

Menéndez, C., Martínez, D., Trujillo, L.E., Mazola, Y., González, E., Pérez, E.R., Hernández, L., 2013. Constitutive high-level expression of a codon-optimized β-fructosidase gene from the hyperthermophile *Thermotoga maritima* in *Pichia pastoris*. Appl. Microbiol. Biotechnol. 97, 1201–1212.

Menendez, J., Valdes, I., Cabrera, N., 2003. The *ICL1* gene of *Pichia pastoris*, transcriptional regulation and use of its promoter. Yeast 20, 1097–1108.

Menrad, K., 2003. Market and marketing of functional food in Europe. J. Food Eng. 56, 181–188.

Mussatto, S.I., Aguilar, C.N., Rodrigues, L.R., Teixeira, J. a., 2009. Fructooligosaccharides and β-fructofuranosidase production by *Aspergillus japonicus* immobilized on lignocellulosic materials. J. Mol. Catal. B Enzym. 59, 76–81.

Mussatto, S.I., Mancilha, I.M., 2007. Non-digestible oligosaccharides: A review. Carbohydr. Polym. 68, 587–597.

Mussatto, S.I., Prata, M.B., Rodrigues, L.R., Teixeira, J.A., 2012. Production of fructooligosaccharides and β-fructofuranosidase by batch and repeated batch fermentation with immobilized cells of *Penicillium expansum*. Eur. Food Res. Technol. 235, 13–22.

Mussatto, S.I., Teixeira, J. a., 2010. Increase in the fructooligosaccharides yield and productivity by solid-state fermentation with *Aspergillus japonicus* using agro-industrial residues as support and nutrient source. Biochem. Eng. J. 53, 154–157.

Nemukula, A., Mutanda, T., Wilhelmi, B.S., Whiteley, C.G., 2009. Response surface methodology: Synthesis of short chain fructooligosaccharides with a fructosyltransferase from *Aspergillus aculeatus*. Bioresour. Technol. 100, 2040–5.

Nguyen, Q.D., Mattes, F., Hoschke, A., Rezessy-Szabo, J., Bhat, M.K., 1999. Production, purification and identification of fructooligosaccharides produced by β-fructofuranosidase from *Aspergillus niger* IMI 303386. Biotechnol. Lett. 21, 183–186.

Nishizawa, K., Akajima, M.N., Abetani, H.N., 2001. Kinetic study on transfructosylation by β-fructofuranosidase from *Aspergillus niger* ATCC 20611 and availability of a membrane reactor for fructooligosaccharide production. Food Sci. Technol. Res. 7, 39–44.

Nobre, C., Teixeira, J. a., Rodrigues, L.R., 2015. New trends and technological challenges in the industrial production and purification of fructo-oligosaccharides. Crit. Rev. Food Sci. Nutr. 55, 1444–1455.

O'Callaghan, J., O'Brien, M.M., McClean, K., Dobson, A.D.W., 2002. Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*. J. Ind. Microbiol. Biotechnol. 29, 55–59.

Ohya, T., Morita, M., Miura, M., Kuwae, S., Kobayashi, K., 2002. High-level production of prourokinase-annexin V chimeras in the methylotrophic yeast *Pichia pastoris*. J. Biosci. Bioeng. 94, 467–473.

Olędzka, G., Dąbrowski, S., Kur, J., 2003. High-level expression, secretion, and purification of the thermostable aqualysin I from *Thermus aquaticus* YT-1 in *Pichia pastoris*. Protein Expr. Purif. 29, 223–229.

Paineau, D., Respondek, F., Menet, V., Sauvage, R., Bornet, F., Wagner, A., 2014. Effects of

short-chain fructooligosaccharides on faecal bifidobacteria and specific immune response in formula-fed term infants: a randomized, double-blind, placebo-controlled trial. J. Nutr. Sci. Vitaminol. (Tokyo). 60, 167–75.

Palcic, M.M., 1999. Biocatalytic synthesis of oligosaccharides. Curr. Opin. Biotechnol. 10, 616–624.

Park, J.P., Oh, T.K., Yun, J.W., 2001. Purification and characterization of a novel transfructosylating enzyme from *Bacillus macerans* EG-6. Process Biochem. 37, 471–476.

Porro, D., Sauer, M., Branduardi, P., Mattanovich, D., 2005. Recombinant protein production in yeasts. Mol. Biotechnol. 31, 245–259.

Potvin, G., Ahmad, A., Zhang, Z., 2012. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochem. Eng. J. 64, 91–105.

Prapulla, S.G., Subhaprada, V., Karanth, N.G., 2000. Microbial production of oligosaccharides: a review. Adv. Appl. Microbiol. 47, 299–343.

Quax, T.E.F., Claassens, N.J., Söll, D., van der Oost, J., 2015. Codon Bias as a Means to Fine-Tune Gene Expression. Mol. Cell 59, 149–161.

Rehm, J., Willmitzer, L., Heyer, A.G., 1998. Production of 1-kestose in transgenic yeast expressing a fructosyltransferase from *Aspergillus foetidus*. J. Bacteriol. 180, 1305–1310.

Resina, D., Serrano, A., Valero, F., Ferrer, P., 2004. Expression of a *Rhizopus oryzae* lipase in *Pichia pastoris* under control of the nitrogen source-regulated formaldehyde dehydrogenase promoter. J. Biotechnol. 109, 103–113.

Ritsema, T., Smeekens, S., 2003. Fructans: Beneficial for plants and humans. Curr. Opin. Plant Biol. 6, 223–230.

Roberfroid, M.B., 1999. Caloric value of inulin and oligofructose. J. Nutr. 129, 1436S–1437S.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2005. Recent trends in the microbial

production, analysis and application of fructooligosaccharides. Trends Food Sci. Technol. 16, 442–457.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2004. Production of fructosyl transferase by *Aspergillus oryzae* CFR 202 in solid-state fermentation using agricultural by-products. Appl. Microbiol. Biotechnol. 65, 530–537.

Sears, I.B., O'Connor, J., Rossanese, O.W., Glick, B.S., 1998. A versatile set of vectors for constitutive and regulated gene expression in *Pichia pastoris*. Yeast 14, 783–790.

Singh, R.S., Singh, R.P., 2010. Production of fructooligosaccharidesfrom inulin by endoinulinases and their prebiotic potential. Food Technol. Biotechnol. 48, 435-450

Spohner, S.C., Czermak, P., 2016. Heterologous expression of *Aspergillus terreus* fructosyltransferase in *Kluyveromyces lactis*. N. Biotechnol. 33, 473–479.

Stahl, C.H., Wilson, D.B., Lei, X.G., 2003. Comparison of extracellular *Escherichia coli* AppA phytases expressed in *Streptomyces lividans* and *Pichia pastoris*. Biotechnol. Lett. 25, 827–831.

Stanbury, P.F., Whitaker, A., Hall, S.J., 2003. Principles of Fermentation Technology, 2nd ed. Butterworth-Heinemann.

Stapleton, P.C., O'Brien, M.M., O'Callaghan, J., Dobson, A.D.W., 2004. Molecular cloning of the cellobiose dehydrogenase gene from *Trametes versicolor* and expression in *Pichia pastoris*. Enzyme Microb. Technol. 34, 55–63.

Stratton, J., Chiruvolu, V., Meagher, M.M., 1998. High cell-density fermentation, in: Higgins, D.R., Cregg, J.M. (Eds.), Methods in Molecular Biology. Humana Press, Totowa, New Jersey, pp. 107–120.

Trinh, L.B., Phue, J.N., Shiloach, J., 2003. Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from *Pichia pastoris*. Biotechnol. Bioeng. 82, 438–444.

Trollope, K.M., Görgens, J.F., Volschenk, H., 2015. Semirational directed evolution of loop regions in *Aspergillus japonicus* β-fructofuranosidase for improved fructooligosaccharide production. Appl. Environ. Microbiol. 81, 7319–7329.

Trujillo, L.E., Arrieta, J.G., Dafhnis, F., García, J., Valdés, J., Tambara, Y., Pérez, M., Hernández, L., 2001. Fructo-oligosaccharides production by the *Gluconacetobacter diazotrophicus* levansucrase expressed in the methylotrophic yeast *Pichia pastoris*. Enzyme Microb. Technol. 28, 139–144.

Van Hijum, S.A.F.T., Van Geel-Schutten, G.H., Rahaoui, H., Van der Maarel, M.J.E.C., Dijkhuizen, L., 2002. Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. Appl. Environ. Microbiol. 68, 4390–4398.

Vandáková, M., Platková, Z., Antošová, M., Báleš, V., Polakovič, M., 2004. Optimization of cultivation conditions for production of fructosyltransferase by *Aureobasidium pullulans*. Chem. Pap. 58, 15–22.

Vassileva, A., Chugh, D.A., Swaminathan, S., Khanna, N., 2001. Expression of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris* using the *GAP* promoter. J. Biotechnol. 88, 21–35.

Vega-Paulino, R.J., Zúniga-Hansen, M.E., 2012. Potential application of commercial enzyme preparations for industrial production of short-chain fructooligosaccharides. J. Mol. Catal. B Enzym. 76, 44–51.

Vega, R., Zúniga-Hansen, M.E., 2011. Enzymatic synthesis of fructooligosaccharides with high 1-kestose concentrations using response surface methodology. Bioresour. Technol. 102, 10180–10186.

Velázquez-Hernández, M.L., Baizabal-Aguirre, V.M., Bravo-Patiño, A., Cajero-Juárez, M., Chávez-Moctezuma, M.P., Valdez-Alarcón, J.J., 2009. Microbial fructosyltransferases

and the role of fructans. J. Appl. Microbiol. 106, 1763–1778.

Woo, S.H., Park, S.H., Lim, H.K., Jung, K.H., 2005. Extended operation of a pressurized 75-L bioreactor for shLkn-1 production by *Pichia pastoris* using dissolved oxygen profile control. J. Ind. Microbiol. Biotechnol. 32, 474–480.

Yamawaki, S., Matsumoto, T., Ohnishi, Y., Kumada, Y., Shiomi, N., Katsuda, T., Lee, E.K., Katoh, S., 2007. Production of single-chain variable fragment antibody (scFv) in fed-batch and continuous culture of *Pichia pastoris* by two different methanol feeding methods. J. Biosci. Bioeng. 104, 403–407.

Yanai, K., Nakane, A., Kawate, A., Hirayama, M., 2001. Molecular cloning and characterization of the fructooligosaccharide-producing β-fructofuranosidase gene from *Aspergillus niger* ATCC 20611. Biosci. Biotechnol. Biochem. 65, 766–73.

Yang, H., Wang, Y., Zhang, L., Shen, W., 2016. Heterologous expression and enzymatic characterization of fructosyltransferase from *Aspergillus niger* in *Pichia pastoris*. N. Biotechnol. 33, 164–170.

Yoshikawa, J., Amachi, S., Shinoyama, H., Fujii, T., 2008. Production of fructooligosaccharides by crude enzyme preparations of β-fructofuranosidase from *Aureobasidium pullulans*. Biotechnol. Lett. 30, 535–539.

Yoshikawa, J., Amachi, S., Shinoyama, H., Fujii, T., 2007. Purification and some properties of beta-fructofuranosidase I formed by *Aureobasidium pullulans* DSM 2404. J. Biosci. Bioeng. 103, 491–493.

Yun, J.W., 1996. Fructooligosaccharides—Occurrence, preparation, and application. Enzyme Microb. Technol. 19, 107–117.

Yun, J.W., Kim, D.H., Song, S.K., 1997. Enhanced production of fructosyltransferase and glucosyltransferase by substrate-feeding cultures of *Aureobasidium pullulans*. J. Ferment. Bioeng. 84, 261–263.

Yun, J.W., Lee, M.G., Song, S.K., 1994. Batch production of high-content fructo-oligosaccharides from sucrose by the mixed-enzyme system of β-fructofuranosidase and glucose oxidase. J. Ferment. Bioeng. 77, 159–163.

Zhang, J., Liu, C., Xie, Y., Li, N., Ning, Z., Du, N., Huang, X., Zhong, Y., 2017. Enhancing fructooligosaccharides production by genetic improvement of the industrial fungus *Aspergillus niger* ATCC 20611. J. Biotechnol. 249, 25–33.

Zhang, W., Bevins, M.A., Plantz, B.A., Smith, L.A., Meagher, M.M., 2000. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnol. Bioeng. 70, 1–8.

Zhong, X., Peng, L., Zheng, S., Sun, Z., Ren, Y., Dong, M., Xu, A., 2004. Secretion, purification, and characterization of a recombinant *Aspergillus oryzae* tannase in *Pichia pastoris*. Protein Expr. Purif. 36, 165–169.

Zhou, W.J., Yang, J.K., Mao, L., Miao, L.H., 2015. Codon optimization, promoter and expression system selection that achieved high-level production of *Yarrowia lipolytica* lipase in *Pichia pastoris*. Enzyme Microb. Technol. 71, 66–72.

Chapter 3 **Research objectives**

The aim of the study is to optimize the heterologous expression of the β-fructofuranosidase enzyme from *A. niger* ATCC 20611 in *P. pastoris* through bioprocess optimization, together with the subsequent optimization of short-chain fructooligosaccharides production from industrial sugar streams using the enzyme.

3.1 Objective one (Chapter 4): To produce a yeast expression system for the optimum production of β-fructofuranosidase

It has been shown that improvement of the production strain in a bioprocess could have a significant cost reduction without substantial capital outlay. Additionally, a superior production strain will invariably result in a superior production process, which makes the optimization of the strain worthwhile. Various molecular techniques can be applied to improve the expression of recombinant proteins from *P. pastoris* but due to the variation in results from these techniques each has to be evaluated on a case by case basis. Therefore, the first objective is to construct and optimize a *P. pastoris* strain for the heterologous expression of the fopA and fopA_V1 enzyme derived from *A. niger* 20611 through strain selection, promoter selection and codon optimization as these have not been demonstrated in literature for these enzymes. The first step will be to determine the best *P. pastoris* host strain for the expression of the enzyme. We have selected DSMZ 70382 from the German Collection of Microorganisms and Cell Cultures and X-33 from Thermo Fischer Scientific. The former was selected due to the freedom to use it commercially, but it is less well known than the latter. The X-33 strain has been through significantly more development for recombinant expression but is hampered by commercial licensing. A further attempt to improve the expression of fopA and fopA_V1 in *P. pastoris* two different promoters, *AOX1* and *GAP*, will be compared in the superior strain determined in the comparison mentioned above. Previous studies have shown that the level of

expression of recombinant proteins, with regards to these two promoters, varies with neither these promoters being the best in all cases (Boer et al., 2000; Delroisse et al., 2005; Döring et al., 1998; Vassileva et al., 2001). Therefore, a comparison of the expression of these two enzymes with these promoters is necessary since it has not yet been reported. Finally, the *fopA* gene will be codon-optimized for improved expression by two different companies, GeneArt® and ATUM. Differences in enzyme expression due to codon optimization by these two companies have not yet been shown for fopA and the expression of fopA_V1 in *P. pastoris* has also not been attempted.

3.2 Objective two (Chapter 5): Optimize the production of the β-fructofuranosidase enzymes by using different fed-batch methods

The second objective is the bioprocess optimization of the enzyme production by the strains exhibiting the highest volumetric activity in objective 3.1, for both fopA and fopA_V1, under control of the *GAP* promoter from objective 1, in bioreactors by using two different fed-batch feeding strategies. A previous study expressed the codon-optimized fopA enzyme in *P. pastoris* using a DO-stat feeding strategy to compare the *AOX1* and *GAP* promoters using defined and semi-defined media (Anane et al., 2016) which resulted in a volumetric activity of ~4 500 U/ml and ~9 000 U/ml for DNA 2.0 codon-optimized (now known as ATUM) fopA enzyme under control of the *GAP* and *AOX1* promoters, respectively. In this study, we will further optimize the expression in bioreactors by comparing the DO-stat feeding strategy to that of a constant feeding strategy, for both fopA and fopA_V1. The strains with *GAP* promoters will be used because it avoids the use and storage of the substantial quantities of volatile and flammable methanol in future large-scale commercial operations. The previous study that engineered the native fopA enzyme to obtain fopA_V1 only showed enzyme production at millilitre scale, with enzyme characterisation in terms of thermostability, specific activity and feedback inhibition from glucose (Trollope et al., 2015). The present objective will evaluate differences

in production levels between the two enzymes in 10 L bioreactors under optimised bioprocess conditions as a comparison of these two feeding strategies for the fopA and fopA_V1 in *P. pastoris* have not been reported.

3.3 Objective three (Chapter 6): Evaluate the difference between the native and an engineered β-fructofuranosidase enzyme to produce a specific scFOS composition

Response surface methodology (RSM) has been used to optimize reaction conditions for FOS production for a fructosyltransferase enzyme and to target the increased production of a specific FOS (Nemukula et al., 2009; Vega and Zúniga-Hansen, 2011). A previous study engineered the native fopA enzyme to improve the production of FOS. The engineered enzyme, fopA_V1, exhibited increased thermostability, increased specific activity and decreased glucose inhibition which resulted in a 22% decrease in reaction time to achieve a targeted scFOS composition (37% GF2, 53% GF3 and 10% GF4). However, the study did not optimize the reaction conditions for either the native fopA enzyme or the new fopA_V1 variant (Trollope et al., 2015). Therefore, the objective is to initially optimize the reaction conditions to produce scFOS from laboratory grade sucrose with RSM for both fopA and fopA_V1, separately, with regards to temperature and enzyme dosage, to fully characterise differences in the biocatalytic performances of these enzymes and to then apply these conditions to industrial sugar streams. These conditions were chosen due to the improved thermostability and specific activity shown by the fopA_V1 enzyme relative to the native fopA enzyme (Trollope et al., 2015). The scFOS composition of a leading commercial scFOS product, Actilight®, will be used as a benchmark to evaluate which of these enzymes is superior in their ability to produce scFOS. The viability of these enzymes to produce the target scFOS composition from industrial sugar streams was also assessed, to determine the impact of impurities present in either crude industrial sugars or

refinery molasses, on the biocatalytic activity of the enzymes. The production of scFOS will also be done at a larger scale to evaluate the effect of scale-up on the reaction.

3.4 References

Anane, E., van Rensburg, E., Görgens, J.F., 2016. Comparison of constitutive and inducible β-fructofuranosidase production by recombinant *Pichia pastoris* in fed-batch culture using defined and semi-defined media. South African J. Chem. Eng. 22, 17–22.

Boer, H., Teeri, T.T., Koivula, A., 2000. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7a secreted from *Pichia pastoris* using two different promoters. Biotechnol. Bioeng. 69, 486–494.

Delroisse, J.M., Dannau, M., Gilsoul, J.J., El Mejdoub, T., Destain, J., Portetelle, D., Thonart, P., Haubruge, E., Vandenbol, M., 2005. Expression of a synthetic gene encoding a *Tribolium castaneum* carboxylesterase in *Pichia pastoris*. Protein Expr. Purif. 42, 286–294.

Döring, F., Klapper, M., Theis, S., Daniel, H., 1998. Use of the glyceraldehyde-3-phosphate dehydrogenase promoter for production of functional mammalian membrane transport proteins in the yeast *Pichia pastoris*. Biochem Biophys Res Commun 250, 531–535.

Nemukula, A., Mutanda, T., Wilhelmi, B.S., Whiteley, C.G., 2009. Response surface methodology: Synthesis of short chain fructooligosaccharides with a fructosyltransferase from *Aspergillus aculeatus*. Bioresour. Technol. 100, 2040–5.

Trollope, K.M., Görgens, J.F., Volschenk, H., 2015. Semirational directed evolution of loop regions in *Aspergillus japonicus* β-fructofuranosidase for improved fructooligosaccharide production. Appl. Environ. Microbiol. 81, 7319–7329.

Vassileva, A., Chugh, D.A., Swaminathan, S., Khanna, N., 2001. Expression of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris* using the *GAP* promoter. J. Biotechnol. 88, 21–35.

Vega, R., Zúniga-Hansen, M.E., 2011. Enzymatic synthesis of fructooligosaccharides with high 1-kestose concentrations using response surface methodology. Bioresour. Technol. 102, 10180–10186.

Chapter 4 Heterologous expression of the codon-optimized *Aspergillus fijiensis* β-fructofuranosidase in *Pichia pastoris*

Gerhardt Coetzee, Jacques J. Smith and Johann F. Görgens

Department of Process Engineering, Stellenbosch University, Stellenbosch, South Africa

Objective of dissertation in this chapter

This chapter addresses objective one, which focusses on the construction of a yeast expression system for the optimum production of the β-fructofuranosidase enzyme from *A. fijiensis* ATCC 20611 by evaluating two different *P. pastoris* strains, two different promoters and two different companies for codon-optimization. The comparison of the fopA enzyme production between two *P. pastoris* strains (X-33 and DSMZ 70382), the comparison of the differences in enzyme expression when the *fopA* gene is codon-optimized by ATUM and Geneart® and the comparison of the production of the two different codon-optimized enzymes under control of the *GAP* and *AOX1* promoters have not yet been reported. Therefore, the first step was to select the best strain between *P. pastoris* X-33 and *P. pastoris* DSMZ 70382 for the expression and production of the Geneart® codon-optimized fopA enzyme with the DSMZ 70382 strain subsequently proving to be superior. The *P. pastoris* DSMZ 70382 strain was selected for the expression of the two different codon-optimized genes under the control of the two promoters. The *AOX1* promoter proved to be superior in both shake flasks and the bioreactors while the ATUM codon-optimized gene expression was superior under control of both the *GAP* and *AOX1* promoters in the bioreactors when compared to Geneart®. The fopA enzyme was purified from culture supernatant and the specific activity determined along with the protein concentration of the various bioreactor cultivations of the different strains. The preferred strain

from this chapter will be used for objective two. Therefore, all the objectives are addressed in this chapter as set out in Chapter 3.

Candidate declaration

In chapter 4 page numbers 51 to 74 of this dissertation, the type and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Planning of experiments	70
Executing experiments	80
Interpretation of results	70
Writing and reviewing of the chapter	80

The listed co-authors have contributed to chapter 4 page numbers 51 to 74 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution (%)
Jacques J. Smith		Planning of experiments	20
		Executing experiments	20
		Interpretation of results	20
		Writing and reviewing of the chapter	10
Johann F. Görgens		Planning of experiments	10
		Interpretation of results	10
		Writing and reviewing of the chapter	10

Signature of candidate: 

Date: 27 January 2019

Declaration by co-authors

The undersigned hereby confirm that

- The declaration above accurately reflects the type and extent of the contributions of the candidates and co-authors to chapter 4 page numbers 51 to 74 in the dissertation,
- No other authors contributed to chapter 4 page numbers 51 to 74 in the dissertation besides those specified above, and
- Potential conflicts of interest have been revealed to all interested parties and that necessary arrangements have been made to use the material in chapter 4 page numbers 51 to 74 of this dissertation.

Institutional affiliation	Date	Signature
Geosyntec Consultants Inc	16/12/2018	

Abstract

Fructooligosaccharides (FOS) are prebiotic compounds added to functional foods for their various health properties. They are commercially produced through the transfructolysation of sucrose by β-fructofuranosidases from organisms such as *Aureobasidium pullulans* or *Aspergillus niger*. This study used recombinant technology to overexpress the *Aspergillus fijiensis* β-fructofuranosidase-encoding gene in *Pichia pastoris* under the transcriptional control of the alcohol oxidase (*AOX1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAP*) gene promoters using two versions of codon-optimized *fopA* copies. In shake flasks, the volumetric enzyme activities of the two variants were similar when expressed under control of the same promoter (11.7 U/ml and 12.7 U/ml for the respective *GAP* strains and 95.8 U/ml and 98.6 U/ml for the respective *AOX1* strains), but the *AOX1* promoter displayed the highest production levels for both codon-optimized genes. When cultivated in bioreactors, the *AOX1* promoter outperformed the *GAP* promoter for both codon-optimized genes in *P. pastoris* (13 702 U/ml and 2 718 U/ml for the *AOX1* promoter for ATUM and Geneart®, respectively and 6 057 U/ml and 1790 U/ml for the *GAP* promoter for ATUM and Geneart®, respectively), while the enzyme activity from the ATUM optimized gene was higher than the one from Geneart®, irrespective of the promoter used.

4.1 Introduction

Fructooligosaccharides (FOS) are popular prebiotic compounds due to their biological and functional properties and are widely used in functional foods. They are low-calorie and non-cariogenic with various health benefits such as decreasing the levels of phospholipids, triglycerides and cholesterol in the blood, improving the absorption of various minerals in the gut and stimulating the growth of *Bifidobacteria* in the human colon (Lu et al., 2013; Maiorano et al., 2008). The supplementation of food products with FOS therefore drives the demand for large-scale production. FOS can be isolated from foods such as onions, Jerusalem artichokes, wheat, rye and garlic or produced enzymatically either by synthesis from sucrose by using fructosyltransferases (EC 2.4.1.9) and β-fructofuranosidases (FFase; EC 3.2.1.26), or by partial hydrolysis of inulin using endo-inulinases (EC 3.2.1.7) (Maiorano et al., 2008; Singh and Singh, 2010). The FOS produced by FFase result in inulin type FOS (GF_n) with β-(2→1)-linked fructose units with a degree of polymerization (DP) of 2-8 (n = 1-7) (Roberfroid et al., 2010).

In addition, FFases are produced by a variety of microbial hosts, of which those originating from *Aureobasidium pullulans* and *Aspergillus* spp. are predominantly sourced for commercial FOS production (Maiorano et al., 2008). Although a number of other promising sources of FFases for FOS production have been reported (Dhake and Patil, 2007; Jedrzejczak-Krzepkowska et al., 2011; Nguyen et al., 2005; Yoshikawa et al., 2007), only a few recombinant FFases for the express purpose of producing FOS are known. Rehm et al. (1998) expressed the sucrose:sucrose 1-fructosyltransferase (1-SST) gene of *Aspergillus foetidus* in *Saccharomyces cerevisiae* to produce 1-kestose, whereas, 1-kestose and 1-nystose were produced by expressing the *Gluconacetobacter diazotrophicus* levansucrase gene (*lsdA*) and the fructosyltransferase (*ftf*) gene from *Lactobacillus reuteri* in *P. pastoris* and *Escherichia coli*, respectively (Trujillo et al., 2001; Van Hijum et al., 2002). More recently,

Spohner and Czermak (2016) expressed and secreted a codon-optimized version of the putative *Aspergillus terreus* β-fructofuranosidase ATEG 04996 which was able to produce FOS comprising of 1-kestose (GF2), nystose (GF3) and 1^F-fructofuranosyl nystose (GF4) with small amounts of GF5 and GF6.

Although *S. cerevisiae* is a well-known yeast expression system, it has been plagued by plasmid instability, low protein yields and hyperglycosylation of proteins (Buckholz and Gleeson, 1991). Alternative systems were developed in other yeasts, including the methylotrophic yeast *P. pastoris*. This yeast exhibits several advantages such as the ability to grow to high cell densities and fast growth on minimal media with no production of intrinsic endotoxins or viral DNA. It secretes low levels of endogenous proteins, which simplifies the purification of the secreted recombinant protein and it has the ability to perform eukaryotic post-translational modifications such as glycosylation and the formation of disulphide bonds (Çelik and Çalık, 2012; Vogl and Glieder, 2013).

Our primary interest in this study was to maximize the expression of a functional fopA enzyme in *P. pastoris*. Different approaches for gene sequence optimization were compared using the different promoters and hosts, and the best performing transformants were evaluated in bioreactor cultures.

4.2 Materials and Methods

4.2.1 Strains and media

The *E. coli* DH5α strain was used as host to maintain and amplify newly constructed plasmids as well as expression vectors required for yeast strain construction. The *E. coli* DH5α parental strain and transformants were grown at 37°C on Luria Bertani (LB) agar plates (0.5% yeast extract, 1% tryptone, 0.5% NaCl) supplemented with either 25 µg/ml zeocin, 50 µg/ml

ampicillin or 100 µg/ml kanamycin, as required. Bacteria were grown in liquid LB media and all plasmid preparations for cloning, transformation was performed using cetyltrimethyl ammonium bromide (CTAB) (Del Sal et al., 1988). Routine culturing and maintenance of *P. pastoris* DSMZ 70382 and X-33 was performed using YPD (1% yeast extract, 2% tryptone, 1% glucose) agar plates or broth and incubated at 30°C. Strains and plasmids used in this study are summarized in Table 4-1.

4.2.2 Construction of expression cassettes

All DNA manipulations were performed as outlined by Sambrook et al. (1989). The initial reports on the isolation of *fopA* and the enzyme indicate that it is from *Aspergillus niger* ATCC 20611 (Hidaka et al., 1988; Hirayama et al., 1989; Yanai et al., 2001), but the strain has been reclassified as *A. fijiensis* by the curators of the ATCC culture collection (http://www.lgcstandards-atcc.org). A synthetic construct containing the *Trichoderma reesei* endoxylanase II (*xln2*) secretion signal (Saarelainen et al., 1993) fused to the mature coding sequence of the *fopA* β-fructofuranosidase (GenBank accession no. AB046383) without the native *fopA* secretion signal was codon-optimized for expression in *P. pastoris* by GeneArt® (Regensburg, Germany). The synthetic *xln2-fopA* was provided on a cloning plasmid pMK-RQ-*fopA* with the appropriate 5' and 3' restriction sites for sub-cloning into *P. pastoris* expression plasmids.

For expression in *P. pastoris* X-33 and DSMZ 70382, the *xln2-fopA* construct was excised from the GeneArt® cloning plasmid pMK-RQ-*fopA* using *Bst*BI and *Xho*I and cloned in-frame into corresponding sites of plasmids pPICZC and pGAPZαB that allowed for inducible (pPICZC-*xln2-fopA*) and constitutive (pGAPZαB-*xln2-fopA*) gene expression, respectively.

Table 4-1. Microbial strains used in this study.

Strains and plasmids	Relevant genotype or construct	Reference or source
Escherichia coli DH5α	fhuA2Δ(argF-lacZ)U169 *phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs, Midrand, South Africa
Pichia pastoris DSMZ 70382	Type strain	Leibniz Institute DSMZ – German Collection of microorganisms and cell cultures
Pichia pastoris X33	Wild-type strain	Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, USA
pMK-RQ-*fopA_G*	*kanR Col E1 fopA_G*	GeneArt® (Regensburg, Germany)
pPICZC	*Sh ble* $AOX1_P$-*mfα*-$AOX1_T$	Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, USA
pGAPZαB	*Sh ble* GAP_P-*mfα*-$AOX1_T$	Invitrogen, Life Technologies, Thermo Fisher Scientific, Waltham, USA
pPICZ-*fopA_G*	*Sh ble* $AOX1_P$-$xln2_S$-*fopA_G*-$AOX1_T$	This study
pGAPZαB-*fopA_G*	*Sh ble* GAP_P-$xln2_S$-*fopA_G*-$AOX1_T$	This study
pJ901-*fopA_A*	*Sh ble* $AOX1_P$-$xln2_S$-*fopA_A*-$AOX1_T$	This study; ATUM (Menlo Park, USA)
pJ905-*fopA_A*	*Sh ble* GAP_P-$xln2_S$-*fopA_A*-$AOX1_T$	This study; ATUM (Menlo Park, USA)
PX33-GG	*P. pastoris* X33 with pGAPZαB-*fopA_G*	This study
PX33-AG	*P. pastoris* X33 with pPICZ-*fopA_G*	This study
P70382-GG	*P. pastoris* DSMZ 70382 with pGAPZαB-*fopA_G*	This study
P70382-AG	*P. pastoris* DSMZ 70382 with pPICZ-*fopA_G*	This study
P70382-GD2	*P. pastoris* DSMZ 70382 with pJ905-*fopA_A*	This study
P70382-AD2	*P. pastoris* DSMZ 70382 with pJ901-*fopA_A*	This study

ATUM (Menlo Park, CA, USA) provided synthetic *P. pastoris* expression plasmids harbouring the alternative codon-optimized *fopA* gene expressed under the transcriptional control of either *AOXI* or *GAP* promoters (pJ901-*fopA* and pJ905-*fopA*, respectively), which were transformed into *P. pastoris* DSMZ 70382 only.

4.2.3 Yeast transformation

The *P. pastoris* strains were transformed with *Sac*I-linearised plasmids using electroporation according to Becker and Guarente (1991). The cells were pulsed for approximately 5 ms with a field strength of 1.5 kV, 200 Ω and 25 µF using a Bio-Rad Gene Pulser® II (Bio-Rad, Hercules, CA, USA). Following electroporation, the cells were allowed to recover for 1 h at 30 °C in a 750 µL YPD and 250 µl 1 M sorbitol mixture followed by plating onto YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 1.5% agar) supplemented with zeocin and incubated at 30 °C for 3 to 5 days.

4.2.4 Protein production in shake flasks

The *P. pastoris* X-33 and DSMZ 70382 transformants were cultured in triplicate in 20 ml buffered glycerol-complex media (BMGY: 1% glycerol, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base and 4×10^{-5}% biotin) to generate biomass. When sufficient biomass was obtained (A_{600} ~10) after approximately 16 h, a sufficient volume of culture to yield an initial A_{600} of 1 was transferred to 75 ml buffered methanol-complex media (BMMY: 0.5% methanol, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base and 4×10^{-5}% biotin) in 500 ml baffled. For constitutive expression, X-33 and DSMZ 70382 transformants were inoculated directly into 75 ml BMGY in 500 ml baffled flasks. Cultures were incubated at 30 °C with agitation at 200 rpm for both biomass generation and protein production. The inducible cultures were supplemented with methanol to a final concentration of 0.5% and the constitutive cultures with

glycerol to a final concentration of 1% after 24 and 48 h. Strains transformed with the respective vector backbones without the codon-optimized *fopA* served as negative controls. The supernatants of each transformant were collected after 72 h of culturing to determine the enzyme activity.

4.2.5 Bioreactor cultivations

Fermentation basal salt medium, supplemented with 1% casamino acids and PTM_1 trace salts, was used as culture media as described by the *Pichia* fermentation process guidelines (Invitrogen Corporation, 2002). The fermentations were performed in 1.3 L Bioflo 110 bioreactors (New Brunswick Scientific, Edison, NJ, USA) at 30°C, pH 5 (maintained with 28% ammonium hydroxide), aeration rate of 1.0 volume of oxygen per volume of fermentation culture per minute (vvm), and 30% dissolved oxygen (DO) (controlled by a cascade effect between agitation (200-1000 rpm) and sparging O_2 when required). Fermentations were performed as per the Pichia fermentation process guidelines (Invitrogen Corporation, 2002) with the following additions: During the glycerol fed-batch phase for the strains containing the *AOX1* promoter, glycerol was fed on-demand (feeding started when DO reached 30% and stopped when the DO was below 30%) for 24 h, followed by the methanol fed-batch phase. The methanol was fed continuously at an initial rate of 1.8 ml/h/L of initial fermenter volume for 1 hr, after which the feed rate was incrementally increased to 3.6, 5.4, 7.3, 9.1 and 10.9 ml/h/L. The feed rate of 5.4 and 9.1 ml/h/L lasted for an hour each, while 3.6 and 7.3 ml/h/L lasted for 2 hours. The last feed rate of 10.9 ml/h/L was maintained for the remainder of the fermentation. For strains containing the *GAP* promoter, the glycerol fed-batch phase the DO-stat was applied for the duration of the fermentation after the glycerol batch phase at a rate of 18.15 ml/h/L of initial fermentation volume.

Samples were taken throughout the fermentations to monitor the A_{600}, wet cell weight and dry cell weight. Enzyme activities were determined at the points of maximum biomass concentration obtained during the fermentation.

4.2.6 Enzyme activity assay

The supernatant of yeast cultures was collected by centrifugation, filter sterilized (0.22 µm filters) and stored at 4 °C. The FFase activity was determined by preparing 100 g/L sucrose in a 50 mM citrate phosphate buffer (pH 5.5) and 750 µl of the substrate solution was equilibrated at 40 °C for 10 min where after 250µl of culture supernatant was added to a final concentration of 25% (v/v) and incubated for 60 min. The reaction was terminated by adding 36% perchloric acid (PCA) to a final concentration of 2.14% (v/v), followed by the addition of 7 N KOH to precipitate the proteins prior to chemical analysis. The samples were diluted appropriately and subjected to HPLC analysis using an external glucose standard calibration as previously described (van Wyk et al., 2013). The amount of glucose liberated during the enzyme reaction was indicative of global FFase activity, with one unit of enzyme defined as the amount of enzyme required to produce 1 µmol glucose per minute under the described conditions (Hidaka et al., 1988).

4.2.7 Quantifying specific enzyme activity

FopA enzyme was purified from the cell-free supernatant obtained from the bioreactor cultures using an ÄKTA purifier (GE Healthcare Life Sciences, MA, USA) with a Superose® 12 FPLC column and eluted using 50 mM citrate phosphate buffer (pH 5.5). The collected fractions were analyzed for enzyme activity and protein content. The 2D-Quant Kit (GE Healthcare Life Sciences, MA, USA) was used to determine the protein concentration using bovine serum albumin (BSA) as protein standard. The specific activity for duplicate fractions was calculated based on the protein concentrations and volumetric activities determined for these fractions.

The enzyme concentrations for the bioreactor supernatants were derived from the maximum volumetric activity from the respective supernatants and the calculated specific activity for the enzyme.

4.3 Results

4.3.1 Identifying the preferred host strain

The production levels from the codon-optimized *fopA* gene from GeneArt® were first evaluated in *P. pastoris* X-33 and *P. pastoris* DSMZ 70382. The effect of inducible (*AOX1* promoter, methanol carbon source) and constitutive (*GAP* promoter, glycerol carbon source) expression on the extracellular fopA enzyme activity levels were examined in shake flasks at 72 h (Fig. 4-1). The lowest activity was detected with the PX33-AG strain (3.2 ± 2.75 U/ml), whereas there was no significant difference in activity between PX33-GG, P70382-AG and P70382-GG (11.3 ± 1.7 U/ml, 12.1 ± 2.9 U/ml and 12.0 ± 2.1 U/ml, respectively). There was a slight decrease in activity of fopA produced in *P. pastoris* DSMZ 70382 under constitutive compared to induced expression. This initial investigation indicated that fopA can readily be produced in *P. pastoris* with the *P. pastoris* DSMZ 70382 strain being the preferred candidate for fopA production showing no substantial difference between induced and constitutive production as well as not being restricted for commercial use.

Following the confirmation of a preferred host for fopA production, the enzyme production levels of the GeneArt® optimised *fopA* gene was compared to the codon-optimized *fopA* gene synthesized by ATUM. The two optimized genes were expressed under control of either the inducible *AOX1* promoter (P70382-AD2 and P70382-AG) or the constitutive *GAP* promoter (P70382-GD2 and P70382-GG). The P70382-GG strain was used as a reference strain and produced activity of 11.7 ± 1.98 U/ml (Fig. 4-2A). Expression of the P70382-GD2 transformants displayed activity of 10.9 ± 2.65 U/ml to 12.7 ± 0.85 U/ml, which was

comparable to that of the reference strain. In contrast, the best P70382-AD2 transformant showed activity of 98.6 ± 5.94 U/ml, which was 8-times higher than that of its GAP counterpart and slightly better than the control strain P70382-AG (Fig. 4-2B).

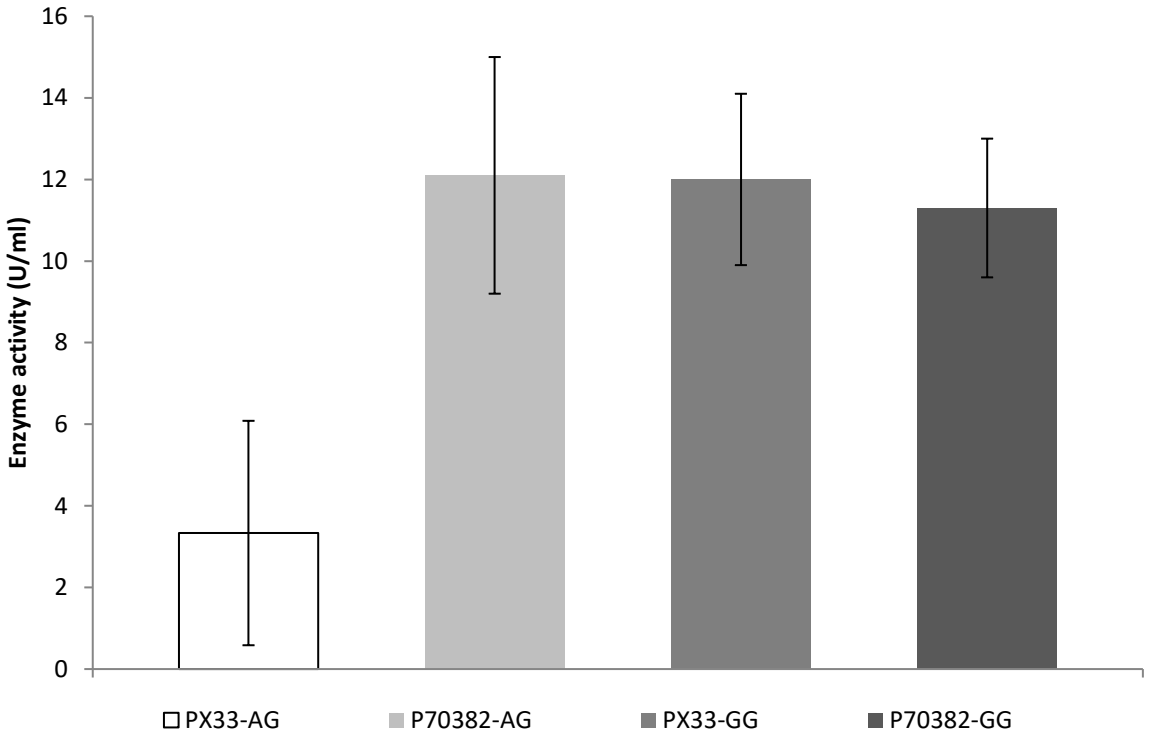


Figure 4-1. Comparison of the volumetric enzyme activity of *P. pastoris* X-33 (PX33) and *P. pastoris* DSMZ 70382 (P70382) containing the codon-optimized fopA gene (Geneart®) under transcriptional control of the alcohol oxidase (AG) and glyceraldehyde 3-phosphate dehydrogenase (GG) promoter, respectively. Cultivations were performed in shake flasks at 30 °C for 72 h at an agitation rate of 200 rpm.

4.3.2 Enzyme production in bioreactors

The *P. pastoris* DSMZ 70382 GAP (P70382-GG and P70382-GD2) and AOX (P70382-AG and P70382-AD2) transformants exhibiting the maximum activity in the shake flask experiments were further evaluated in 1.3 L bioreactors (Fig. 4-3). A similar trend in FFase production levels was observed in bioreactors as in shake flasks, with higher volumetric activities for the *fopA* genes expressed under the *AOX1* promoter in relation to the *GAP*

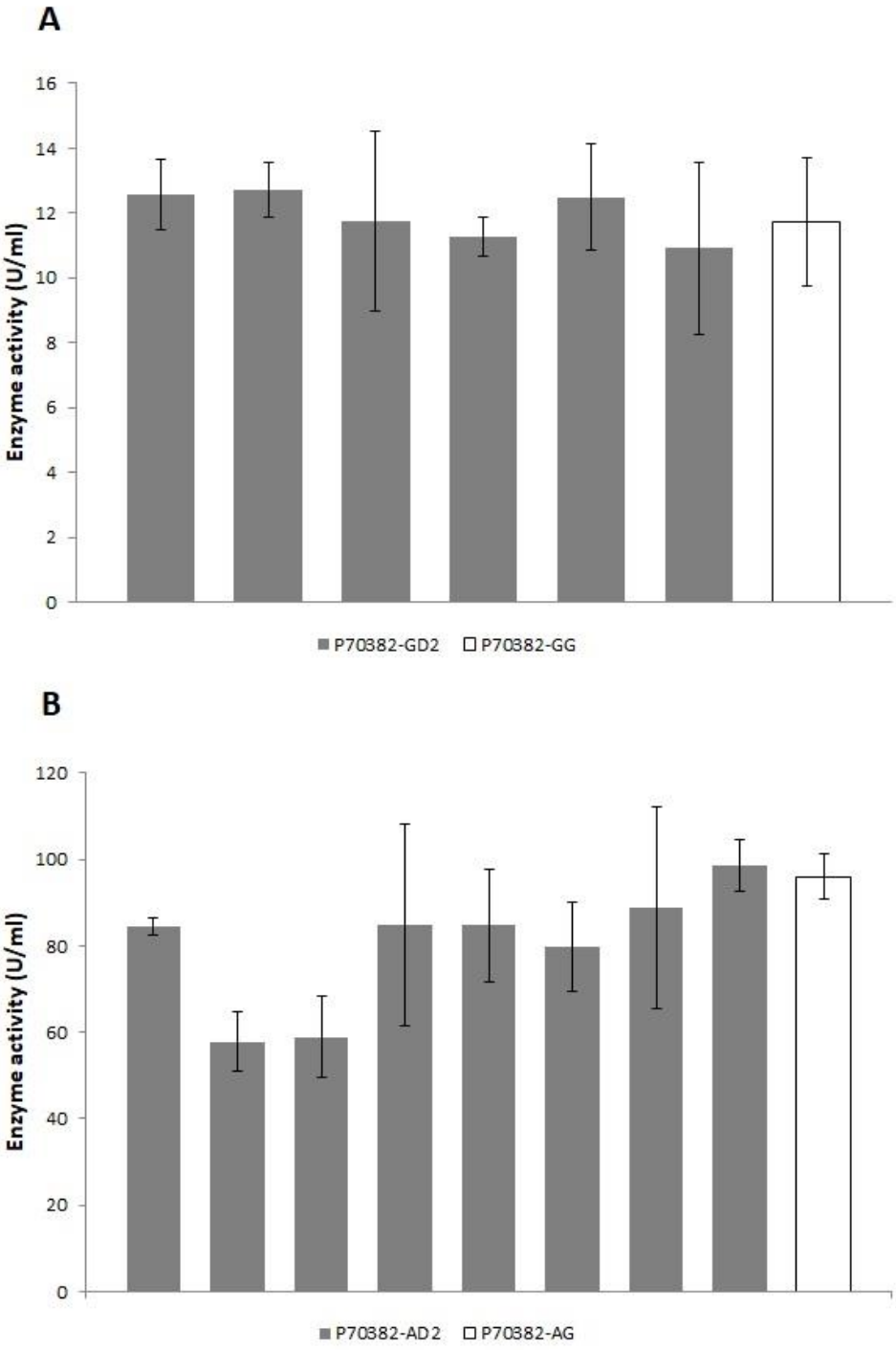


Figure 4-2. Volumetric enzyme activity of positive *P. pastoris* transformants with the codon-optimized fopA gene from ATUM (grey bars) under control of the glyceraldehyde 3-phosphate dehydrogenase (*GAP*) promoter (A) and under control of the alcohol oxidase (*AOX1*) promoter (B) relative to the control containing the codon-optimized fopA gene from Geneart® (white bars) cultivated in shake flasks for 72 hrs at 30 °C at an agitation rate of 200 rpm.

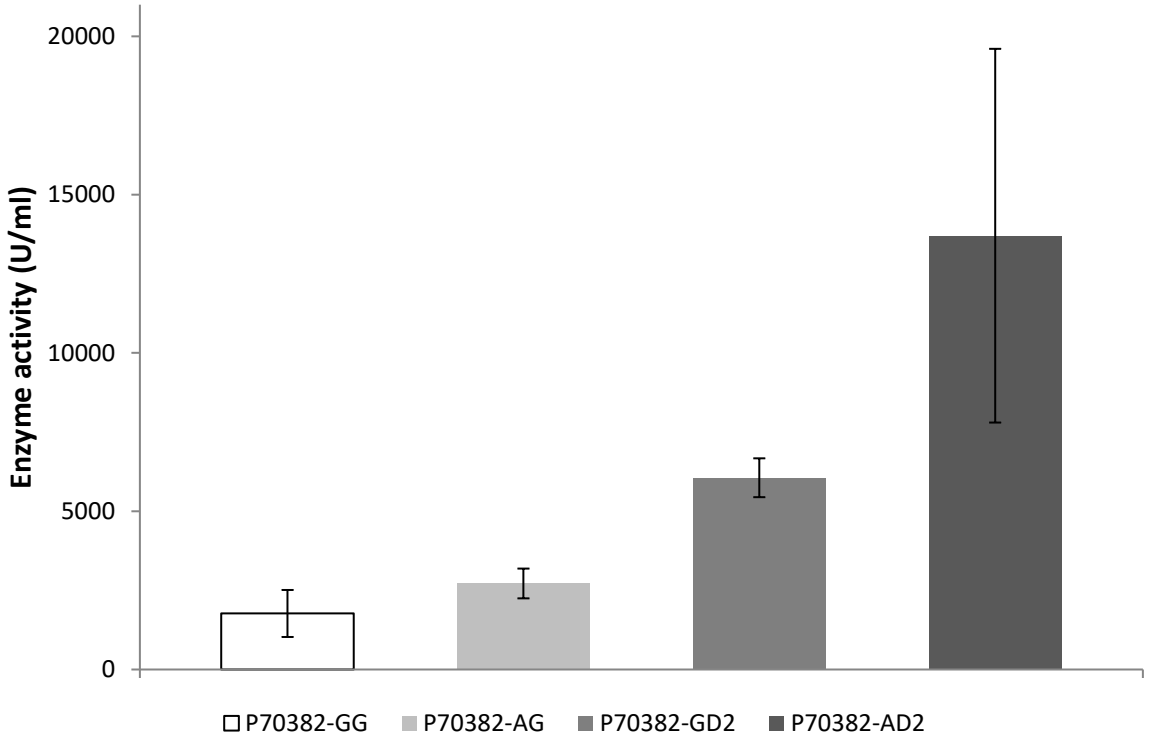


Figure 4-3. Volumetric enzyme activity of *P. pastoris* AOX1fopA (Geneart®; P70382-AG), *P. pastoris* GAPfopA (Geneart®; P70382-GG), *P. pastoris* AOX1fopA (ATUM; P70382-AD2) and *P. pastoris* GAPfopA (ATUM; P70382-GD2) cultivated in 1.3 L fermenters in triplicate. Enzyme activity was determined at the point of maximum biomass.

promoter (Figs. 4-2A, 4-2B and 4-3). Enzyme activities of 1 790 ± 718 U/ml and 6 057 ± 613 U/ml were obtained for the P70382-GG and P70382-GD2 strains, respectively. The *P. pastoris* AOX strains performed better with 2 718 ± 470 U/ml and 13 702 ± 5902 U/ml for the P70382-AG strain and P70382-AD2 strain, respectively. A significant difference was observed in the activities obtained in bioreactors between the two different codon-optimized genes.

The specific activity of fopA in the supernatants of bioreactor cultures was determined using the 2D Quant protein quantification assay (Table 4-2). The calculated specific activity for fopA was 3 518 U/mg. The P70382-AD2 strain produced the highest amount of protein at 3.90 g/L with the lowest for P70382-GG at 0.51 g/L.

Table 4-2. Protein concentrations in the supernatant of 1.3 L bioreactors derived from the maximum activities of the cultivated strains after determining the specific activity of fopA with 2D Quant protein assay. P70382-GG and P70382-AG are the Geneart® codon-optimized genes under control of the *GAP* and *AOX1* promoters, respectively while P70382-GD2 and P70382-AD2 are the ATUM codon-optimized genes under control of the *GAP* and *AOX1* promoters, respectively.

Protein concentration (g/L)			
P70382-GG	P70382-AG	P70382-GD2	P70382-AD2
0.51 ± 0.20	0.77 ± 0.13	1.72 ± 0.17	3.90 ± 1.68

4.4 Discussion

Several *P. pastoris* host strains are available for the expression of heterologous proteins (Ahmad et al., 2014). These individual strains have been developed to address certain complications encountered in protein expression and application such as proteolysis, which leads to reduced protein yield, and glycosylation, which can result in decreased serum half-life and allergic reactions in humans. Furthermore, the use of some of the commercial strains are restricted by patents and material ownership policy (Ahmad et al., 2014; Ballou, 1990). For this reason, we evaluated the potential of the wild-type *P. pastoris* strain X-33 and the type strain DSMZ 70382 to express and produce fopA for the production of FOS. The analysis of the ATUM codon-optimized gene was limited to expression in the *P. pastoris* DSMZ 70382 strain, as the use of this strain in an industrial capacity is not proprietary and it showed good performances in initial screening phases (data not shown). DSMZ 70382 type strain showed substantially higher volumetric activity when the Geneart® codon-optimized gene was expressed under control of the *AOX1* promoter compared to the X-33 strain. However, there was little difference in enzyme activity between the two strains when the gene was expressed under control of the *GAP* promoter (12.0 ± 2.1 U/ml for the DSMZ 70382 strain and 11.3 ± 1.7

U/ml for the X-33 strain). Ang et al. (2016) showed that *P. pastoris* KM71H strain obtained higher levels of the human DNA topoisomerase I (2.26 ng/ml) compared to that of the *P. pastoris* X-33 strain (0.75 ng/ml), while Blanchard et al. (2008) found that strain *P. pastoris* GS115 could express biologically active *N*-glycosylated ^{15}N-labeled human chorionic gonadotropin (phCG) in contrast to *P.pastoris* X-33. Therefore, to optimise the production of a heterologous protein the selection of a strain is critical and thus the evaluation of different strains can have a significant effect.

In the bioreactors, the codon-optimized *fopA* from ATUM showed higher activity than its counterpart from Geneart® (Fig. 4-4). Since the genes from the respective suppliers were expressed under the control of the same promoters and in the same host strains, the difference in expression could be attributed to different algorithms used by the suppliers for codon-optimization. The differences are illustrated in the gene alignments in Appendix A-1. Increased expression by utilizing codon-optimized genes in *P. pastoris* has been demonstrated previously, with the possible advantages including the avoidance of premature termination of translation by depletion of specific aminoacyl-tRNA and also the termination of transcription if DNA contains a high proportion of AT bases (Daly and Hearn, 2005). Sinclair and Choy (2002) expressed the human glucocerebrosidase protein in *P. pastoris* and found that the levels of the translated product were very low despite having sufficient levels of transcripts. After codon optimization, the amount of protein expressed increased 10.6-fold. Furthermore, Yang and Liu (2010) increased the expression of the *A. niger*, *lip2*, lipase encoding gene in *P. pastoris* and subsequently improved enzyme activity and protein concentration from 16.5 U/ml to 191 U/ml and 29.3 mg/L to 154 mg/L, respectively, through codon optimization performed by ATUM. In the present study the *P. pastoris* strains harbouring the respective optimized genes under the same promoters produced similar enzyme activities in shake flasks, with 11.7 ± 1.98 and 12.7 ± 0.85 U/ml for the Geneart® and ATUM genes, respectively, under

the *GAP* promoter and 95.8 ± 5.32 and 98.6 ± 5.94 U/ml under control of the *AOX1* promoter (Figs. 4-2A and 4-2B). Significant differences were observed in activity between the *P. pastoris* strains with the same genes and promoters in bioreactors when compared to the same strains in shake flasks (Figs 4-2A, 4-2B and 4-3). Therefore, selecting the best transformants from shake flask data alone does not appear to be sufficient; bioreactor cultivations are also required to determine production levels for preferred transformants due to superior temperature, pH and DO control.

It is also evident that fopA activity was higher in strains expressing the genes under control of the *AOX1* promoter than for the *GAP* promoter, in both shake flasks and bioreactors. In shake flasks (Figs. 4-2A and 4-2B), the Geneart® gene under control of the *GAP* promoter displayed an activity of 11.7 ± 1.98 U/ml in contrast to 95.8 ± 5.32 U/ml for the *AOX1* promoter, which was similar for the ATUM gene (12.7 ± 0.85 U/ml and 98.6 ± 5.94 U/ml for the *GAP* and *AOX1* promoters, respectively). The bioreactors (Fig. 4-4) showed the same trend with the Geneart® gene (1 790 ± 718 U/ml and 2 718 ± 470 U/ml for the *GAP* and *AOX1* promoters, respectively) as well as for the ATUM gene (6 057 ± 613 U/ml and 13702 ± 5902 U/ml for the *GAP* and *AOX1* promoters, respectively). Previous comparisons of the *GAP* or *AOX1* promoters for recombinant/heterologous protein production in *P. pastoris* produced varied results: functional mammalian transport proteins displayed 5-fold higher levels for the *GAP* promoter relative to the *AOX1* promoter (Döring et al., 1998), while *GAP* produced 2-fold higher protein levels than *AOX1* in shake flasks (Delroisse et al., 2005). Conversely, Boer et al. (2000) showed increased yield from *AOX1* expressing a cellobiohydrolase and Vassileva et al. (2001) reported higher levels of hepatitis B surface antigen from an *AOX1* integrant compared to a *GAP* integrant in shake flasks.

The *P. pastoris* strains were able to produce fopA in the supernatant in the g/L range with a maximum of 3.90 g/L produced by the P70382-AD2 (Table 4-2), compared to P70382-AG

strain that only produced a maximum of 0.77 g/L. Protein production and secretion in a bioreactor by *P. pastoris* has been shown to be as high as 14.8 g/L for mouse gelatin (Werten et al., 1999).

The data presented here indicate that *P. pastoris* is a promising expression platform for the production of fopA as it is able to efficiently produce and secrete the recombinant protein. The codon-optimization of the gene and the evaluation of different promoters and hosts was also shown to be necessary for optimum protein production.

4.5 References

Ahmad, M., Hirz, M., Pichler, H., Schwab, H., 2014. Protein expression in *Pichia pastoris*: Recent achievements and perspectives for heterologous protein production. Appl. Microbiol. Biotechnol. 98, 5301–5317.

Ang, R.P., Teoh, L.S., Chan, M.K., Miswan, N., Khoo, B.Y., 2016. Comparing the expression of human DNA topoisomerase I in KM71H and X33 strains of *Pichia pastoris*. Electron. J. Biotechnol. 21, 9–17.

Ballou, C.E., 1990. Isolation, characterization, and properties of *Saccharomyces cerevisiae mnn* mutants with nonconditional protein glycosylation defects. Methods Enzymol. 185, 440–470.

Becker, D.M., Guarente, L., 1991. High-efficiency transformation of yeast by electroporation. Methods Enzymol. 194, 182–187.

Blanchard, V., Gadkari, R.A., George, A.V.E., Roy, S., Gerwig, G.J., Leeflang, B.R., Dighe, R.R., Boelens, R., Kamerling, J.P., 2008. High-level expression of biologically active glycoprotein hormones in *Pichia pastoris* strains - Selection of strain GS115, and not X-33, for the production of biologically active N-glycosylated 15N-labeled phCG. Glycoconj. J. 25, 245–257.

Boer, H., Teeri, T.T., Koivula, A., 2000. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7a secreted from *Pichia pastoris* using two different promoters. Biotechnol. Bioeng. 69, 486–494.

Buckholz, R.G., Gleeson, M.A., 1991. Yeast systems for the commercial production of heterologous proteins. Nat. Biotechnol. 9, 1067–1072.

Çelik, E., Çalık, P., 2012. Production of recombinant proteins by yeast cells. Biotechnol. Adv.

30, 1108–1118.

Daly, R., Hearn, M.T.W., 2005. Expression of heterologous proteins in *Pichia pastoris*: A useful experimental tool in protein engineenring and production. J. Mol. Recognit. 18, 119–138.

Del Sal, G., Manfioletti, G., Schneider, C., 1988. A one-tube plasmid DNA mini-preparation suitable for sequencing. Nucleic Acids Res. 16, 9878.

Delroisse, J.M., Dannau, M., Gilsoul, J.J., El Mejdoub, T., Destain, J., Portetelle, D., Thonart, P., Haubruge, E., Vandenbol, M., 2005. Expression of a synthetic gene encoding a *Tribolium castaneum* carboxylesterase in *Pichia pastoris*. Protein Expr. Purif. 42, 286–294.

Dhake, A.B., Patil, M.B., 2007. Effect of substrate feeding on production of fructosyltransferase by *Penicillium purpurogenum*. Brazilian J. Microbiol. 38, 194–199.

Döring, F., Klapper, M., Theis, S., Daniel, H., 1998. Use of the glyceraldehyde-3-phosphate dehydrogenase promoter for production of functional mammalian membrane transport proteins in the yeast *Pichia pastoris*. Biochem Biophys Res Commun 250, 531–535.

Hidaka, H., Hirayama, M., Sumi, N., 1988. A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 52, 1181–1187.

Hirayama, M., Sumi, N., Hidaka, H., 1989. Purification and properties of a fructooligosaccharide-producing β-fructofuranosidase from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 53, 667–673.

Invitrogen Corporation, 2002. *Pichia* fermentation process guidelines.

Jedrzejczak-Krzepkowska, M., Tkaczuk, K.L., Bielecki, S., 2011. Biosynthesis, purification

and characterization of β-fructofuranosidase from *Bifidobacterium longum* KN29.1. Process Biochem. 46, 1963–1972.

Lu, L., Wu, J., Song, D., Zhao, H., Gu, G., Guo, Y., Lan, J., Xiao, M., 2013. Purification of fructooligosaccharides by immobilized yeast cells and identification of ethyl β-D-fructofuranoside as a novel glycoside formed during the process. Bioresour. Technol. 132, 365–369.

Maiorano, A.E., Piccoli, R.M., Da Silva, E.S., De Andrade Rodrigues, M.F., 2008. Microbial production of fructosyltransferases for synthesis of pre-biotics. Biotechnol. Lett. 30, 1867–1877.

Nguyen, Q.D., Rezessy-Szabo, J.M., Bhat, M.K., Hoschke, A., 2005. Purification and some properties of β-fructofuranosidase from *Aspergillus niger* IMI303386. Process Biochem. 40, 2461–2466.

Rehm, J., Willmitzer, L., Heyer, A.G., 1998. Production of 1-kestose in transgenic yeast expressing a fructosyltransferase from *Aspergillus foetidus*. J. Bacteriol. 180, 1305–1310.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M.-J., Léotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P.D., Neyrinck, A.M., Meheust, A., 2010. Prebiotic effects: metabolic and health benefits. Br. J. Nutr. 104, S1-63.

Saarelainen, R., Paloheimo, M., Fagerström, R., Suominen, P.L., Nevalainen, K.M.H., 1993. Cloning, sequencing and enhanced expression of the *Trichoderma reesei* endoxylanase II (pI 9) gene *xln2*. MGG Mol. Gen. Genet. 241, 497–503.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd

ed. Cold Spring Harbor Laboratory Press, New York.

Sinclair, G., Choy, F.Y.M., 2002. Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast, *Pichia pastoris*. Protein Expr. Purif. 26, 96–105.

Singh, R.S., Singh, R.P., 2010. Production of fructooligosaccharides from inulin by endoinulinases and their prebiotic potential. Food Technol. Biotechnol. 48, 435–450.

Spohner, S.C., Czermak, P., 2016. Heterologous expression of *Aspergillus terreus* fructosyltransferase in *Kluyveromyces lactis*. N. Biotechnol. 33, 473–479.

Trujillo, L.E., Arrieta, J.G., Dafhnis, F., García, J., Valdés, J., Tambara, Y., Pérez, M., Hernández, L., 2001. Fructo-oligosaccharides production by the *Gluconacetobacter diazotrophicus* levansucrase expressed in the methylotrophic yeast *Pichia pastoris*. Enzyme Microb. Technol. 28, 139–144.

Van Hijum, S.A.F.T., Van Geel-Schutten, G.H., Rahaoui, H., Van der Maarel, M.J.E.C., Dijkhuizen, L., 2002. Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. Appl. Environ. Microbiol. 68, 4390–4398.

van Wyk, N., Trollope, K.M., Steenkamp, E.T., Wingfield, B.D., Volschenk, H., 2013. Identification of the gene for β-fructofuranosidase from *Ceratocystis moniliformis* CMW 10134 and characterization of the enzyme expressed in *Saccharomyces cerevisiae*. BMC Biotechnol. 13, 100.

Vassileva, A., Chugh, D.A., Swaminathan, S., Khanna, N., 2001. Expression of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris* using the *GAP* promoter. J. Biotechnol. 88, 21–35.

Vogl, T., Glieder, A., 2013. Regulation of *Pichia pastoris* promoters and its consequences for protein production. N. Biotechnol. 30, 385–404.

Werten, M.W.T., Van Den Bosch, T.J., Wind, R.D., Mooibroek, H., De Wolf, F.A., 1999. High-yield secretion of recombinant gelatins by *Pichia pastoris*. Yeast 15, 1087–1096.

Yanai, K., Nakane, A., Kawate, A., Hirayama, M., 2001. Molecular cloning and characterization of the fructooligosaccharide-producing β-fructofuranosidase gene from *Aspergillus niger* ATCC 20611. Biosci. Biotechnol. Biochem. 65, 766–73.

Yang, J., Liu, L., 2010. Codon optimization through a two-step gene synthesis leads to a high-level expression of *Aspergillus niger lip2* gene in *Pichia pastoris*. J. Mol. Catal. B Enzym. 63, 164–169.

Yoshikawa, J., Amachi, S., Shinoyama, H., Fujii, T., 2007. Purification and some properties of beta-fructofuranosidase I formed by *Aureobasidium pullulans* DSM 2404. J. Biosci. Bioeng. 103, 491–493.

Chapter 5 Process development using different glycerol fed-batch methods for the production of a novel engineered β-fructofuranosidase enzyme in *Pichia pastoris*

Authors: Gerhardt Coetzee, María del Prado García Aparicio, Eugéne van Rensburg and Johann F. Görgens

Department of Process Engineering, Stellenbosch University, Stellenbosch, South Africa

Objective of dissertation in this chapter

This chapter addresses objective two, which focusses on determining the optimized processing conditions for maximised production of a native (GAPfopA) and a novel engineered version (GAPfopA_V1) of the β-fructofuranosidase enzyme from *A. fijiensis* ATCC 20611. The engineered version of this enzyme has only previously been expressed and produced in *Saccharomyces cerevisiae*. Two *P. pastoris* strains, one from Chapter 4 containing the ATUM codon-optimized native enzyme, and the other constructed in this chapter with the engineered version are evaluated in bench top bioreactors for the production of the two enzyme variants under control of the *GAP* promoter. The effects of two different glycerol fed-batch methods on the physiology of the two strains will also be evaluated through measurement of physiological parameters (growth rate, biomass yields and productivities) since this will ultimately affect the expression and production of the two enzymes. After 155 h the DO-stat cultivation produced less biomass than the constant feed at 55 h for both strains because of a lower growth rate but produced a higher volumetric activity. However, due to the shorter cultivation time, the constant feed cultivations produced higher volumetric productivities, which is advantageous for an industrial production process. Both the feeding methods produced higher volumetric activities for the GAPfopA strain than for the GAPfopA_V1 strain. The enzymes produced

here will be evaluated in Chapter 5 for the production of scFOS from industrial sugar streams. Consequently, all objectives set out in Chapter 3 were addressed here.

Candidate declaration

In chapter 5 page numbers 75 to 104 of this dissertation, the type and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Planning of experiments	70
Executing experiments	80
Interpretation of results	70
Writing and reviewing of the chapter	70

The listed co-authors have contributed to chapter 5 page numbers 75 to 104 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution (%)
María del Prado García Aparicio		Planning experiments	20
		Executing experiments	20
		Writing and reviewing of chapter	10
Eugéne van Rensburg		Interpretation of results	20
		Writing and reviewing of the chapter	10
Johann F. Görgens		Planning of experiments	10
		Interpretation of results	10
		Writing and reviewing of the chapter	10

Signature of candidate:

Date: 27 January 2019

Declaration by co-authors

The undersigned hereby confirm that

- The declaration above accurately reflects the type and extent of the contributions of the candidates and co-authors to chapter 5 page numbers 75 to 104 in the dissertation,
- No other authors contributed to chapter 5 page numbers 75 to 104 in the dissertation besides those specified above, and
- Potential conflicts of interest have been revealed to all interested parties and that necessary arrangements have been made to use the material in chapter 5 page numbers 75 to 104 of this dissertation.

Institutional affiliation	Date	Signature
Department of Process Engineering	27/01/2019	

Abstract

The fopA enzyme from *Aspergillus niger* ATCC 20611 has been extensively used to produce fructooligosaccharides. The optimization of this process has previously focused predominantly on process development, but molecular techniques are increasingly employed. An engineered version of the fopA enzyme with increased thermostability, specific activity and reduced glucose inhibition has been previously expressed and produced in *Saccharomyces cerevisiae*. In this study, the native (GAPfopA) and engineered (GAPfopA_V1) versions of the fopA enzyme were expressed in *Pichia pastoris* under control of the GAP promoter and the production evaluated in 10 L bioreactors using DO-stat and constant feed methods. The DO-stat cultivations had overall lower biomass concentration (119.73 g/L and 118.39 g/L for GAPfopA and GAPfopA_V1 for DO-stat and 133.86 g/L and 132.44 g/L for GAPfopA and GAPfopA_V1 for constant feed) but produced the highest maximum volumetric activity for the native GAPfopA enzyme (2 129 U/ml) compared to that of GAPfopA_V1 (1 686 U/ml). The GAPfopA produced the highest volumetric enzyme activity for both the DO-stat and constant feed methods (2 129 U/ml and 1686 U/ml for GAPfopA and GAPfopA_V1 for DO-stat and 1 413 U/ml and 1 222 U/ml for GAPfopA and GAPfopA_V1 for constant feed). However, overall the constant feed fermentations produced higher volumetric productivity for both strains (23.96 x 10^3 and 20.72 x 10^3 U/L/h for GAPfopA and GAPfopA_V1, respectively) due to a reduction in process time of 96 h. Therefore, increased biomass growth with constant feed fermentations using glycerol does not produce higher maximum enzyme activity but resulted in higher volumetric productivity, which is more advantageous for an industrial process.

Highlights

Constant feed fermentations produced higher biomass growth

Increased biomass did not result in increased volumetric activity

Shorter process time produced higher volumetric productivity for constant feed method

Keywords: DO-stat, constant feed, *GAP*, *Pichia pastoris*, fopA

5.1 Introduction

The methylotrophic yeast *Pichia pastoris* has successfully been used as a recombinant host for the expression of an increasing number and variety of heterologous proteins, such as *Escherichia coli* phytase, mouse endostatin and human antigen-binding fragment (Chen et al., 2004; Garcia-Ortega et al., 2013; Trinh et al., 2003). This yeast offers many advantages heterologous protein expression host, which include eukaryotic post-translational modification, growing to high cell-densities on minimal media by which the product yield can be maximised, high level of protein expression and secretion, and ease of genetic manipulation (Cereghino et al., 2002).

Fed-batch fermentations with the *GAP* promoter usually consist of two phases, namely an initial glycerol batch phase (GB) followed by a glycerol fed-batch phase (GFB) where the glycerol substrate is fed continuously to the culture at a predetermined rate. The feeding methods used during fed-batch phase could either be DO-stat feed, constant feed or exponential (Çalık et al., 2015). The DO-stat regime has the main advantage of maintaining the dissolved oxygen (DO) tension at a level that would sustain an active, healthy culture for extended periods of time, thereby avoiding oxygen-limited growth where oxygen demand for exceeds the system's ability to supply, thus leading to hypoxic conditions that affect cell viability and product formation (Chung, 2000). Exponential feed has the advantage of maintaining a constant specific growth rate (μ), which has been directly or indirectly linked to protein production (Çalık et al., 2015; Potvin et al., 2012). Constant feed fermentations have been used to successfully express various proteins (Baumann et al., 2008; Goodrick et al., 2001; Zhang et al., 2007). However, there is currently considerable variation on the constant feed rates used and the effect of constant feed on productivity of a bioprocess needs further investigation (Çalık et al., 2015). Furthermore, both exponential and constant feed are examples of feed forward control and are based on the cell growth models of the specific organism. The μ for these

feeding strategies are nor controlled and the cultures growth characteristics may change as the cultivation progresses, which requires the culture's feed to be restricted below its optimum specific growth rate (Dietzsch et al., 2011).

Fructooligosaccharides (FOS) occur naturally in plants (asparagus, sugar beet, onion and Jerusalem artichoke) and are also produced by various microorganisms. The sugars are considered prebiotic additives that possess various health benefits (reduced cholesterol, enhanced mineral absorption in the gut and promoting the growth of bifidobacteria) and can be used as low calorific sweeteners (Singh et al., 2017; Yun, 1996). The fructan molecules are produced by the transfructosylation of sucrose by β-fructofuranosidases (EC 3.2.1.26) and consists mainly of 1-kestose (GF2), nystose (GF3) and 1^F-fructofuranosyl nystose (GF4) (Yun, 1996).

Industrial production of FOS is catalysed by the β-fructofuranosidase enzyme (fopA) from *Aspergillus niger* ATCC 20611 using a 50% to 60% (w/v) sucrose solution as substrate at a temperature between 50 and 60 °C (Nishizawa et al., 2001). Conventional approaches for improving FOS production mostly focused on screening for enzymes with enhanced transfructosylating activity (Chávez et al., 1997; Ghazi et al., 2007) or process development for both enzyme and FOS production (Balasubramaniem et al., 2001; Sheu et al., 2002). However, molecular techniques for altering enzyme function have received increasing attention. For example, the fopA enzyme from *A. niger* was engineered for increased thermostability, specific activity and reduced glucose inhibition and expressed in *Saccharomyces cerevisiae* (Trollope et al., 2015). More recently, Zhang et al. (2017) homologously expressed another fopA engineered enzyme in *A. niger* ATCC 20611. In both these studies, the protein was produced at small scale in shake flasks.

In this study, the *GAP* promoter was selected due to the use of glycerol being preferred for industrial processes with regards to safety and acceptability for food-production as opposed to methanol. The difference in production levels of a native (GAPfopA) and a novel engineered fopA (GAPfopA_V1) enzyme (Trollope et al., 2015) under control of *GAP* promoter expressed in *P. pastoris* was evaluated at a larger scale in bioreactors using the DO-stat and constant feed fed-batch methods.

5.2 Materials and Methods

5.2.1 Microbial strains, media and plasmids

Escherichia coli DH5α [fhuA2Δ(argF-lacZ)U169 phoAglnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1hsdR17] (New England Biolabs, Midrand, South Africa) served as host for plasmid amplification. Cells were grown at 37 °C in low salt Luria Bertani broth supplemented with 25 µg/ml zeocin. The *Pichia pastoris* strain (GAPfopA) containing the native codon-optimised gene (ATUM, Newark, USA) under control of the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter was obtained from the culture collection of the Department of Process Engineering, Stellenbosch University, South Africa. The *P. pastoris* strain DSMZ 70382 (CBS704) was selected as expression host for the production of the engineered FFase. *P. pastoris* cells were grown in yeast peptone dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% glucose) supplemented with sorbitol and zeocin (100 to 1000 µg/ml), as appropriate. Solid media were supplemented with 15 g/L agar. For enzyme expression in shake flasks *P. pastoris* transformants were cultivated in buffered glycerol complex medium (BMGY) consisting of 1% glycerol, 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6, 1.34% yeast nitrogen base and $4X10^{-5}$ % biotin. Zeocin was purchased from Melford Laboratories Ltd (Chelsworth, UK). All other chemicals were

purchased from Sigma-Aldrich (South Africa) or Merck (South Africa) with the yeast nitrogen base obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

5.2.2 DNA cloning and yeast transformation

The four amino acid substitution (F140Y-A178P-G321N-Q490S) variant of the *Aspergillus japonicus fopA* FFase (GenBank accession number AB046383) was described previously (Trollope et al., 2015). The fopA_V1 coding sequence, fused to the *Trichoderma reesei* endoxylanase 2 (*xln2*) secretion signal, was cloned from the pJ227 cloning vector as a 2027 bp EcoRI-XhoI fragment into the *P. pastoris* expression vector pJ905 harbouring the *GAP* promoter (ATUM, Newark, USA). The pJ905_fopA_V1 plasmid was linearised with XmaJI (Thermo Fisher Scientific, Waltham, USA) prior to yeast transformation (Lin-Cereghino et al., 2005). The electroporation was carried out in electroporation cuvettes (gap, 2.0 mm) in a Gene Pulser® II electroporator (Bio-Rad Laboratories, Hercules, CA, USA) with charging voltage of 1500 V, resistance of 200 Ω and capacitance of 25 μF. After the electroporation, the cells were incubated in YPD supplemented with 1 M sorbitol for 3 h before plating on selective media at 30 ˚C for up to 3 days until colonies formed.

Transformants were transferred to YPD plates (1% yeast extract, 2% peptone, 2% glucose and 1.3% agar) and then re-streaked on YPD plates increasing concentrations of zeocin. Positive transformants were further confirmed by colony PCR with primers specific to *fopA_V1*. The sequences of the primers were 5'-ACGCATGTCATGAGATTATTGG-3'and 5'-GCAAATGGCATTCTGACATCC-3' (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa).

5.2.3 Transformant screening

The screening of the transformants were derived from the method of Krettler et al. (2013). Single colonies of the transformants were inoculated into test tubes containing 5 ml of BMGY

medium and grown overnight at 30 °C on a rotating wheel. The respective cultures were then inoculated into 500 ml baffled shake flasks containing 100 ml of BMGY medium and incubated overnight at 30 °C at 200 rpm. When the cultures reached an OD_{600} >= 10 the fractional volume of the culture to be harvested was calculated for inoculating the small-scale expression culture (100 ml BMGY in 500 ml baffled flasks) at OD_{600} = 1. This volume was centrifuged at 3 000 g for 3 min and resuspended in YP medium (1% yeast extract, 2% peptone) and then inoculated into the baffled flasks in triplicate and incubated for 72 h at 30 °C on an orbital shaker adjusted to 200 rpm. Samples were withdrawn at 24 h intervals and the same volume of glycerol added to the culture as was withdrawn to a final concentration of 1% at every interval. The was supernatant kept at 4 °C for determining the enzyme activity.

5.2.4 Bioreactor cultivations

Pichia pastoris cultivations were carried out in Bioflo 110 bioreactors (New Brunswick Scientific Co. Inc., Edison NJ, USA) fitted with a glass reactor vessel with a total volume of 10 L and a working volume of 8 L. Temperature was controlled using a heating jacket and cooling coil and the reactor was fitted with a combination glass pH electrode and polarographic DO probe (all Mettler Toledo, Sandton, South Africa). The Biocommand version 3.30 Plus software (New Brunswick Scientific Co. Inc.) was used for monitoring DO, temperature, pH and agitation and feed rate control. Fermentations were performed as described in *Pichia* Fermentation Guidelines (Invitrogen Corporation, 2002) (Thermo Fisher Scientific, Waltham, MA, USA) with modifications noted below. Starter cultures were prepared by inoculating several colonies from YPD agar plates into test tubes containing 8 ml buffered minimal glycerol (BMG) culture medium consisting of 1.34% yeast nitrogen base, 1% glycerol, 1.64 µM biotin, 100 mM potassium phosphate buffer (pH 6.0). The culture was incubated for 24 h at 30 °C before inoculating 2 L Erlenmeyer shake flasks containing 400 ml fresh BMG medium to a final biomass concentration equal to an optical density (OD) of 0.1 measured at 600 nm

using a spectrophotometer (Biochrom WPA Lightwave II, Harvard Bioscience, Holliston, MA, USA). These cultures were grown for a further 18 h on an orbital shaker adjusted to 200 rpm at 30 °C to a biomass concentration equal to OD_{600} of between 6 and 8. The entire volume of these cultures was used to inoculate the bioreactor to a final volume of 4 L, resulting in an initial biomass concentration equal to an OD_{600} of between 0.6 and 0.8 at the start of the batch phase.

Basalt salt medium (BSM), supplemented with 1% casein hydrolysate and PTM_1 trace salts consisting of (per L) 6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin and 5.0 ml H_2SO_4 was used as culture media in the bioreactors. Cultivations were carried out at 30 °C and 28% ammonium hydroxide (Sigma-Aldrich) was added to maintain the culture at pH 5.0. Atmospheric air was sparged at an aeration rate of 1.0 vvm to maintain the dissolved oxygen (DO) tension above 30% of saturation. To maintain this DO at high biomass concentrations when the rate of oxygen consumption can exceed the oxygen transfer rate, the agitation rate (200 to 1000 rpm) was cascaded to air flow rate, followed by sparging pure O_2.

Two glycerol fed-batch strategies were used in this study, namely a DO-stat and a constant feed strategy. During the DO-stat strategy, a solution of 50% (w/v) glycerol (Scienceworld, South Africa) was supplied to the culture at a rate of 18.15 ml/h per litre initial fermentation volume. Since depletion of the glycerol carbon source would result in an increase in the DO, the pumps automatically fed the culture at the abovementioned rate when the DO exceeded 35% of saturation. Conversely, since the DO will decrease as carbon was supplied to the culture, the same threshold of 35% of saturation was used to switch the pumps off to prevent overfeeding of the culture. During the constant feeding strategy, a solution of 50% (w/v) glycerol was supplied to the culture at a rate of 18.15 ml/h/L of initial fermentation volume, which commenced at the end of the batch phase. In each strategy, the cultivations were

terminated when a final volume of 8 L was reached. Samples were withdrawn throughout the fermentations, centrifuged at 13 000 rpm for 3 minutes and filtered through 0.22 um syringe filters, and analyzed for volumetric enzyme activity, dry cell weight (DCW) concentration and glycerol concentration. Graphs of the DCW concentration and volumetric enzyme activity over time for the respective cultivations are illustrated in Appendices B-1 and B-2.

5.2.5 Sample analysis and calculations

Dry cell weight concentration was determined by centrifuging (Prism™ Microcentrifuge, Labnet International, Edison, NJ, USA) a 2 ml sample of culture supernatant at 13 000 rpm for 3 min. The sample was resuspended in dH_2O, centrifuged again and dried in an oven at 60 ºC for approximately 24 h (until weight stabilized) and converted to DCW per liter of whole broth. Maximum growth rate (μ_{max}) was determined graphically by plotting the natural logarithm of the biomass concentration (g/L) during batch phase against fermentation time and determining the gradient of the exponential growth phase. The biomass yield (Y_{XS}) and product yield (Y_{PS}) were determined by plotting the total biomass (g) and total enzyme activity (U), respectively, against the mass of substrate consumed (g) and determining the slope of the plots. Biomass productivity (Q_X) was determined as follows:

$$Qx = \frac{X}{V \cdot t}$$

Where X is the total biomass (g) at time t (h) and volume V (L). Volumetric productivity (Q_P) was determined with the following equation:

$$Qp = \frac{P}{V \cdot t}$$

Where P is the total product (U) at time t (h) and volume V (L).

5.2.6 Enzyme activity assay

The activity of the FFase enzyme was determined using 100 g/L sucrose (Merck) as substrate prepared in 50 mM citrate phosphate buffer (pH 5.5). The substrate solution (0.75 ml) was equilibrated at 40 °C for 10 min prior to adding the culture supernatant (0.25 ml) containing the enzyme to a final concentration of 25% (v/v). After incubation at 40 °C for 60 min, the reaction was terminated by addition of 35% (w/v) perchloric acid (PCA) to a final concentration of 2.14% (v/v) followed by the addition of 7 M KOH to precipitate the proteins prior to HPLC analysis (Hidaka et al., 1988). Negative controls consisted of all the assay constituents except either sucrose or enzyme which was replaced with 50 mM citrate phosphate buffer (pH 5.5).

The glucose concentration was determined using a Dionex Ultimate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Coulochem III electrochemical detector controlled by Chromeleon 6.8 Chromatography Data System software (Thermo Fisher Scientific). The HPLC was fitted with a CarboPac PA1 (4 × 250 mm) analytical column coupled to a PA1 (4 × 50 mm) guard column (Thermo Fisher Scientific). 10 µl samples were injected and eluted according to the method of van Wyk et al. (2013). The concentration of glucose liberated during the assays was indicative of enzyme activity. A unit of enzyme was defined as the amount of enzyme required to produce 1 µmol glucose per minute under the described conditions (Hidaka et al., 1988).

5.2.7 Glycerol concentration

The glycerol concentration was analysed by HPLC (Finnigan Surveyor, Thermo Fisher Scientific) using a Rezex RHM-Monosaccharide column (Phenomenex, Torrance, CA, USA) fitted with a guard column. A Refractive Index (RI) detector (Thermo Fisher Scientific, Finnigan Surveyor) was used to quantify the glycerol by integrating the area under the peak of

the eluted substance, confirmed using a series of standards of a known range of concentrations. The Rezex RHM-Monosaccharide column was maintained at 60 °C and 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 ml/min for 25 minutes. Samples were acidified with 10% H_2SO_4 to a final concentration of 0.5 % (v/v) and filtered through 0.22 µm nitrocellulose filters (Membrane Solutions, Kent, WA, USA) prior to analysis.

5.2.8 SDS-PAGE analysis

SDS-PAGE was performed according to the method of Laemmli (1970) using a separating gel with an 8% polyacrylamide concentration and a stacking gel with 5% concentration. The gels were silver stained according to Merril et al. (1981).

5.3 Results

5.3.1 Screening of *P. pastoris* transformants

The *P. pastoris* strains transformed with the *fopA_V1* gene were screened for on selective YPD plates containing zeocin and positive transformants confirmed with PCR. Seven confirmed transformants were subsequently screened in shake flasks for the strain producing the highest volumetric activity compared to the GAPfopA reference strain (Fig. 5-1). Whereas five of the seven transformants exhibited marked enzyme activity, no activity was detected for strains G100.1 and G250.3 in spite of confirmed transformation of the *fopA_V1* gene using PCR. Strain G250.2 exhibited the highest activity 121.8 U/ml after 48 h, which is 35% higher than that of the reference strain, which produced a maximum activity of 79.3 U/ml in the same time period. Strain G250.2 was consequently selected for further study and is henceforth referred to as strain GAPfopA_V1.

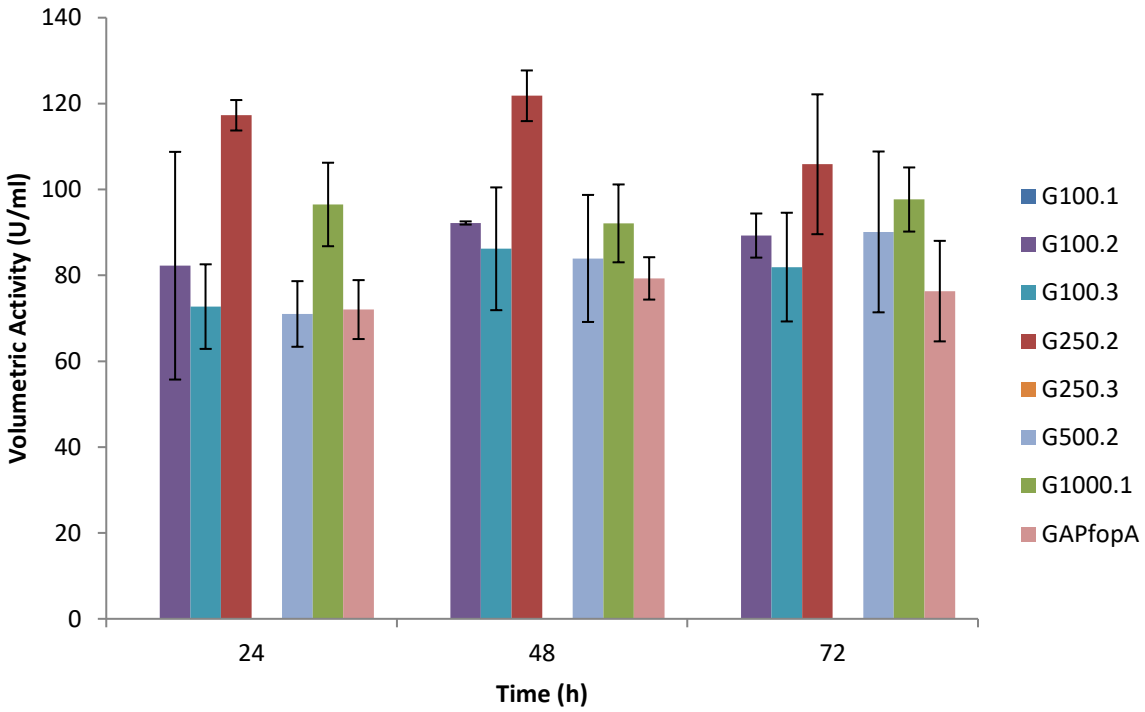


Figure 5-1. Volumetric enzyme activity of *P. pastoris* transformants containing the *GAPfopA_V1* gene relative to the control with the *GAPfopA* gene in shake flasks. Cultivations were performed at 30 °C over 72 h at an agitation rate of 200 rpm. Error bars denote the standard error of triplicate experiments.

5.3.2 Bioreactor cultivations

The cultivation and enzyme production parameters for strains GAPfopA and GAPfopA_V1 were grown in bioreactors using either a DO-stat or a constant feeding strategy are shown in Table 5-1. For each strategy, the maximum volumetric activity recorded during the course of the cultivation was reported, which corresponded to 155 and 59 h for DO-stat and constant feed, respectively. Generally, the maximum specific growth rate (μ_{max}) during the batch phase and biomass concentration were virtually identical for the two strains for both the feeding strategies. Therefore, the respective cultures were in a similar metabolic state from the start of the fed-batch phase and would not impact on the rest of the cultivation.

Table 5-1. Fermentation results for the two strains, GAPfopA and GAPfopA_V1, cultivated in 10 L bioreactors fed with two different substrate feeding strategies, DO-stat and constant feed.

		Batch phase			Fed-batch phase				
Feed method	Enzyme	μ_{max} (h^{-1})	DCW conc. (g/L)	[a] Q_P (x 1000) (U/L/h)	[a] Volumetric activity (U/ml)	Y_{PS} (U/g)	[a] DCW conc. (g/L)	Y_{XS} (g/g)	[a] Q_X (g/L/h)
DO-stat	GAPfopA	0.21 ± 0.02	27.89 ± 0.01	13.74 ± 5.27	2129.25 ± 816.39	11485.15 ± 7697.35	119.73 ± 1.99	0.49 ± 0.00	0.77 ± 0.01
	GAPfopA_V1	0.19 ± 0.04	27.32 ± 1.13	10.88 ± 1.37	1686.91 ± 212.29	9892.10 ± 865.36	118.39 ± 10.69	0.47 ±0.01	0.76 ± 0.07
Constant	GAPfopA	0.24 ± 0.02	26.69 ± 0.08	23.96 ± 0.44	1413.36 ± 26.18	5670.00 ± 592.41	133.86 ± 3.92	0.55 ± 0.03	2.27 ± 0.07
	GAPfopA_V1	0.20 ± 0.03	27.99 ± 2.42	20.72 ± 2.30	1222.70 ± 135.69	4587.50 ± 74.10	132.44 ± 12.95	0.54 ± 0.03	2.24 ± 0.22

Mean ± standard deviation, n = 2

μ_{max} = maximum specific growth rate; DCW = dry cell weight; Q_P = volumetric productivity; Y_{PS} = product yield; Y_{XS} = biomass yield; Q_{XS} = biomass productivity

[a] Analyses were done at the maximum volumetric activity for DO-stat and constant feed (155 h and 59 h, respectively)

The faster substrate feed rate for the constant feed cultivations resulted in a higher rate of enzyme production. This is reflected in the volumetric productivity (Q_P) for the constant feed cultivations that were higher for both strains compared to the DO-stat method (23.96 x 10^3 and 20.72 x 10^3 U/L/h for GAPfopA and GAPfopA_V1 for constant feed and 13.74 x 10^3 and 10.88 x 10^3 U/L/h for DO-stat). However, a faster rate of enzyme production did not result in maximum volumetric activity. There is considerable variation in the volumetric activity for DO-stat feed as a result of considerable variability between the replicates of the DO-stat cultivations, which is evident through the large standard deviations. This is a result of the considerable variation in the DO level, which leads to inconsistent feed of the substrate. Despite this variation in the values the trend for volumetric activity tend to be higher for the GAPfopA strain compared to the GAPfopA_V1 strain (2 129.25 and 1 686.91 U/ml, respectively) with the higher volumetric activity reflected in the higher enzyme production for the GAPfopA strain (fig 5-2B).

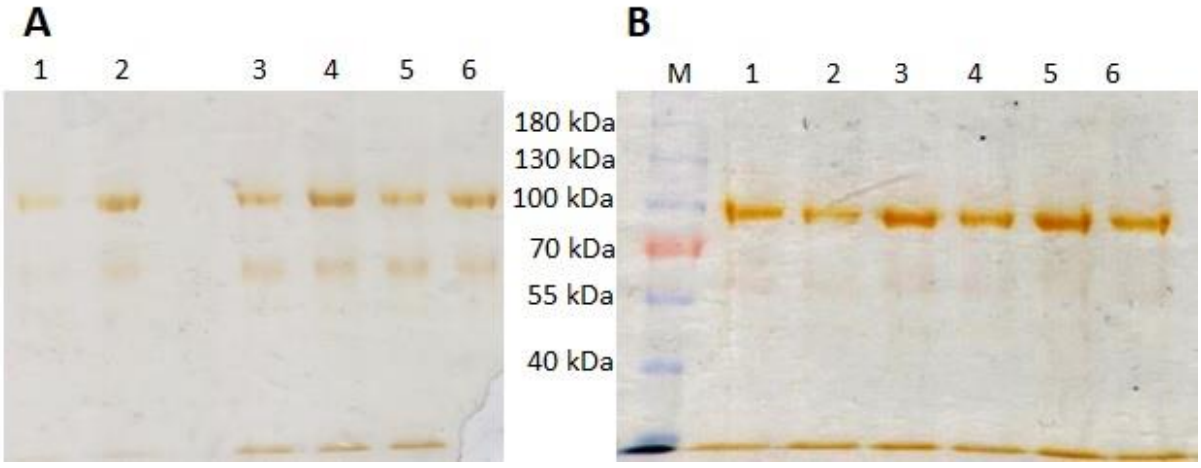


Figure 5-2. SDS-PAGE of the crude supernatant of the cultivations of two different feeding strategies: (A) constant feed with odd-numbered lanes representing GAPfopA_V1 and even-numbered lanes GAPfopA. Lanes 1 and 2 (33 h), lanes 3 and 4 (59 h) and lanes 5 and 6 (67 h). (B) DO-stat feed with odd-numbered lanes representing GAPfopA and even-numbered lanes GAPfopA_V1. Lanes 1 and 2 (107 h), lanes 3 and 4 (131 h) and lanes 5 and 6 (155 h). Lane M represents the molecular weight marker.

The cultivations using constant feed, as with the DO-stat feed cultivations, exhibited similar values for the batch phase specific growth rate (0.24 and 0.20 h^{-1}), biomass productivity (2.27 and 2.24 g/L/h), DCW concentration (133.86 and 132.44 g/L) and Y_{xs} (0.55 and 0.54 g/g) for the two different strains (Table 5-1). The trend for volumetric activity for the two strains also reflect those of the DO-stat cultivations with the GAPfopA strain having a higher activity than the GAPfopA_V1 strain (1 413.36 U/ml and 1 222.70 U/ml, respectively), which also correlated with higher protein production for GAPfopA (Table 5-1, fig. 5-2A).

When the constant feed cultivations are compared to the DO-stat cultivations, the former produces a biomass productivity 3x higher (~2.2 g/L/h and ~0.7 g/L/h), a DCW ~14 g/L more and a higher Y_{xs} (maximum of 0.55 and 0.49 g/g) than the latter for both strains (Table 5-1). Despite the increased biomass production for the constant feed fermentations, the maximum volumetric activity is generally higher for the DO-stat fermentations for both the strains.

The fermentation control system was configured to maintain the DO at 30% of saturation irrespective of fed-batch strategy. During DO-stat cultivations, the DO exhibited significant variation from the setpoint during the fed-batch phase, which ranged between approximately 10% and 55% of saturation (fig. 5-3A). Conversely, oscillations in the DO were substantially less pronounced during the fed-batch phase where constant feed was applied and was generally maintained between 20% and 40% of saturation (fig. 5-3B). However, the greater biomass achieved during constant feed necessitated sparging with pure O_2 throughout the fed-batch phase to maintain the required DO.

5.4 Discussion

The purpose of this study was to compare the production of enzyme activity for two different *P. pastoris* strains (GAPfopA and GAPfopA_V1), each harbouring a different version of the

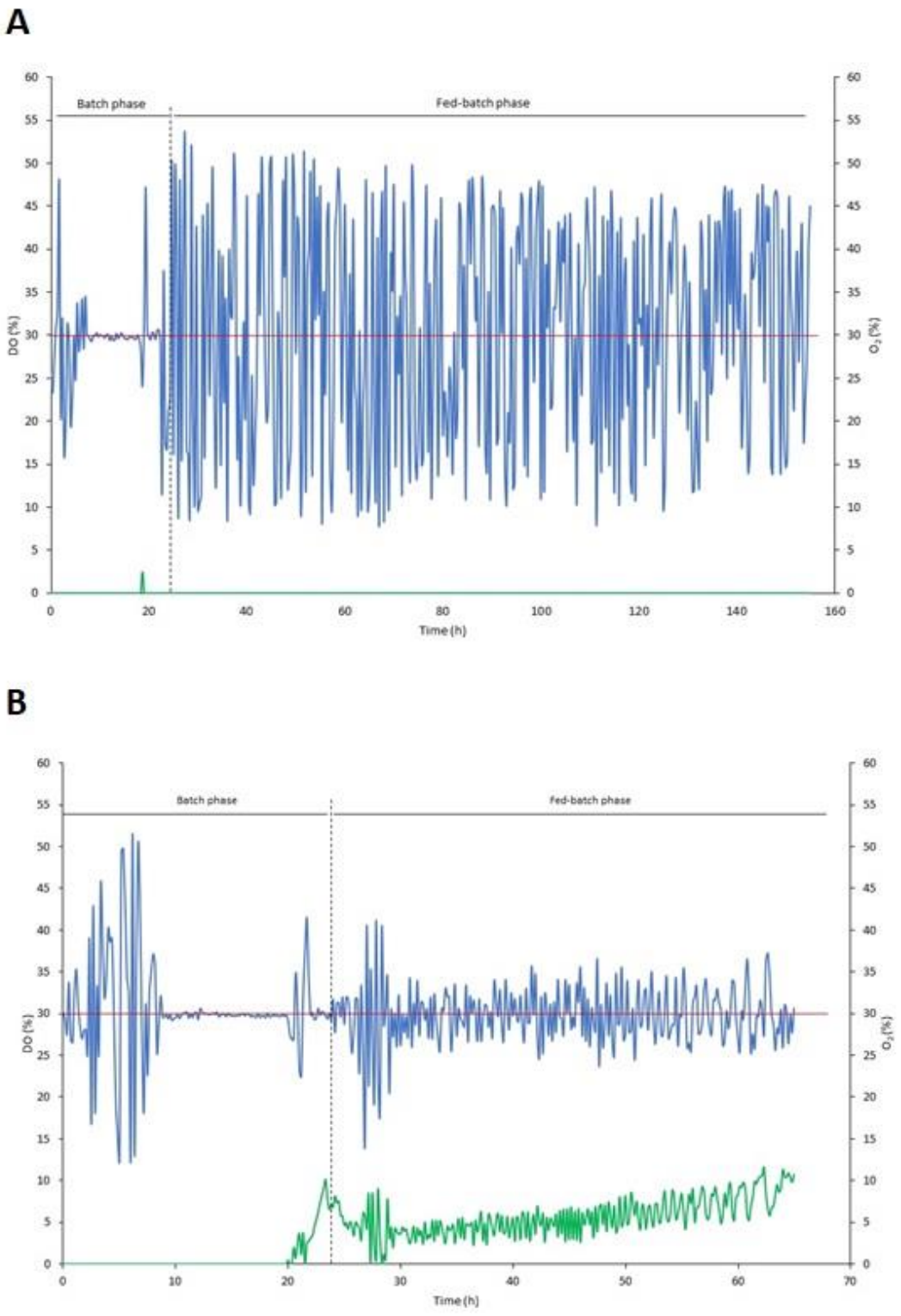


Figure 5-3. Dissolved oxygen (DO) level (blue) and O2 sparged (green) during the DO-stat and constant feed fermentations. The red line indicates the setpoint value at which the DO is controlled.

fopA protein under control of the *GAP* promoter, while employing two different feeding strategies, namely DO-stat and constant feed using glycerol as carbon source.

Relative enzyme production by the two recombinant strains used in this study did not improve consistently when transferred from shake flasks to bioreactors. Furthermore, screening transformants in microtitre plates have been shown to not necessarily be reflective of what occurs in a bioreactor, possibly due to poorly controlled feeding. In shake flasks, the GAPfopA_V1 strain (transformant strain G250.2) produced a higher volumetric activity than the GAPfopA reference strain (121.8 U/ml and 79.3 U/ml, respectively) (fig. 5-1) despite the similar DCW concentrations (data not shown). This result contrasted with results from bioreactor-grown fed-batch cultures where the GAPfopA strain exhibited a higher volumetric enzyme activity (2 129 U/ml) and level of protein production (Table 5-1, fig 5-2A and 5-2B) despite the two strains producing similar biomass concentrations in the DO-stat (119.73 and 118.39 g/L for GAPfopA and GAPfopA_V1, respectively) and constant feed (133.86 and 132.44 g/L for GAPfopA and GAPfopA_V1, respectively) fermentations. Screening in 96-well plates or shake flasks are usually performed using a pulse feeding regime where an initial excess of the substrate is followed by substrate depletion, which leads to inconsistent growth conditions. In bioreactors, substrate feed is more uninterrupted, which avoids substrate depletion, resulting in more consistent biomass growth, which leads to a higher production of recombinant proteins (Looser et al., 2014). Furthermore, the transformant producing the highest amount of product generally suffers from increased metabolic burden of recombinant protein production and is thus not necessarily the best candidate for bioreactor cultivation (Holmes et al., 2009).

The growth performance for the respective strains was within expected levels indicating no abnormalities in the physiological growth of the strains. The maximum growth rates attained by the strains evaluated during the batch phase was between 0.19 h^{-1} and 0.24 h^{-1} (Table 5-1),

which is similar to what has previously been reported for *P. pastoris* (0.15 – 0.20 h^{-1}; Looser et al., 2014). The Y_{xs} (between 0.47 and 0.55 g/g) and biomass concentrations (between 118.39 and 133.86 g/L) for this study (Table 5-1) were also within ranges reported previously.

In terms of culture growth performance, constant feed fed-batch outperformed DO-stat fed-batch cultures due to poor DO control of the latter. Large oscillations resulted in an artificially broad DO band, which led to intermittent feeding and hence, inferior lower biomass accumulation. The constant feed fermentation exhibited higher biomass productivity (maximum of 2.27 and 0.77 g/L/h for constant feed and DO-stat, respectively), biomass yield (maximum of 0.55 and 0.49 g/g for constant feed and DO-stat, respectively) and DCW concentration (maximum of 133.86 and 119.73 g/L for constant feed and DO-stat, respectively) compared to the DO-stat fermentation, which could be attributed to the poor DO-stat feed control resulting from poor DO control. In this study, the DO is controlled with a proportional-integral-derivative (PID) controller, which has been shown to be problematic in controlling DO at high-cell-density fermentations due to the variations in DO being faster during the latter stages of the process when using the DO-stat method (Chung, 2000; Ferreira et al., 2012). Chung (2000) showed that during the fed-batch phase, feeding either methanol or glycerol, the rate of oxygen transfer nears that of the rate of oxygen utilization. This leads to increased oscillations in the metabolic behaviour of *P. pastoris* and results in controller destabilization and loss of culture productivity. Furthermore, the DO level oscillated between ~10% and ~55% (fig. 5-2) because of the poor DO-stat feed control, which created an artificially wide DO band resulting in intermittent feeding and therefore lower biomass growth compared to that of the constant feed fermentation. Lee et al. (2003) evaluated the cell-growth during the glycerol fed-batch phase for derepression of the *AOX1* promoter by feeding glycerol using the DO-stat method. The feed was controlled within a band consisting of two DO setpoints. The two bands evaluated had a lower setpoint of 10% DO and the upper band was either 30% DO or 50% DO.

The feed would start when the DO rose above 10% and would stop when it reached the upper DO setpoint. The study found that the specific growth rate was higher for the narrow band (10% - 30% DO) than for the wider band (10% - 50% DO) due to the more frequent feeding of the former.

No residual glycerol was detected during the fed-batch cultivations, which implied that the cultures were maintained under carbon-limited conditions and that biomass growth was not inhibited by the substrate accumulation. However, the constant feed fermentations required supplementary pure O_2 throughout the fed-batch phase to maintain the required level of dissolved oxygen of 30% of saturation to ensure that a dual limitation (carbon and oxygen) was not imposed (fig. 5-3B). On the other hand, the DO-stat fermentation did not require pure O_2 supplementation, except for a brief spike near the end of the batch phase where the culture growth was very active (fig. 5-3A). The culture growth rate was already reduced to such an extent (maximum biomass concentration of 119.73 g/L after 155 h for DO-stat and 133.86 g/L after 59 h for constant feed) due to the poor DO-stat control that the rate of oxygen consumption was low and O_2 supplementation was not required.

Predicting protein production based on biomass growth is not definitive and should be determined on a case by case basis. In this study, the increase in final biomass concentration, biomass productivity and subsequently growth rate in the constant feed fermentations compared to DO-stat fermentations did not result in higher volumetric activity (Table 5-1). The protein production in *P. pastoris* can either be growth associated (Cunha et al., 2004), partially growth associated (Kobayashi et al., 2000) or growth dissociated (Potgieter et al., 2010). However, it is generally accepted that proteins expressed under control of the GAP promoter are primarily growth-associated where the rate of product formation and product titer are correlated to the culture growth rate (Looser et al., 2014; Rebnegger et al., 2014) and biomass concentration (Pal et al., 2006). An increase in the specific growth rate leads to an increase in

the glycolytic flux, higher transcription levels of the gene under *GAP* promoter control (Garcia-Ortega et al., 2016) as well as increases in the specific rate of protein secretion (Rebnegger et al., 2014). This contradictory finding in this study could possibly be attributed to saturation of the secretory pathways and subsequent degradation of the accumulated intracellular product (Idiris et al., 2010; Kuo et al., 2015), in spite of increased levels of growth-associated protein expression at higher growth rates. Li et al. (2001) also showed that decreasing the fermentation temperature decreased the growth rate, which increased the production of herring antifreeze protein, possibly due to improved protein folding, and could explain the higher volumetric activity for the GAPfopA and GAPfopA_V1 strains at the lower growth rates.

The DO-stat fermentation produced a higher maximum volumetric activity than the constant feed fermentation, but the shorter process time of the constant feed fermentation resulted in a higher volumetric productivity (maximum of 23.96 x 10^3 U/L/h for constant feed and 13.74 x 10^3 U/L/h for DO-stat). A higher volumetric productivity is advantageous for manufacturing processes because it results in a higher enzyme production per unit volume of bioreactor, and therefore a more efficient and cost-effective application of equipment capital (Looser et al., 2014). Menéndez et al. (2013) recombinantly expressed and produced the β-fructosidase enzyme from *Thermotoga maritima* in *P. pastoris* using a constant feed fed-batch fermentation and produced an enzyme productivity of 3 347 U/L/h while Martínez et al. (2014) produced the same enzyme in *P. pastoris*, which resulted in a volumetric productivity of 5 735 U/L/h and 4 662 U/L/h for a constant and exponential feed, respectively.

In conclusion, the GAPfopA and GAPfopA_V1 enzymes were successfully expressed and produced by *P. pastoris* to high titre using fed-batch culture. The DO-stat fermentation was characterised by poor DO control and slow substrate feed rates, which had a marked effect on culture growth in terms of biomass accumulation. However, despite this inferior performance, the DO-stat cultures outperformed the constant feed cultures as evident from higher volumetric

enzyme activity although the extended process time to reach this higher level of enzyme production, as evident from SDS-PAGE analysis, resulted in lower volumetric enzyme productivity. Further evaluation is required to determine the optimum growth rate for the maximum production of the enzymes. Finally, the constant feed method exhibited better reproducibility and higher productivity despite a marginally lower enzyme production.

5.5 References

Balasubramaniem, A.K., Nagarajan, K.V., Paramasamy, G., 2001. Optimization of media for β-fructofuranosidase production by *Aspergillus niger* in submerged and solid state fermentation. Process Biochem. 36, 1241–1247.

Baumann, K., Maurer, M., Dragosits, M., Cos, O., Ferrer, P., Mattanovich, D., 2008. Hypoxic fed-batch cultivation of *Pichia pastoris* increases specific and volumetric productivity of recombinant proteins. Biotechnol. Bioeng. 100, 177–183.

Çalık, P., Ata, Ö., Güneş, H., Massahi, A., Boy, E., Keskin, A., Öztürk, S., Zerze, G.H., Özdamar, T.H., 2015. Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters. Biochem. Eng. J. 95, 20–36.

Cereghino, G.P.L., Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Curr. Opin. Biotechnol. 13, 329–332.

Chávez, F.P., Rodriguez, L., Díaz, J., Delgado, J.M., Cremata, J. A., 1997. Purification and characterization of an invertase from *Candida utilis*: Comparison with natural and recombinant yeast invertases. J. Biotechnol. 53, 67–74.

Chen, C.C., Wu, P.H., Huang, C.T., Cheng, K.J., 2004. A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of an *Escherichia coli* phytase. Enzyme Microb. Technol. 35, 315–320.

Chung, J.D., 2000. Design of metabolic feed controllers: Application to high-density fermentations of *Pichia pastoris*. Biotechnol. Bioeng. 68, 298–307.

Cunha, A.E., Clemente, J.J., Gomes, R., Pinto, F., Thomaz, M., Miranda, S., Pinto, R., Moosmayer, Donner, P., Carrondo, M.J.T., 2004. Methanol induction optimization for scFv antibody fragment production in *Pichia pastoris*. Biotechnol. Bioeng. 86, 458–467.

Dietzsch, C., Spadiut, O., Herwig, C., 2011. A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for *Pichia pastoris*. Microb. Cell Fact. 10, 14.

Ferreira, A.R., Ataíde, F., Von Stosch, M., Dias, J.M.L., Clemente, J.J., Cunha, A.E., Oliveira, R., 2012. Application of adaptive DO-stat feeding control to *Pichia pastoris* X33 cultures expressing a single chain antibody fragment (scFv). Bioprocess Biosyst. Eng. 35, 1603–1614.

Garcia-Ortega, X., Adelantado, N., Ferrer, P., Montesinos, J.L., Valero, F., 2016. A step forward to improve recombinant protein production in *Pichia pastoris*: From specific growth rate effect on protein secretion to carbon-starving conditions as advanced strategy. Process Biochem. 51, 681–691.

Garcia-Ortega, X., Ferrer, P., Montesinos, J.L., Valero, F., 2013. Fed-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive *GAP* promoter. Biochem. Eng. J. 79, 172–181.

Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Ferrer, M., Ballesteros, A., Plou, F.J., 2007. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus*. J. Biotechnol. 128, 204–211.

Goodrick, J.C., Xu, M., Finnegan, R., Schilling, B.M., Schiavi, S., Hoppe, H., Wan, N.C., 2001. High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system. Biotechnol. Bioeng. 74, 492–497.

Hidaka, H., Hirayama, M., Sumi, N., 1988. A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 52, 1181–1187.

Holmes, W.J., Darby, R.A.J., Wilks, M.D.B., Smith, R., Bill, R.M., 2009. Developing a scalable model of recombinant protein yield from *Pichia pastoris*: The influence of culture conditions, biomass and induction regime. Microb. Cell Fact. 8.

Idiris, A., Tohda, H., Kumagai, H., Takegawa, K., 2010. Engineering of protein secretion in yeast: strategies and impact on protein production. Appl. Microbiol. Biotechnol. 86, 403–417.

Invitrogen Corporation, 2002. *Pichia* fermentation process guidelines.

Kobayashi, K., Kuwae, S., Ohya, T., Ohda, T., Ohyama, M., Tomomitsu, K., 2000. High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, *Pichia pastoris*, based on optimal methanol feeding strategy. J. Biosci. Bioeng. 90, 280–288.

Krettler, C., Reinhart, C., Bevans, C.G., 2013. Expression of GPCRs in *Pichia pastoris* for structural studies., 1st ed, Methods in enzymology. Elsevier Inc.

Kuo, T.C., Shaw, J.F., Lee, G.C., 2015. Improvement in the secretory expression of recombinant *Candida rugosa* lipase in *Pichia pastoris*. Process Biochem. 50, 2137–2143.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lee, C.Y., Lee, S.J., Jung, K.H., Katoh, S., Lee, E.K., 2003. High dissolved oxygen tension enhances heterologous protein expression by recombinant *Pichia pastoris*. Process Biochem. 38, 1147–1154.

Li, Z., Xiong, F., Lin, Q., D'Anjou, M., Daugulis, A.J., Yang, D.S.C., Hew, C.L., 2001. Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. Protein Expr. Purif. 21, 438–445.

Lin-Cereghino, J., Wong, W.W., Xiong, S., Giang, W., Luong, L.T., Vu, J., Johnson, S.D., Lin-Cereghino, G.P., 2005. Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*. Biotechniques 38, 44–48.

Looser, V., Bruhlmann, B., Bumbak, F., Stenger, C., Costa, M., Camattari, A., Fotiadis, D., Kovar, K., 2014. Cultivation strategies to enhance productivity of *Pichia pastoris*: A review. Biotechnol. Adv. 33, 1177–1193.

Martínez, D., Menéndez, C., Echemendia, F.M., Pérez, E.R., Trujillo, L.E., Sobrino, A., Ramírez, R., Quintero, Y., Hernández, L., 2014. Complete sucrose hydrolysis by heat-killed recombinant *Pichia pastoris* cells entrapped in calcium alginate. Microb. Cell Fact. 13, 87.

Menéndez, C., Martínez, D., Trujillo, L.E., Mazola, Y., González, E., Pérez, E.R., Hernández, L., 2013. Constitutive high-level expression of a codon-optimized β-fructosidase gene from the hyperthermophile *Thermotoga maritima* in *Pichia pastoris*. Appl. Microbiol. Biotechnol. 97, 1201–1212.

Merril, C.R., Goldman, D., Sedman, S.A., Ebert, M.H., 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in crebrospinal fluid proteins. Science (80-.). 211, 1437–1438.

Nishizawa, K., Akajima, M.N., Abetani, H.N., 2001. Kinetic study on transfructosylation by β-fructofuranosidase from *Aspergillus niger* ATCC 20611 and availability of a membrane reactor for fructooligosaccharide production. Food Sci. Technol. Res. 7, 39–44.

Pal, Y., Khushoo, A., Mukherjee, K.J., 2006. Process optimization of constitutive human granulocyte-macrophage colony-stimulating factor (hGM-CSF) expression in *Pichia pastoris* fed-batch culture. Appl. Microbiol. Biotechnol. 69, 650–657.

Potgieter, T.I., Kersey, S.D., Mallem, M.R., Nylen, A.C., D'Anjou, M., 2010. Antibody expression kinetics in glycoengineered *Pichia pastoris*. Biotechnol. Bioeng. 106, 918–927.

Potvin, G., Ahmad, A., Zhang, Z., 2012. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochem. Eng. J. 64, 91–105.

Rebnegger, C., Graf, A.B., Valli, M., Steiger, M.G., Gasser, B., Maurer, M., Mattanovich, D., 2014. In *Pichia pastoris*, growth rate regulates protein synthesis and secretion, mating and stress response. Biotechnol. J. 9, 511–525.

Sheu, D.-C., Duan, K.-J., Cheng, C.-Y., Bi, J.-L., Chen, J.-Y., 2002. Continuous production of high-content fructooligosaccharides by a complex cell system. Biotechnol. Prog. 18, 1282–1286.

Singh, S.P., Jadaun, J.S., Narnoliya, L.K., Pandey, A., 2017. Prebiotic oligosaccharides: Special focus on fructooligosaccharides, its biosynthesis and bioactivity. Appl. Biochem. Biotechnol. 183, 613–635.

Trinh, L.B., Phue, J.N., Shiloach, J., 2003. Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from *Pichia pastoris*. Biotechnol. Bioeng. 82, 438–444.

Trollope, K.M., Görgens, J.F., Volschenk, H., 2015. Semirational directed evolution of loop regions in *Aspergillus japonicus* β-fructofuranosidase for improved fructooligosaccharide production. Appl. Environ. Microbiol. 81, 7319–7329.

van Wyk, N., Trollope, K.M., Steenkamp, E.T., Wingfield, B.D., Volschenk, H., 2013. Identification of the gene for β-fructofuranosidase from *Ceratocystis moniliformis* CMW 10134 and characterization of the enzyme expressed in *Saccharomyces cerevisiae*. BMC Biotechnol. 13, 100.

Yun, J.W., 1996. Fructooligosaccharides—Occurrence, preparation, and application. Enzyme Microb. Technol. 19, 107–117.

Zhang, A.L., Zhang, T.Y., Luo, J.X., Chen, S.C., Guan, W.J., Fu, C.Y., Peng, S.Q., Li, H.L., 2007. Constitutive expression of human angiostatin in *Pichia pastoris* by high-density cell culture. J. Ind. Microbiol. Biotechnol. 34, 117–122.

Zhang, J., Liu, C., Xie, Y., Li, N., Ning, Z., Du, N., Huang, X., Zhong, Y., 2017. Enhancing fructooligosaccharides production by genetic improvement of the industrial fungus *Aspergillus niger* ATCC 20611. J. Biotechnol. 249, 25–33.

Chapter 6 Evaluation of the performance of an engineered β-fructofuranosidase from *Aspergillus fijiensis* to produce short-chain fructooligosaccharides from industrial sugar streams

Gerhardt Coetzee, Eugéne van Rensburg and Johann F. Görgens

Department of Process Engineering, Stellenbosch University, Stellenbosch, South Africa

Objective of dissertation in this chapter

This chapter addresses objective three, which focusses on the evaluation of the difference in the ability of the native and engineered enzyme, produced in Chapter 5, to produce a targeted scFOS composition from industrial sugar streams and scale-up production of scFOS from table sugar. First, the optimum conditions for the production of scFOS by these enzymes had to be determined as this has not yet been reported. Therefore, the optimum conditions with regards to temperature and enzyme dosage were determined by RSM to produce a scFOS composition similar to a commercial FOS product from laboratory grade sucrose. Since the engineered enzyme has only previously been evaluated on laboratory-grade sucrose the enzyme was evaluated on industrial sugar streams as possible low-cost substitutions. The optimized conditions were therefore applied to A-molasses and refinery molasses with both enzymes. Both these enzymes were able to produce the target scFOS composition from A-molasses albeit with an increased reaction time due to the impurities contained in the substrate. Neither enzyme could produce the required scFOS composition from refinery molasses. The production of scFOS from laboratory grade sucrose in shake flasks was replicated in a scaled-up reaction with table sugar in a bioreactor with the latter producing the required scFOS composition in a shorter time period. Furthermore, the improved thermostability and subsequent shorter reaction

times because of this characteristic of the engineered enzyme was confirmed here. Thus, all objectives were met as set out in Chapter 3.

Candidate declaration

In chapter 6 page numbers 105 to 137 of this dissertation, the type and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Planning of experiments	80
Executing experiments	100
Interpretation of results	70
Writing and reviewing of the chapter	70

The listed co-authors have contributed to chapter 6 page numbers 105 to 137 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution (%)
Eugéne van Rensburg		Planning of experiments	10
		Interpretation of results	20
		Writing and reviewing of the chapter	20
Johann F. Görgens		Planning of experiments	10
		Interpretation of results	10
		Writing and reviewing of the chapter	10

Signature of candidate: 

Date: 27 January 2019

Declaration by co-authors

The undersigned hereby confirm that

- The declaration above accurately reflects the type and extent of the contributions of the candidates and co-authors to chapter 6 page numbers 105 to 137 in the dissertation,

- No other authors contributed to chapter 6 page numbers 105 to 137 in the dissertation besides those specified above, and

- Potential conflicts of interest have been revealed to all interested parties and that necessary arrangements have been made to use the material in chapter 6 page numbers 105 to 137 of this dissertation.

Institutional affiliation	Date	Signature

Abstract

An engineered β-fructofuranosidase, GAPfopA_V1 was compared to the native enzyme, GAPfopA, for the production of short-chain fructooligosaccharides (scFOS) from white and brown sugar (commercial table sugar), and refinery and A-molasses (refinery mill and sugar mill, respectively). The minimum reaction temperature and enzyme dosage required to produce scFOS with a composition similar to that of a commercial prebiotic product, consisting of oligomers with 2 to 4 fructose moieties, were determined using response surface methodology. At 62 °C and 10 U/g sucrose, the GAPfopA_V1 resulted in a 28% and 25% decrease in reaction time required to reach the target scFOS composition, in comparison to the native enzyme, using white and brown sugar as substrate, respectively. Whereas the target scFOS composition could not be reached using refinery molasses, this composition was reached with A-molasses using both enzymes, where GAPfopA had a 6% shorter reaction time than GAPfopA_V1.

Highlights

Engineered enzyme exhibited improved thermostability

Reduced scFOS production time with engineered enzyme

Industrial sugars feasible for fructooligosaccharide production

Keywords: Short-chain fructooligosaccharides (scFOS); *Aspergillus fijiensis*; β-fructofuranosidase

6.1 Introduction

Fructooligosaccharides (FOS) occur naturally in plants (asparagus, sugar beet, onion and Jerusalem artichoke) and are also produced by various bacteria, such as *Bacillus macerans* and *Arthrobacter* sp. and fungi, including *Aspergillus niger*, *A. japonicus* and *Aureobasidium pullulans* (Sangeetha et al., 2005a; Yun, 1996). These oligosaccharides function as prebiotics with proven health benefits for humans and animals. Typical benefits include improved mineral absorption, protection against colon cancer, the bifidogenic effect, positive effects on lipid metabolism and the control of type II diabetes (Hidaka et al., 1986; Roberfroid, 2007; Singh and Singh, 2010). scFOS is produced from the transfructosylation of sucrose by β-fructofuranosidases (EC 3.2.1.26) and consists mainly of 1-kestose (GF2), nystose (GF3) and 1^F-fructofuranosyl nystose (GF4) (Yun, 1996). Beghin Meiji is the major producer of oligofructose from sugar beet and marketer of Actilight®. The powdered version of this product (Actilight 950P®) consists of 95% scFOS with a composition of 37% GF2, 53% GF3 and 10% GF4 (Paineau et al., 2014; Respondek et al., 2014). The health benefits of scFOS have been demonstrated *in vivo* and *in vitro* (Gibson et al., 2004; Yen et al., 2011) with several clinical trials showing health benefits of specifically Actilight 950P® (Lecerf et al., 2015; Paineau et al., 2014; Respondek et al., 2014).

The general reaction mechanism for fructosyltransferase enzymes is a double-displacement mechanism involving two steps, namely hydrolysis and transfer of a sugar monomer (Chuankhayan et al., 2010). Sucrose is first hydrolyzed to produce a free glucose, while the fructose is retained to form a fructosyl-enzyme intermediate. In the second step, the fructose moiety is transferred to an acceptor, which can be either a fructan (sucrose, GF2 or GF3) or water. When water is the acceptor the result is hydrolysis, which produces glucose and fructose while the fructan acceptor leads to FOS as a product. GF2 is produced first since sucrose is

usually the initial acceptor. However, GF2 then decreases as another fructose monomer is added to form GF3, which in turn becomes an acceptor to form GF4. The transfructosylating reaction predominantly occurs at sucrose concentrations above 200 g/L (Kim et al., 1996), whereas the same enzyme has a hydrolytic action at sucrose concentrations below 5 g/L.

Commercial FOS is produced in a 20 h batch process by the transfructosylation of sucrose with a 50% to 60% (w/v) sucrose solution at pH 5.5 to 6.0 and temperatures between 50 and 60 °C (Nishizawa et al., 2001). However, this process has limitations with regards to enzyme inhibition and enzyme stability, which restricts the maximum attainable FOS yield. At temperatures above 50 °C, the long-term stability of the enzyme becomes compromised even when immobilised (Yun and Song, 1999). The maximum FOS yield using sucrose solutions of 55% to 80% (w/v) were between 50% to 60% of the total mass of sugars in the reaction due to the inhibitory effect of the released glucose (Ghazi et al., 2007; Sangeetha et al., 2004).

Given the commercial value of FOS, various approaches were followed in the past to improve production efficiency. In some instances, research focused on either screening for novel enzymes with improved transfructosylating capability (Chávez et al., 1997; Ghazi et al., 2007), optimising the production of the native enzymes with known transfructosylating capability (Balasubramaniem et al., 2001; Vandáková et al., 2004) or optimising the FOS production process (Sangeetha et al., 2005b; Sheu et al., 2002). Furthermore, there have been several studies on the use of by-products of the sugar production process (Ghazi et al., 2006; Rehman et al., 2016) and other agro-industrial wastes as substrates for the production of FOS and/or transfructosylating enzymes (Mussatto and Teixeira, 2010; Sangeetha et al., 2004) with the aim to reduce production costs.

The β-fructofuranosidase (fopA) from *A. fijiensis* (previously known as *A. niger* ATCC 20611) has been used extensively for commercial scFOS production due to a high transfructosylating

to hydrolytic activity ratio and strong regiospecificity when using sucrose (Yanai et al., 2001). Molecular techniques have been used to improve the production of this enzyme with homologous expression (Zhang et al., 2017).

Engineering the fructosyltransferase enzyme to alter its function was generally a successful strategy to optimise and change the composition of FOS products (Beine et al., 2008; De Abreu et al., 2013). More recently Trollope et al. (2015) engineered the β-fructofuranosidase, also referred to as fopA, from *A. japonicus* through semi-rational directed evolution and successfully increased the specific activity and thermostability of the enzyme, while also demonstrating decreased glucose inhibition compared to the native enzyme. The thermostability of the improved variant was demonstrated with isothermal denaturation (ITD) and differential scanning fluorimetry (DSF). However, the results of these techniques are more theoretical and do not necessarily reflect what would occur in an actual scFOS production process.

In this study, an engineered enzyme (GAPfopA_V1) from *A. fijiensis* (Trollope et al., 2015) was compared to the native enzyme (GAPfopA) to demonstrate its improved ability to produce scFOS using the composition of Actilight® as reference. Moreover, industrial substrates were used in addition to refined sugar to demonstrate the suitability of producing a high-value product from an industrial waste stream. Response surface methodology (RSM) based on a central composite design (CCD) was used to optimise scFOS production in terms of minimum temperature and enzyme dosage, and allow comparison of enzymes under optimised conditions.

6.2 Methods

6.2.1 Materials

The *Pichia pastoris* strains containing the respective codon-optimised genes (ATUM, Newark, CA, USA) under control of the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter were maintained in the culture collection of the Department of Process Engineering, and the Department of Microbiology, Stellenbosch University, South Africa. 1^F-fructofuranosylnystose (Wako Chemicals GmbH, Neuss, Germany), 1-kestose and nystose (Carbosynth Limited, Berkshire, UK) served as standards for high-performance liquid chromatography (HPLC) analysis of scFOS produced from sucrose. All other chemicals were purchased from Sigma-Aldrich (South Africa) or Merck (South Africa) with the yeast nitrogen base obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

6.2.2 Bioreactor cultivations

A starter culture was prepared by inoculating a single colony into a test tube containing 4 ml buffered minimal glycerol (BMG) culture medium consisting of 1.34% yeast nitrogen base, 1% glycerol, 1.64 µM biotin, 100 mM potassium phosphate buffer (pH 6.0). The culture was incubated for 24 h at 30 °C before inoculating 250 ml Erlenmeyer shake flasks containing 40 ml fresh BMG medium to a final biomass concentration equating to an optical density (OD) of 0.1 measured at 600 nm. These cultures were grown for a further 18 h on an orbital shaker adjusted to 200 rpm to a biomass concentration equal to OD_{600} of between 6 and 8. The entire volume of these cultures was used to inoculate the bioreactor to a final volume of 400 ml, resulting in an initial biomass concentration equal to an OD_{600} of 0.6-0.8 at the start of the batch phase.

The cultivations were carried out in Bioflo 110 bioreactors (New Brunswick Scientific Co. Inc., Edison NJ, USA) fitted with a glass reactor vessel with a total volume of 1.3 L and a working volume of 1 L. The Biocommand version 3.30 Plus software (New Brunswick Scientific Co. Inc.) was used for monitoring DO, temperature, pH and agitation and feed rate control. Fermentations were performed as described in Pichia Fermentation Guidelines (Invitrogen Corporation, 2002) (Thermo Fisher Scientific, Waltham, MA, USA) with modifications noted below.

Fermentation basalt salt medium (BSM), supplemented with 1% casein hydrolysate and PTM_1 trace salts was used as culture media in the bioreactors. Cultivations were carried out at 30 °C and the pH was maintained at pH 5.0 using 28% ammonium hydroxide (Sigma-Aldrich). Atmospheric air was sparged at an aeration rate of 1.0 vvm to maintain the dissolved oxygen tension (DOT) above 30% of saturation. A DO-stat strategy was followed during the glycerol fed-batch phase where a solution of 50% (w/v) glycerol (Scienceworld, South Africa) was fed at a rate of 18.15 ml/h/L of initial fermentation volume when the DOT exceeded 35%, which indicated that the carbon substrate in the culture medium was depleted. The same threshold was used to switch pumps off when during glycerol feeding when the DOT decreased below 35% of saturation, which prohibited overfeeding of the culture. The glycerol fed-batch phase was maintained for 72 h after the initial batch phase. To harvest the enzymes from the culture supernatant, the whole culture volume was centrifuged batch-wise using a fixed angle rotor benchtop centrifuge (Avanti J-E, Beckman Coulter, Pinelands, South Africa) for 3 min at 3000 rpm and filtered through a 22 µm syringe filter (Pall South Africa (Pty) Limited) to sterilize sample and stored at 4 °C.

6.2.3 Enzyme activity assay

The enzyme activity was determined using 100 g/L sucrose (Merck) as substrate prepared in 50 mM citrate phosphate buffer (pH 5.5). The substrate solution was equilibrated at 40 °C for 10 min prior to adding the culture supernatant containing the enzyme to a final concentration of 25% (v/v). After incubation at 40 °C for 60 min, the reaction was stopped by addition of 35% (w/v) perchloric acid (PCA) to a final concentration of 2.14% (v/v) followed by the addition of 7 M KOH to precipitate the proteins prior to chemical analysis (Hidaka et al., 1988). Reactions consisting of all the assay constituents except either sucrose or enzyme served as negative controls.

6.2.4 Sugar analyses

The samples containing the sugars of interest (sucrose, glucose, fructose and scFOS) were analysed on a Dionex Ultimate 3000 system equipped with a Coulochem III electrochemical detector controlled by Chromeleon 6.8 Chromatography Data System software. The columns used were a CarboPac PA1 (4 × 250 mm) analytical column coupled to a PA1 (4 × 50 mm) guard column. The samples (10 µl) were eluted according to van Wyk et al. (2013) The concentration of glucose liberated during the assays was indicative of enzyme activity. A unit of enzyme was defined as the amount of enzyme required to produce 1 µmol glucose per minute under the described conditions (Hidaka et al., 1988).

6.2.5 Optimisation and validation of scFOS production

scFOS was produced using a 60% sucrose solution (w/v) prepared in a 50 mM citrate phosphate buffer (pH 5.0). The substrate solution was equilibrated for 2 min at temperatures as described in the text using a temperature-controlled water bath with an orbital shaker (Gyratory Water Bath Shaker, New Brunswick Scientific Co. Inc.) adjusted to 120 rpm before adding the

enzyme-containing supernatant at dosages as described in the text. Temperatures and enzyme dosages were based on factorial levels determined by a statistical design (see below). The reaction was incubated for 8 h with samples withdrawn every 2 h prior to terminating the reaction using the PCA/KOH method described above. Validation experiments were performed as described above with values determined from desirability analysis. All incubations were done in triplicate.

6.2.6 Central composite design

The production of scFOS was optimised using response surface methodology (RSM) with a two-factor central composite design (CCD) where temperature and enzyme dosage were the treatment variables. The response variables were 1-kestose, nystose and 1F-fructofuranosyl-nystose. The factoral design space ranged between 57 °C ≤ A ≤ 67 °C and 8 U/g sucrose ≤ B ≤ 12 U/g sucrose, where A represents the temperature and B the enzyme dosage. The ranges were based on the results of Trollope et al. (2015) who produced and characterised the engineered version of the fopA enzyme. A total of 11 experiments for each enzyme was performed (Table 6-1). All statistical analyses were performed using STATISTICA (data analysis software) version 13.3 (TIBCO Software Inc., 2017, USA) and Design Expert® software (Stat-Ease Inc., Minneapolis, USA). A p-value smaller than 0.05 was considered significant to identify main and interaction effects. The polynomial equations based on actual values obtained from the analysis of multiple regression for the GAPfopA and GAPfopA_V1 enzymes are presented in Appendix C.

6.2.7 scFOS production from industrial substrates

The industrial substrates (A-molasses and refinery molasses) were obtained from RCL Foods (Sugar and Milling Division, South Africa) with brown and white table sugar (Selati Sugar, RCL Foods, Sugar and Milling Division Ltd, South Africa) as reference. The substrates were

diluted to 60% (w/v) sucrose in RO water. The diluted substrates were equilibrated to the optimum temperature determined by RSM for 2 min using a temperature-controlled water bath with an orbital shaker (Gyratory Water Bath Shaker, New Brunswick Scientific Co. Inc.) adjusted to 120 rpm. The respective enzyme-containing supernatants were added at the optimum dosage determined by RSM and incubated for 12 h with samples taken every 2 h and treated and analysed as already described. The pH of all the substrates was determined at 0 h and 12 h.

Table 6-1. Central composite design of temperature (A) and enzyme dosage (B) for scFOS production for both GAPfopA and GAPfopA_V1.

Experimental run	Coded values		Natural values	
	X_A	X_B	A (°C)	B (U/g sucrose)
1	0	0	62.00	10.00
2	1	1	67.00	12.00
3	-1	1	57.00	12.00
4	0	0	62.00	10.00
5	1	-1	67.00	8.00
6	0	1.414	62.00	12.83
7	0	-1.414	62.00	7.17
8	-1.414	0	54.93	10.00
9	0	0	62.00	10.00
10	-1	-1	57.00	8.00
11	1.414	0	69.07	10.00

6.2.8 Scale-up production of scFOS

The scale-up production of scFOS was performed in a 20 L New Brunswick Bioflo IV bioreactor (New Brunswick Scientific Co. Inc.). A 60% sucrose solution (w/v) was prepared using white table sugar (Tongaat Hulett Sugar South Africa Ltd) dissolved in a 50 mM citrate phosphate buffer (pH 5.0) to a final volume of 15 L. The substrate solution was equilibrated to the optimum temperature as determined from optimization and agitated at 200 rpm. The required enzyme dosage was added, and the reaction was performed for 12 h and samples were taken every 2 h and samples treated and analysed as described previously.

6.3 Results and discussion

6.3.1 Characterization of processing parameters for scFOS production

The engineered GAPfopA_V1 enzyme was compared to the native enzyme using RSM to determine the minimum temperature and enzyme dosage required to produce scFOS with a composition similar to Actilight® when using high purity sucrose buffered at pH 5.0. These conditions were then used for scFOS production from industrial substrates to assess the potential for valorising sugar mill waste to a high-value prebiotic product. The GAPfopA and GAPfopA_V1 enzymes with volumetric activities of 1202 and 1124 U/mL, respectively, were produced using two recombinant *P. pastoris* strains in bioreactor culture (Chapter 5 in this thesis). Using the reaction conditions recommended by Trollope et al. (2015) as a benchmark, scFOS production at 62 ^{0}C and 10 U/g sucrose with a reaction time of 8 h were used as point of departure.

The results of the CCD experiments for FOS production with GAPfopA and GAPfopA_V1 after 8 h are shown in Tables 6-2 and 6-3, respectively, where the cube points were 5 ^{0}C and 2 U/g sucrose above and below the respective centre points of 62 ^{0}C and 10 U/g sucrose. Both

Table 6-2. Observed responses after 8 h incubation with GAPfopA as percentages of total sugars and percentage of total scFOS.

Experimental run	% of Total sugars						% scFOS		
	Glucose	Sucrose	Fructose	GF2	GF3	GF4	GF2	GF3	GF4
1	32.41	10.66	4.10	21.03	26.11	5.70	39.81	49.41	10.78
2	33.14 ± 0.84	11.15 ± 0.68	6.25 ± 0.92	20.34 ± 0.22	24.55 ± 0.85	4.58 ± 0.01	41.11 ± 0.45	49.62 ± 0.63	9.27 ± 0.18
3	31.37 ± 0.77	10.19 ± 0.83	3.44 ± 0.83	20.63 ± 0.32	29.01 ± 1.10	5.37 ± 0.01	37.51 ± 1.11	52.73 ± 1.27	9.76 ± 0.16
4	33.57	10.27	4.33	20.42	26.47	4.94	39.39	51.07	9.54
5	30.98 ± 0.69	13.05 ± 0.51	6.46 ± 1.18	25.42 ± 0.32	21.16 ± 0.89	2.93 ± 0.15	51.35 ± 0.76	42.74 ± 0.62	5.92 ± 0.14
6	34.04 ± 0.67	9.68 ± 0.39	5.35 ± 0.90	18.10 ± 0.06	26.28 ± 0.57	6.55 ± 0.55	35.56 ± 0.71	51.59 ± 0.08	12.85 ± 0.79
7	31.31 ± 0.07	11.61 ± 0.09	4.90 ± 0.77	25.03 ± 0.51	23.76 ± 0.41	3.40 ± 0.31	47.95 ± 0.41	45.52 ± 0.25	6.53 ± 0.67
8	31.51 ± 0.01	9.94 ± 0.63	3.17 ± 1.00	28.26 ± 1.00	23.46 ± 0.73	3.65 ± 0.10	51.04 ± 0.30	42.36 ± 0.07	6.60 ± 0.38
9	32.80	10.39	4.33	20.22	26.76	5.49	38.54	50.99	10.47
10	29.59 ± 0.09	10.94 ± 0.35	3.22 ± 0.84	27.87 ± 0.08	25.11 ± 0.51	3.27 ± 0.18	49.55 ± 0.21	44.64 ± 0.58	5.81 ± 0.37
11	32.63 ± 1.65	10.47 ± 2.38	6.05 ± 1.18	26.21 ± 0.36	21.96 ± 1.77	2.68 ± 0.22	51.56 ± 1.24	43.15 ± 1.87	5.28 ± 0.63

Mean ± standard deviation, n = 2

Table 6-3. Observed responses after 8 h incubation with GAPfopA_V1 as percentages of total sugars and percentage of total scFOS.

Experimental run	% of Total sugars						% scFOS		
	Glucose	Sucrose	Fructose	GF2	GF3	GF4	GF2	GF3	GF4
1	35.32	10.26	4.34	18.34	25.53	6.21	36.63	50.97	12.40
2	36.71 ± 0.81	7.59 ± 1.63	7.40 ± 1.75	15.95 ± 0.48	24.61 ± 0.72	7.75 ± 0.28	33.00 ± 0.37	50.95 ± 0.52	16.05 ± 0.88
3	33.83 ± 0.77	7.47 ± 1.28	3.70 ± 1.15	18.96 ± 0.11	29.57 ± 0.92	6.48 ± 0.17	34.47 ± 0.60	53.75 ± 1.05	11.78 ± 0.45
4	33.22	10.15	3.98	18.06	28.27	6.32	34.30	53.70	12.00
5	33.71 ± 0.68	9.98 ± 2.22	8.10 ± 3.21	20.22 ± 0.74	23.41 ± 0.84	4.58 ± 0.09	41.94 ± 0.09	48.56 ± 0.05	9.50 ± 0.14
6	36.44 ± 1.18	7.80 ± 1.79	6.28 ± 1.58	15.75 ± 0.11	25.54 ± 0.38	8.20 ± 0.48	31.83 ± 0.40	51.60 ± 0.24	16.56 ± 0.64
7	33.17 ± 1.19	9.85 ± 2.44	6.30 ± 1.81	21.62 ± 0.27	24.75 ± 0.23	4.30 ± 0.06	42.67 ± 0.06	48.85 ± 0.08	8.48 ± 0.02
8	29.50 ± 2.08	9.79 ± 1.36	2.86 ± 1.18	30.42 ± 2.08	22.02 ± 1.79	5.42 ± 0.75	52.61 ± 0.61	38.05 ± 0.06	9.34 ± 0.55
9	33.97	10.10	4.37	18.14	26.01	7.41	35.18	50.44	14.38
10	31.97 ± 0.64	8.50 ± 2.10	3.16 ± 1.44	24.43 ± 0.22	28.49 ± 0.77	3.46 ± 0.58	43.33 ± 0.37	50.53 ± 1.40	6.14 ± 1.03
11	35.32 ± 0.32	9.25 ± 2.96	7.79 ± 2.64	18.67 ± 0.57	24.00 ± 0.81	4.97 ± 0.25	39.19 ± 1.19	50.39 ± 1.71	10.42 ± 0.52

Mean ± standard deviation, n = 2

the enzymes produced ~30% glucose after 8 h, whereas the sucrose concentration, in saccharide weight composition, decreased from 100% at the start of the experiments to between 7% and 14%, depending on the temperatures and enzyme dosages used. This data compared well with the results of Nishizawa et al. (2001) who used the wild-type enzyme from *Aspergillus niger* ATCC 20611 and reported values of 30% glucose and 10-12% sucrose after 6 h. In their study, GF2 also decreased to below 25%, which was similar to our findings.

In all the experimental runs, with the exception of runs 8 and 9 (Tables 6-2 and 6-3), the GAPfopA_V1 enzyme produced an scFOS mixture where the GF2 fraction was lower (33.00% - 43.33% and 37.51% - 51.56% for GAPfopA_V1 and GAPfopA, respectively) and the GF3 (48.56% - 53.75% and 42.74% - 52.73% for GAPfopA_V1 and GAPfopA, respectively) and GF4 (8.48% - 16.56% and 5.92% - 12.85% for GAPfopA_V1 and GAPfopA, respectively) fractions greater than when the GAPfopA enzyme was used, which pointed to a clear improvement in reaction efficiency, since a greater proportion of the GF2 fraction was converted to GF3 and GF3 to GF4 in a constant time interval (Vega and Zúniga-Hansen, 2011). This result was consistent with the results of Trollope et al. (2015) where their study demonstrated greater thermostability by the V1 variant. This thermostability resulted in a greater level of sustained enzyme activity at the reaction temperature used to catalyse an improved conversion, leading to higher concentrations of GF3 and GF4 and hence, lower GF2 concentrations. The effect of the improved thermostability of the GAPfopA_V1 variant was especially evident in run 11 that was conducted at 69.07 °C (10% greater than the benchmark temperature), where the GAPfopA_V1 enzyme produced 10.42% GF4, which was almost twice the quantity produced by GAPfopA at the same conditions. This trend was also evident in runs 2 and 5 at a temperature of 67 °C, irrespective of enzyme dosage. This data supported the findings of Hirayama et al. (1989), where the purified native fopA enzyme was reported to

have a temperature optimum of 50 to ~ 60 °C but rapidly lost ~50% of its activity when the temperature was increased to 70 °C.

Response surface plots and related analysis of variance (ANOVA) of the regression models are shown in fig. 6-1 and table 6-4, respectively. The surface plots illustrate the dynamics of the conversion of GF2 to GF3 and of GF3 to GF4 as well as the increased thermostability of the GAPfopA_V1 enzyme compared to the native enzyme. An increase in enzyme dosage resulted in a clear decrease in GF2 (from 47.97% to 33.89% at 62 °C for GAPfopA and from 41.05% to 29.32% at 63 °C for GAPfopA_V1) and an increase in GF3 (from 45.28% to 53.61% at 62 °C for GAPfopA and from 50.67% to 53.91% at 63 °C for GAPfopA_V1) and GF4 (from 6.76% to 12.50% at 62 °C for GAPfopA and from 8.28% to 16.76% at 63 °C for GAPfopA_V1) after an 8 h reaction time for both enzymes. Since a higher enzyme to substrate ratio will result in a faster reaction the linear response of the enzyme dosage was generally significant ($p < 0.05$; Table 6-4), which suggested that the optimum enzyme dosage to minimize GF2 and maximize GF3 and GF4 was not within the range of enzyme dosages evaluated. However, there was no significant effect for enzyme dosage for the GF3 response for GAPfopA_V1 (Table 6-4; fig. 6-1D). This is possibly due to the increased efficiency with which GAPfopA_V1 converts GF3 to GF4 because of the improved thermostability of the enzyme. Further kinetic analysis would be required to confirm this hypothesis.

A clear temperature optimum (~62 °C) was reached with regards to minimizing GF2 and maximizing GF3 and GF4, which was particularly evident from the significant quadratic effect for GAPfopA (Table 6-4). However, from the response surface plots for GAPfopA_V1 (fig. 6-1B, 6-1D and 6-1F) it is clear that the temperature optimum is higher (~63 °C) than for the native GAPfopA enzyme. Since the same temperature ranges were used to demonstrate the thermostability of the enzymes, the quadratic effect for GAPfopA_V1 was borderline

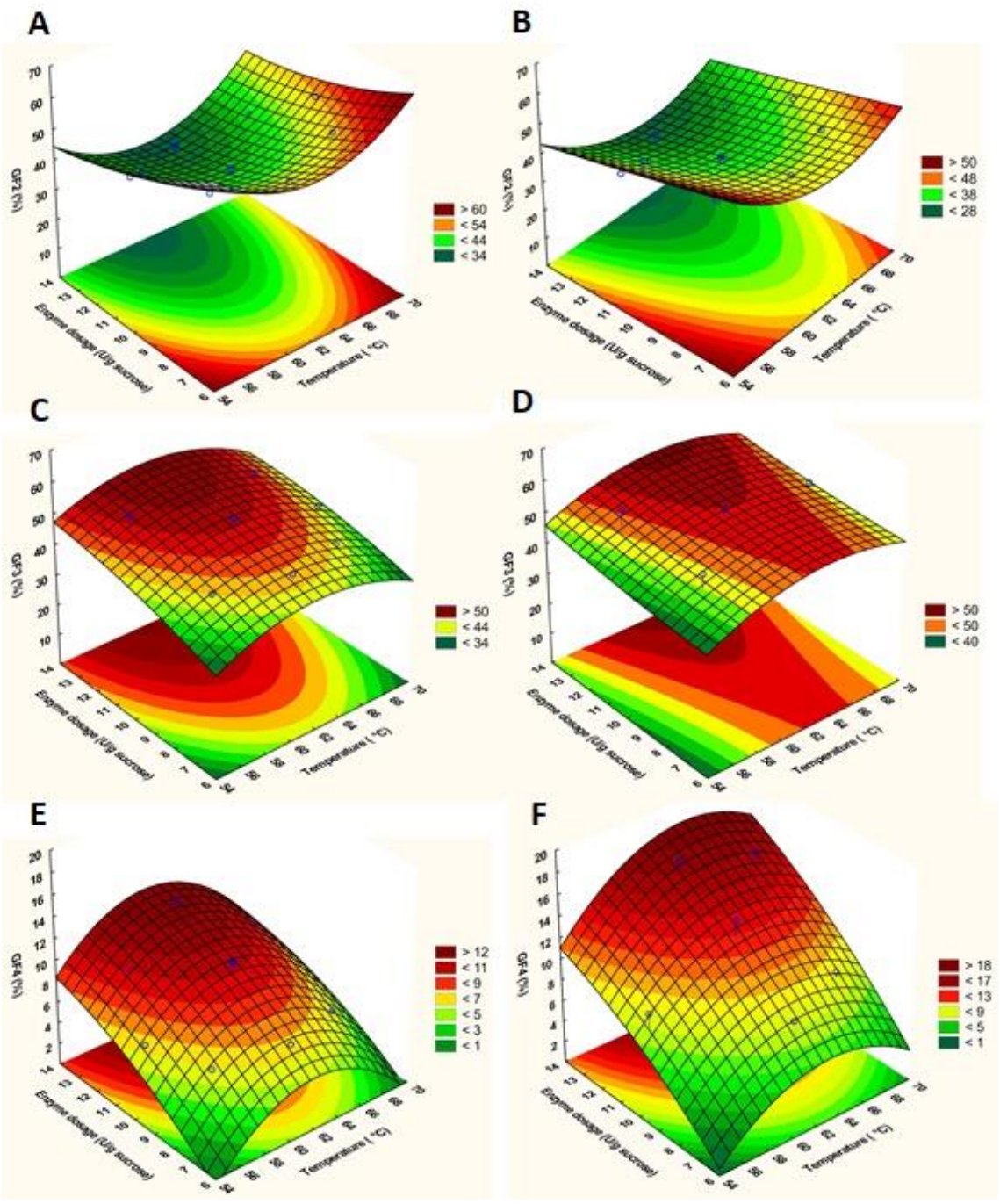


Figure 6-1. Response surface and contour plots at an 8 h reaction time for FOS percentage as a function of enzyme dosage and temperature for (A), (C) and (E) as percentage GF2, GF3 and GF4, respectively, produced by GAPfopA and (B), (D) and (F) produced by GAPfopA_V1.

Table 6-4. ANOVA of the model terms best fitting the relationship between the independent variables and responses.

Enzyme	Response variable	Independent variable	*p*-value	Adj. R^2
GAPfopA	GF2%	Temperature (Q)	0.002	0.93
		Enzyme dosage (L)	0.002	
	GF3%	Temperature (Q)	0.013	0.82
		Enzyme dosage (L)	0.012	
	GF4%	Temperature (Q)	0.015	0.94
		Enzyme dosage (L)	0.012	
GAPfopA_V1	GF2%	Temperature (L)	0.022	0.77
		Temperature (Q)	0.011	
		Enzyme dosage (L)	0.010	
	GF3%	Temperature (Q)	0.055[a]	0.23
		Enzyme dosage (L)	0.194	
	GF4%	Temperature (Q)	0.097[b]	0.88
		Enzyme dosage (L)	0.023	

Q = Quadratic; L =Linear
Lack of fit for all model terms *p*-value ≥ 0.05

[a] Borderline significant; [b] Marginally significant at 90% confidence level

significant. To encompass the optimum conditions for GAPfopA_V1, the enzyme dosage and temperature ranges would have to be adjusted, which will most likely result in a significant quadratic response during ANOVA analysis. However, the validation data (see below) showed that the responses were still sufficient to predict the optimum conditions whereby the target GF2, GF3 and GF4 fractions could be achieved. The increased thermostability was further confirmed in the higher fraction GF4 for GAPfopA_V1 compared to GAPfopA (12.93% and 10.26%, respectively) after an 8 h reaction time. This would indicate a faster reaction for the former compared to the latter.

6.3.2 Desirability analysis

A desirability analysis was performed for GAPfopA and GAPfopA_V1 to determine the minimum temperature and enzyme dosage required to produce scFOS with a composition similar to Actilight®. To reduce the number of possible solutions and still be able to produce the target composition, the range selected for the analysis was ±3% from the defined percentages for the respective scFOS sugars to replicate Actilight®. The area within the band in Fig. 6-2 illustrates the different possible combinations of enzyme dosages and temperatures required to produce the scFOS combination mentioned above. Based on this analysis, the minimum amount enzyme required for GAPfopA and GAPfopA_V1 to produce the target scFOS composition was 10 U/g sucrose combined with a minimum temperature of 62 °C.

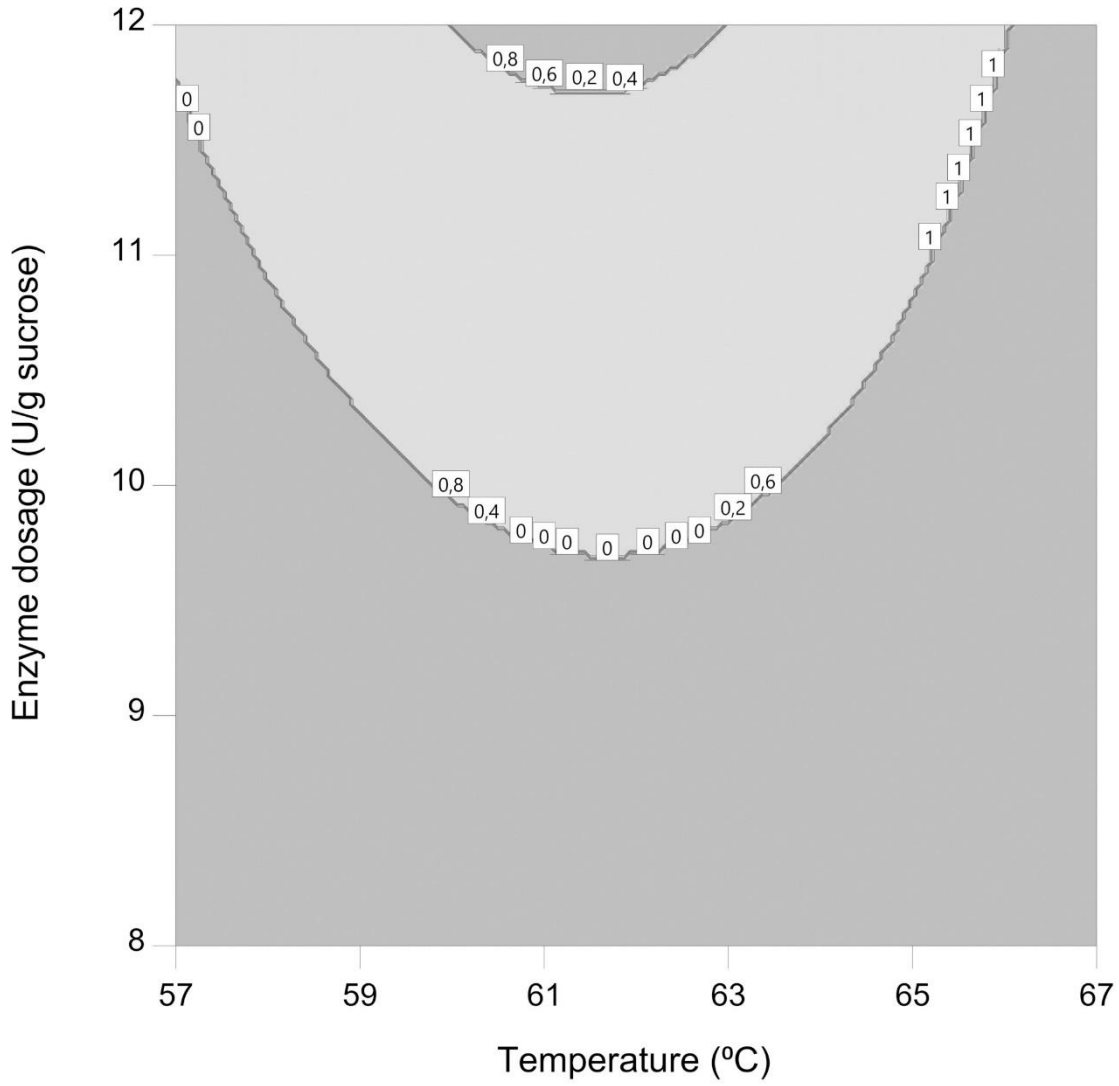


Figure 6-2. Graph of desirability analyses performed to determine the minimum combination of enzyme dosage and temperature required for GAPfopA to produce a scFOS composition similar to Actilight® (± 3%) after an 8 h reaction.

6.3.3 Model validation

The conditions determined by desirability analysis were used to produce the target scFOS composition from buffered high purity sucrose in shake flasks. Reaction profiles depicted in Fig. 6-3 showed that a 10% GF4 composition (similar to Actilight®) was obtained at 6.5 and 7.8 h for GAPfopA_V1 and GAPfopA, respectively, which were longer than the respective times of 5.5 and 7.0 h reported by Trollope et al. (2015). Therefore, reaction time for the GAPfopA_V1 variant compared to the native enzyme decreased by 16% under our conditions,

while Trollope et al. (2015) reported a decrease of 22%. However, compared to Trollope et al. (2015) who carried out the reaction at pH 5.5, reactions in the present study was carried out at

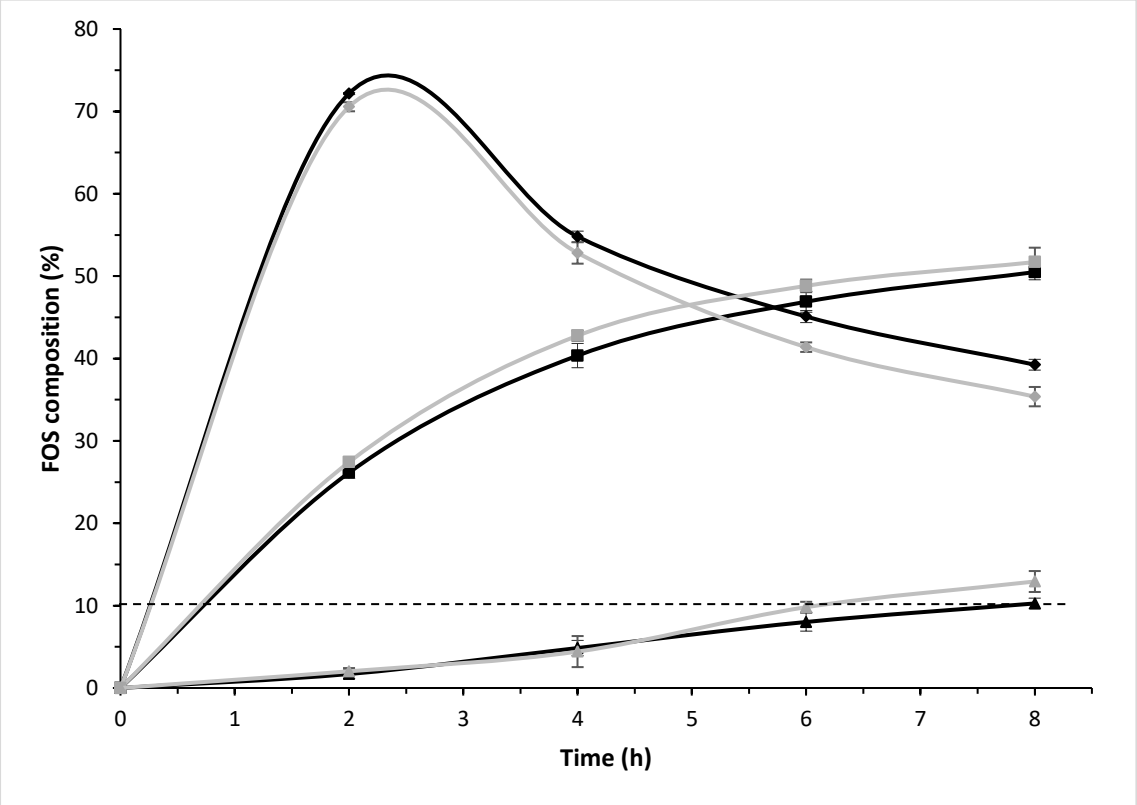


Figure 6-3. Production of scFOS from 60% (w/v) sucrose as a function of time using GAPfopA (black) and GAPfopA_V1 (grey). The reaction was performed at 62.0 °C and pH 5.0 at an enzyme dosage of 10 U/g sucrose at an agitation rate of 120 rpm. The percentage GF2 (♦), percentage GF3 (■) and percentage GF4 (▲) are shown where error bars denote the standard error of triplicate experiments.

a lower pH value of pH 5.0. Differences in the expression host could possibly also account for this discrepancy, where Trollope et al. (2015) used *Saccharomyces cerevisiae*, which has markedly different post-translational modification characteristics compared to *P. pastoris* (Cregg et al., 2000).

Furthermore, it was shown that at the optimum conditions used, the scFOS composition can be tailored by terminating the reaction at specific time points (Fig. 6-3). Nemukula et al. (2009) isolated a fructosyltransferase from *A. aculeatus* and optimised the conditions (pH, temperature, reaction time, enzyme and sucrose concentrations) for fructooligosaccharide

production, while Vega and Zúniga-Hansen (2011) evaluated the temperature and the concentrations of sucrose and enzyme to preferentially produce high concentrations of 1-kestose and both these studies concluded that the reaction time played a critical role in the composition of the scFOS produced.

6.3.4 scFOS production using industrial substrates under optimised conditions

The conditions determined in section 6.3.2 was used to ascertain the ability of these enzymes to produce scFOS from industrial substrates, since this has not yet been demonstrated for these enzymes (Table 6-5). Compared to the high purity sucrose and buffered conditions used previously (Fig. 6-3), both enzymes produced scFOS with the same composition as Actilight® using brown sugar and a composition closely resembling Actilight® when using white sugar. Compared to GAPfopA, reaction times with GAPfopA_V1 were 28% and 25% shorter when using respectively white and brown sugar as substrates (Table 6-5). Neither enzyme produced the required scFOS composition when using refinery molasses as substrate, while on the other hand, the target composition was indeed reached with both GAPfopA and GAPfopA_V1 using A-molasses. This difference in producing the required scFOS composition between the two substrates is possibly due to a pH effect. The optimum pH for these enzymes generally ranges between pH 5.00 and 5.50. Since these reactions were carried out in an unbuffered medium, the pH value on refinery molasses remained at ~pH 4.50 throughout the reaction, i.e. below the optimum pH value of the enzyme, whereas conversion of sucrose in A-molasses occurred at pH 5.68 and 5.47 between the start and end of the reaction. It is, therefore, conceivable that the lower pH of the refinery molasses was responsible for the poorer performance.

All the substrates with both enzymes were slower in achieving the scFOS compositions reported in Table 6-5 compared to the ideal buffered and high purity sucrose conditions used

Table 6-5. The reaction time required to produce the percentage scFOS that closely resembles Actilight® from industrial type substrates. All substrates diluted to 60% sucrose (w/v) and the reaction performed for 12 h at 62.0 °C with an enzyme dosage of 10 U/g sucrose and an agitation rate of 120 rpm.

Enzyme	Substrate	%GF2	%GF3	%GF4	Reaction time (h)
GAPfopA	White sugar	38.8 ± 0.2	51.4 ± 0.1	10.0 ± 0.0	10.6 ± 0.2
	Brown sugar	37.0 ± 0.6	53.3 ± 0.4	10.0 ± 0.0	10.7 ± 0.5
	Refinery	40.7 ± 0.3	49.1 ± 0.3	10.0 ± 0.0	10.4 ± 0.1
	A-molasses	36.6 ± 0.0	53.4 ± 0.1	10.1 ± 0.0	9.7 ± 0.2
GAPfopA_V1	White sugar	38.2 ± 0.2	50.9 ± 0.3	10.0 ± 0.0	7.6 ± 0.1
	Brown sugar	36.5 ± 0.3	53.1 ± 0.3	10.0 ± 0.0	8.0 ± 0.2
	Refinery	40.9 ± 0.1	48.6 ± 0.2	10.0 ± 0.0	10.3 ± 0.1
	A-molasses	37.5 ± 0.1	52.2 ± 0.2	10.0 ± 0.0	10.3 ± 0.1

Mean ± standard deviation, n = 3

in section 3.3 (Table 6-5). Molasses contains various elements that can be inhibitory to enzymes, such as ash (7-15% in molasses) and various trace metals (Al, As, Cu, Fe, Mg, Mn and Zn) (Teclu et al., 2009). The concentrations of these metals tend to decrease in the product as the sugar production process progresses and is much higher in the crude juices than in the final sugar product (Mohamed, 1999). Hirayama et al. (1989) found that some of these metals Al, Cu, Mg, Mn and Zn inhibit the transfructosylating activity of the native fopA enzyme with Mn having the largest effect at a 12% reduction of the enzyme activity.

6.3.5 Scale-up production of scFOS

The reactions with the respective enzymes in the bioreactors were faster than that of the shake flasks, but this could mainly be due to superior mixing. Data illustrating the scaled-up production of scFOS using the optimised conditions (Section 6.3.2) in a 20 L bioreactor are shown in Fig. 6-4. The increased thermostability illustrated in shake flasks (Fig. 6-3) was replicated in the 20 L bioreactor with the fraction GF2 decreasing and the fraction GF3 and GF4 increasing faster with the GAPfopA_V1 enzyme compared to GAPfopA. A scFOS composition similar to Actilight® was produced in a bioreactor at 5.6 h for GAPfopA_V1 (36.8% GF2, 53.6% GF3 and 10.0% GF4) compared to GAPfopA at 6.9 h (37.5% GF2, 52.4% GF3 and 10.0% GF4), which equates to 19% less time required to achieve the target scFOS composition. In shake flasks, GAPfopA_V1 delivered a scFOS composition close to Actilight® (37.6% GF2, 51.3% GF3 and 10.9% GF4) after 7 h, whereas a similar composition of 39.4% GF2, 50.2% GF3 and 10.3% GF4 was reached after more than 8 h using the native enzyme.

The maximum scFOS yield from sucrose in an industrial setup is limited to a range of 55 – 60% due to the inhibitory effect of glucose (Sangeetha et al., 2005a). The total scFOS produced for GAPfopA at 6.9 h and for GAPfopA_V1 at 5.6 h was 55.01% and 54.94%, respectively (data not shown), which is at the lower theoretical value possible for batch reactions. The GAPfopA_V1 enzyme was engineered for some relief from glucose inhibition (Trollope et al., 2015) but in this study, the total scFOS yield was similar to that of GAPfopA. Despite the similar values in total scFOS yield at the point where the required scFOS profile is reached, the scFOS productivity differs due to the longer time it takes for GAPfopA to reach this scFOS profile (58.86 g/L h and 47.83 g/L h for GAPfopA_V1 and GAPfopA, respectively). The productivity is less than what Vega and Zúniga-Hansen (2011) achieved in a batch system (91.9

g/L h), but similar to what Vega-Paulino and Zúniga-Hansen (2012) achieved at 52.5 – 55.9 g/L h and higher than that of Detofol et al. (2015) at 3.16 x 10^3g/m^3 h in a batch basket reactor.

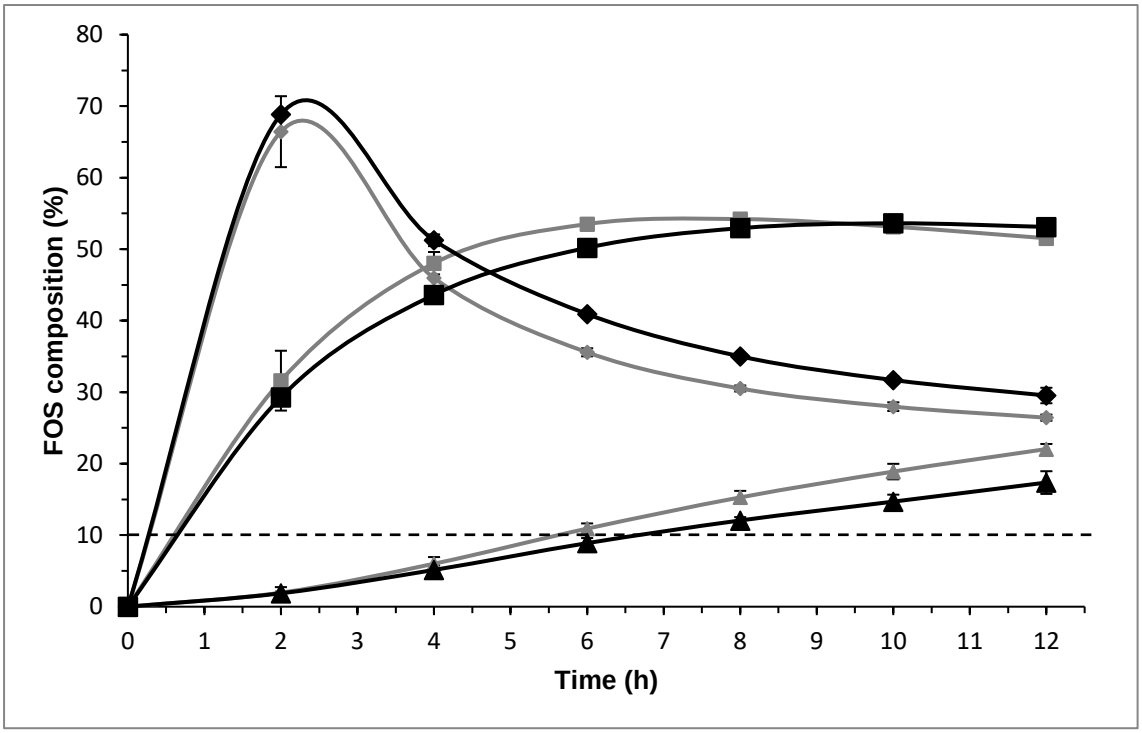


Figure 6-4. Production of scFOS over a 12 h time period with GAPfopA (black) and GAPfopA_V1 (grey) in a 20 L bioreactor. The reaction was performed at 62.0 °C, pH 5.0 with an enzyme dosage of 10 U/g sucrose stirred at 200 rpm. The percentage GF2(♦), percentage GF3 (■) and percentage GF4 (▲) are shown. Error bars denote standard errors (n = 3).

6.4 Conclusions

An increased thermostability of the engineered β-fructofuranosidase enzyme had a markedly beneficial effect on process productivity when producing scFOS from sucrose as substrate. Compared to GAPfopA, the improved thermostability of GAPfopA_V1 was demonstrated at pilot scale, which resulted in a 19% decrease in reaction time at 62 °C, 10 U/g sucrose to achieve the required scFOS composition. Industrial sugar refinery intermediate processing streams are suitable for production of commercial grade scFOS for both enzymes from refinery and A-molasses. However, a limit in impurities that the enzymes will tolerate was demonstrated because the target scFOS composition was not produced from refinery molasses.

Acknowledgements

The authors gratefully acknowledge Nico Stolz (RCL foods, Sugar and Milling Division) for providing samples of refinery and A-molasses.

6.5 References

Balasubramaniem, A.K., Nagarajan, K.V., Paramasamy, G., 2001. Optimization of media for β-fructofuranosidase production by *Aspergillus niger* in submerged and solid state fermentation. Process Biochem. 36, 1241–1247.

Beine, R., Moraru, R., Nimtz, M., Na'amnieh, S., Pawlowski, A., Buchholz, K., Seibel, J., 2008. Synthesis of novel fructooligosaccharides by substrate and enzyme engineering. J. Biotechnol. 138, 33–41.

Chávez, F.P., Rodriguez, L., Díaz, J., Delgado, J.M., Cremata, J. A., 1997. Purification and characterization of an invertase from *Candida utilis*: Comparison with natural and recombinant yeast invertases. J. Biotechnol. 53, 67–74.

Chuankhayan, P., Hsieh, C.Y., Huang, Y.C., Hsieh, Y.Y., Guan, H.H., Hsieh, Y.C., Tien, Y.C., Chen, C. De, Chiang, C.M., Chen, C.J., 2010. Crystal structures of *Aspergillus japonicus* fructosyltransferase complex with donor/acceptor substrates reveal complete subsites in the active site for catalysis. J. Biol. Chem. 285, 23251–23264.

Cregg, J.M., Cereghino, J.L., Shi, J., Higgins, D.R., 2000. Recombinant protein expression in *Pichia pastoris*. Mol. Biotechnol. 16, 23–52.

De Abreu, M., Alvaro-Benito, M., Sanz-Aparicio, J., Plou, F.J., Fernandez-Lobato, M., Alcalde, M., 2013. Synthesis of 6-kestose using an efficient β-fructofuranosidase engineered by directed evolution. Adv. Synth. Catal. 355, 1698–1702.

Detofol, M.R., Aguiar-Oliveira, E., Bustamante-Vargas, C.E., Soares, A.B. de J., Soares, M.B.A., Maugeri, F., 2015. Modeling and simulation of fructooligosaccharides synthesis in a batch basket reactor. J. Biotechnol. 210, 44–51.

Ghazi, I., Fernandez-Arrojo, L., Garcia-Arellano, H., Ferrer, M., Ballesteros, A., Plou, F.J., 2007. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus*. J. Biotechnol. 128, 204–211.

Ghazi, I., Fernandez-Arrojo, L., Gomez De Segura, A., Alcalde, M., Plou, F.J., Ballesteros, A., 2006. Beet sugar syrup and molasses as low-cost feedstock for the enzymatic production of fructo-oligosaccharides. J. Agric. Food Chem. 54, 2964–2968.

Gibson, G.R., Probert, H.M., Loo, J. Van, Rastall, R.A., Roberfroid, M.B., 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev. 17, 259.

Hidaka, H., Eida, T., Takizawa, T., Tokunaga, T., Tashiro, Y., 1986. Effects of fructooligosaccharides on intestinal flora and human health. Bifidobact. Microflora 5, 37–50.

Hidaka, H., Hirayama, M., Sumi, N., 1988. A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 52, 1181–1187.

Hirayama, M., Sumi, N., Hidaka, H., 1989. Purification and properties of a fructooligosaccharide-producing β-fructofuranosidase from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. 53, 667–673.

Invitrogen Corporation, 2002. *Pichia* fermentation process guidelines.

Kim, M.H., In, M.J., Cha, H.J., Yoo, Y.J., 1996. An empirical rate equation for the fructooligosaccharide-producing reaction catalyzed by β-fructofuranosidase. J. Ferment. Bioeng. 82, 458–463.

Lecerf, J.M., Clerc, E., Jaruga, A., Wagner, A., Respondek, F., 2015. Postprandial glycaemic

and insulinaemic responses in adults after consumption of dairy desserts and pound cakes containing short-chain fructo-oligosaccharides used to replace sugars. J Nutr Sci 4, 1–7.

Mohamed, A.E., 1999. Environmental variations of trace element concentrations in Egyptian cane sugar and soil samples (Edfu factories). Food Chem. 65, 503–507.

Mussatto, S.I., Teixeira, J. A., 2010. Increase in the fructooligosaccharides yield and productivity by solid-state fermentation with *Aspergillus japonicus* using agro-industrial residues as support and nutrient source. Biochem. Eng. J. 53, 154–157.

Nemukula, A., Mutanda, T., Wilhelmi, B.S., Whiteley, C.G., 2009. Response surface methodology: Synthesis of short chain fructooligosaccharides with a fructosyltransferase from *Aspergillus aculeatus*. Bioresour. Technol. 100, 2040–5.

Nishizawa, K., Akajima, M.N., Abetani, H.N., 2001. Kinetic study on transfructosylation by β-fructofuranosidase from *Aspergillus niger* ATCC 20611 and availability of a membrane reactor for fructooligosaccharide production. Food Sci. Technol. Res. 7, 39–44.

Paineau, D., Respondek, F., Menet, V., Sauvage, R., Bornet, F., Wagner, A., 2014. Effects of short-chain fructooligosaccharides on faecal bifidobacteria and specific immune response in formula-fed term infants: a randomized, double-blind, placebo-controlled trial. J. Nutr. Sci. Vitaminol. (Tokyo). 60, 167–75.

Rehman, A.U., Kovacs, Z., Quitmann, H., Ebrahimi, M., Czermak, P., 2016. Enzymatic production of fructo-oligosaccharides from inexpensive and abundant substrates using a membrane reactor system. Sep. Sci. Technol. 51, 1537–1545.

Respondek, F., Hilpipre, C., Chauveau, P., Cazaubiel, M., Gendre, D., Maudet, C., Wagner, A., 2014. Digestive tolerance and postprandial glycaemic and insulinaemic responses after consumption of dairy desserts containing maltitol and fructo-oligosaccharides in

adults. Eur. J. Clin. Nutr. 68, 575–80.

Roberfroid, M.B., 2007. Inulin-type fructans : functional food ingredients. J. Nutr. 137, 2493S–2502S.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2005a. Recent trends in the microbial production, analysis and application of fructooligosaccharides. Trends Food Sci. Technol. 16, 442–457.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2005b. Maximization of fructooligosaccharide production by two stage continuous process and its scale up. J. Food Eng. 68, 57–64.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2004. Production of fructo-oligosaccharides by fructosyl transferase from *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77. Process Biochem. 39, 755–760.

Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., 2004. Production of fructosyl transferase by *Aspergillus oryzae* CFR 202 in solid-state fermentation using agricultural by-products. Appl. Microbiol. Biotechnol. 65, 530–537.

Sheu, D.-C., Duan, K.-J., Cheng, C.-Y., Bi, J.-L., Chen, J.-Y., 2002. Continuous production of high-content fructooligosaccharides by a complex cell system. Biotechnol. Prog. 18, 1282–1286.

Singh, R.S., Singh, R.P., 2010. Production of fructooligosaccharides from inulin by endoinulinases and their prebiotic potential. Food Technol. Biotechnol. 48, 435–450.

Teclu, D., Tivchev, G., Laing, M., Wallis, M., 2009. Determination of the elemental composition of molasses and its suitability as carbon source for growth of sulphate-reducing bacteria. J. Hazard. Mater. 161, 1157–1165.

Trollope, K.M., Görgens, J.F., Volschenk, H., 2015. Semirational directed evolution of loop regions in *Aspergillus japonicus* β-fructofuranosidase for improved fructooligosaccharide production. Appl. Environ. Microbiol. 81, 7319–7329.

van Wyk, N., Trollope, K.M., Steenkamp, E.T., Wingfield, B.D., Volschenk, H., 2013. Identification of the gene for β-fructofuranosidase from *Ceratocystis moniliformis* CMW 10134 and characterization of the enzyme expressed in *Saccharomyces cerevisiae*. BMC Biotechnol. 13, 100.

Vandáková, M., Platková, Z., Antošová, M., Báleš, V., Polakovič, M., 2004. Optimization of cultivation conditions for production of fructosyltransferase by *Aureobasidium pullulans*. Chem. Pap. 58, 15–22.

Vega-Paulino, R.J., Zúniga-Hansen, M.E., 2012. Potential application of commercial enzyme preparations for industrial production of short-chain fructooligosaccharides. J. Mol. Catal. B Enzym. 76, 44–51.

Vega, R., Zúniga-Hansen, M.E., 2011. Enzymatic synthesis of fructooligosaccharides with high 1-kestose concentrations using response surface methodology. Bioresour. Technol. 102, 10180–10186.

Yanai, K., Nakane, A., Kawate, A., Hirayama, M., 2001. Molecular cloning and characterization of the fructooligosaccharide-producing β-fructofuranosidase gene from *Aspergillus niger* ATCC 20611. Biosci. Biotechnol. Biochem. 65, 766–73.

Yen, C.H., Kuo, Y.W., Tseng, Y.H., Lee, M.C., Chen, H.L., 2011. Beneficial effects of fructo-oligosaccharides supplementation on fecal bifidobacteria and index of peroxidation status in constipated nursing-home residents - A placebo-controlled, diet-controlled trial. Nutrition 27, 323–328.

Yun, J.W., 1996. Fructooligosaccharides—Occurrence, preparation, and application. Enzyme Microb. Technol. 19, 107–117.

Yun, J.W., Song, S.K., 1999. Enzymatic production of fructooligosaccharides from sucrose, in: Bucke, C. (Ed.), Carbohydrate Biotechnology Protocols. Methods in Biotechnology™. Humana Press, Totowa, New Jersey, pp. 141–151.

Zhang, J., Liu, C., Xie, Y., Li, N., Ning, Z., Du, N., Huang, X., Zhong, Y., 2017. Enhancing fructooligosaccharides production by genetic improvement of the industrial fungus *Aspergillus niger* ATCC 20611. J. Biotechnol. 249, 25–33.

Chapter 7 Conclusions and recommendations

This work has shown that *Pichia pastoris* is a suitable host for the high-level expression and production of functional codon-optimized native (GAPfopA) and engineered (GAPfopA_V1) versions of the fopA enzyme from *Aspergillus fijiensis* ATCC 20611 and that these enzymes can be applied for the batch production of scFOS from a selection of industrial sugar streams. The main conclusions and recommendations of this work will be further discussed here.

7.1 Conclusions

Strain, promotor and codon optimisation strategy was successfully employed to produce a high-yielding fopA enzyme-producing strain. The expression and production of the codon-optimized fopA enzymes showed that the selection of the strain, promoter and codon-optimization algorithm plays an important role in the level of fopA produced. Based on the experimental work described in this study, *P. pastoris* strain DSMZ 70382, the inducible alcohol oxidase (*AOX1*) promoter and codon-optimization of the native *fopA* gene by ATUM proved to be superior in terms of volumetric activity compared to the *P. pastoris* strain X-33, the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter and codon-optimization from GeneArt®.

A strong enzyme producer is not necessarily related to improved economic process performance. Whereas a superior strain is in principle desirable to maximise the production process economic efficiency, especially in terms of equipment size and hence, capital cost, several other cost contributing factors should also be considered. For example, although the *P. pastoris* DSMZ 70382 strain is not protected by a patent, which means that the use of this strain will not incur additional cost from licencing fees, the AOX1 promoter requires methanol for induction, which is hazardous and costly to handle, store and transport in large volumes, which would add substantial operating cost.

Two different glycerol feeding methods (DO-stat and constant feed) were successfully employed to produce the native (GAPfopA) and engineered (GAPfopA_V1) enzymes with the DO-stat method producing the highest volumetric enzyme activity for both. Despite *P. pastoris* DSMZ 70382 GAPfopA strain producing lower volumetric enzyme activity than its *AOX1* controlled counterpart, the *P. pastoris* DSMZ 70382 strain (no licensing fees) with the native ATUM codon-optimized gene (produced higher volumetric enzyme activity) under control of the *GAP* promoter (glycerol a safer substrate to use at large scale) was selected for scale-up production and compared to an engineered version (GAPfopA_V1) of the fopA enzyme, which was harboured by the same strain. Of the two fed-batch methods used, glycerol supplied using a constant feed strategy resulted in a higher rate of biomass accumulation and greater biomass content in a shorter time than DO-stat cultures, but produced comparatively lower maximum volumetric enzyme activity compared to a fed-batch culture where glycerol was supplied using a DO-stat strategy. When comparing the maximum volumetric activity and protein production for the two strains using the same feed method, the GAPfopA strain was higher for both the feeding methods. Nevertheless, the volumetric productivity for the constant feed method was 1.7x and 1.9x higher for GAPfopA and GAPfopA_V1, respectively. Volumetric productivity is the more important factor for industrial processes because the aim is to reduce process time while maximizing product formation.

The increased thermostability of the engineered fopA enzyme (GAPfopA_V1) was demonstrated by a 19% decrease in the time required to produce short-chain fructooligosaccharides (scFOS) with the desired composition of 37% GF2, 53% GF 3 and 10% GF4 using buffered table sugar as substrate.

The application of the recombinant enzyme under optimised conditions was successfully applied to industrial sugar waste streams. An assessment of two streams, namely A-molasses and refinery molasses revealed that the native and engineered enzymes produced the target

scFOS composition from A-molasses. However, refinery molasses proved not suitable, apparently due to the pH of the solution inhibiting both enzymes. Therefore, the viability of using industrial sugar refinery intermediate processing streams was demonstrated for producing scFOS for possible commercial use.

7.2 Recommendations

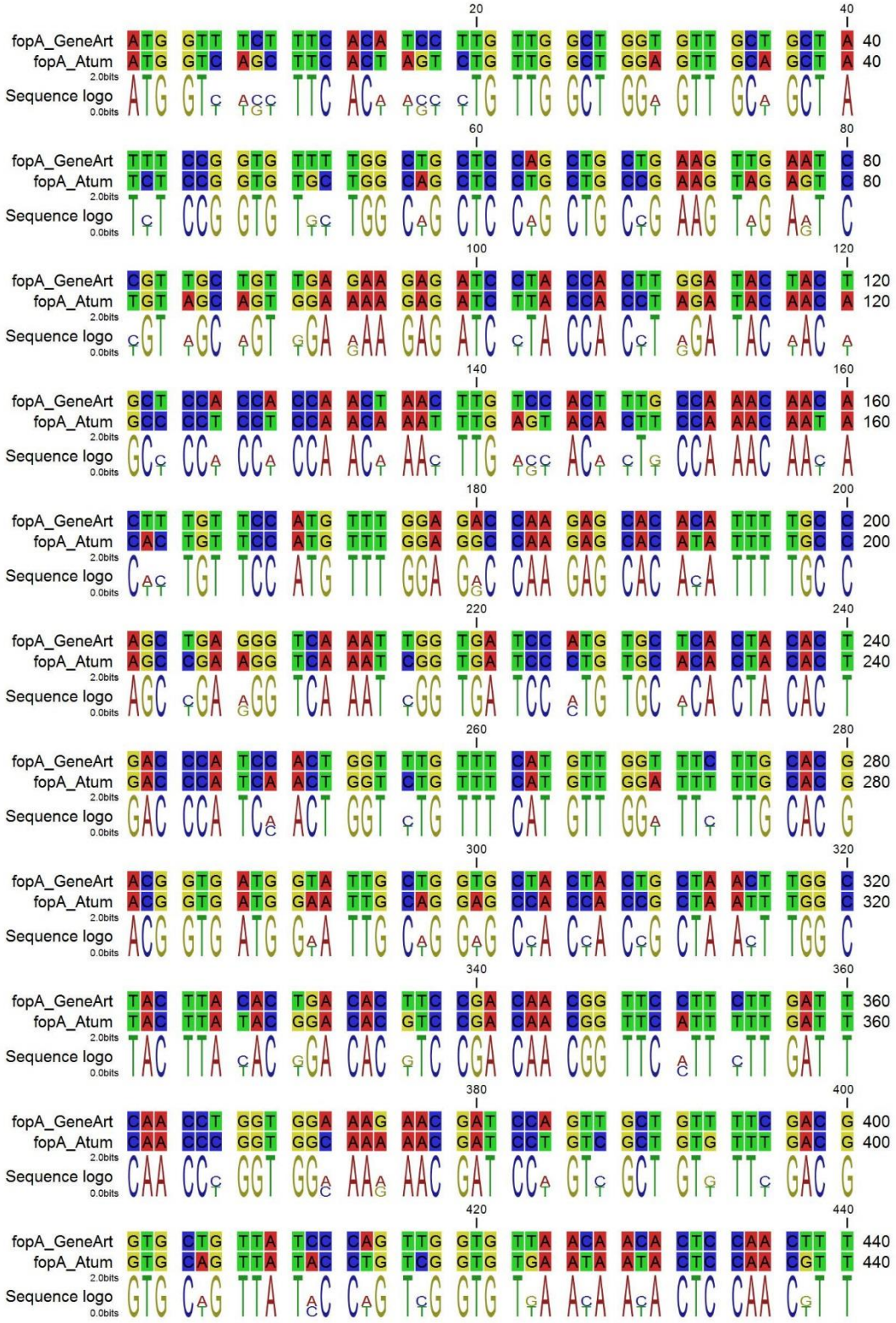
From the research presented, there are several areas available for further study.

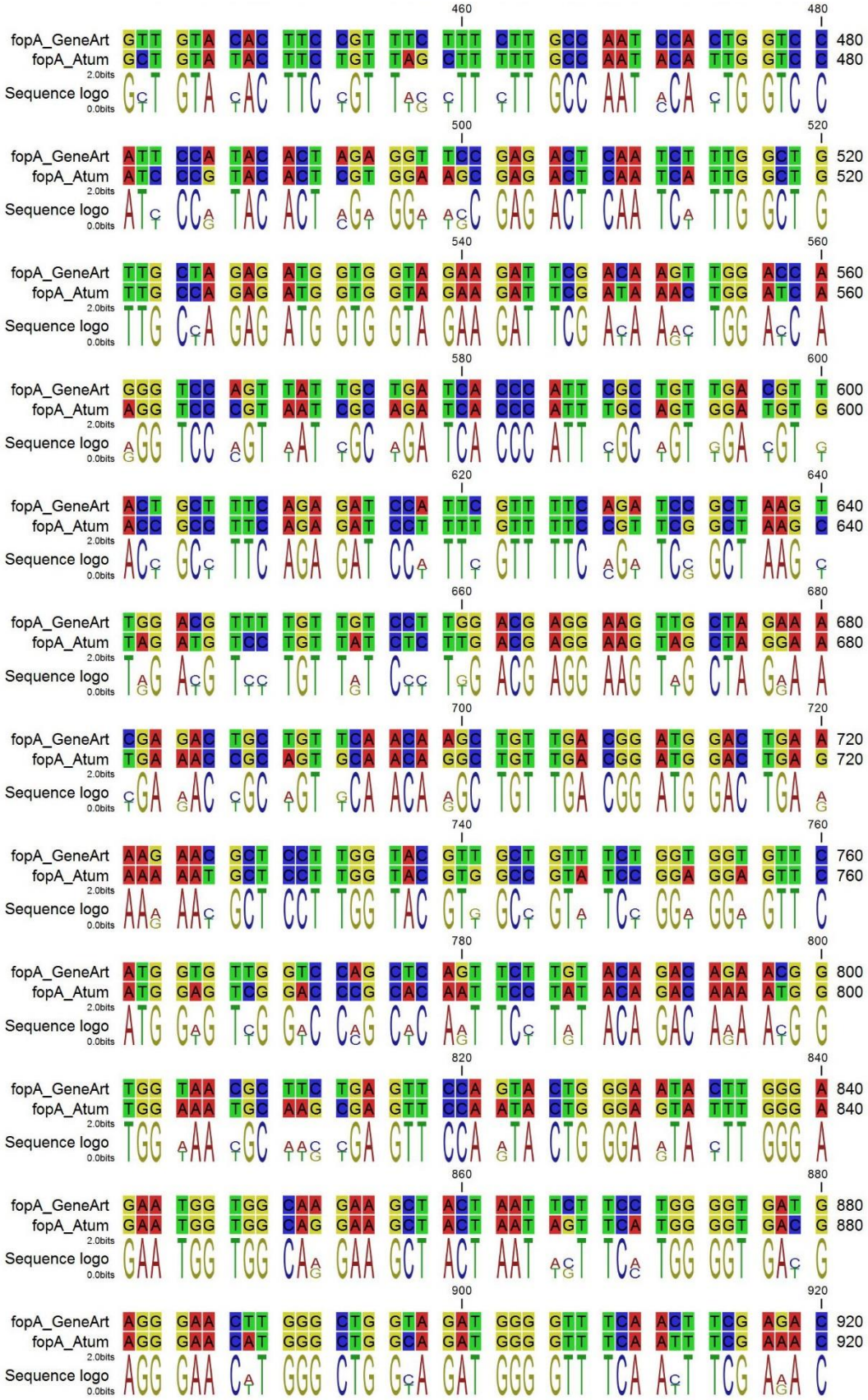
During the production of the enzymes in the scaled-up bioreactors, the DO-stat feed exhibited increasingly poor dissolved oxygen (DO) control. Furthermore, the small-scale bioreactor fermentations showed significantly higher volumetric enzyme activity than the scaled-up fermentations. The fermentations exhibiting slower biomass growth resulted in increased maximum volumetric activity. Thus, determining the optimum growth rate, through either exponential feed fermentations or chemostat, would be advantageous. Additional process development is required to address all these shortcomings.

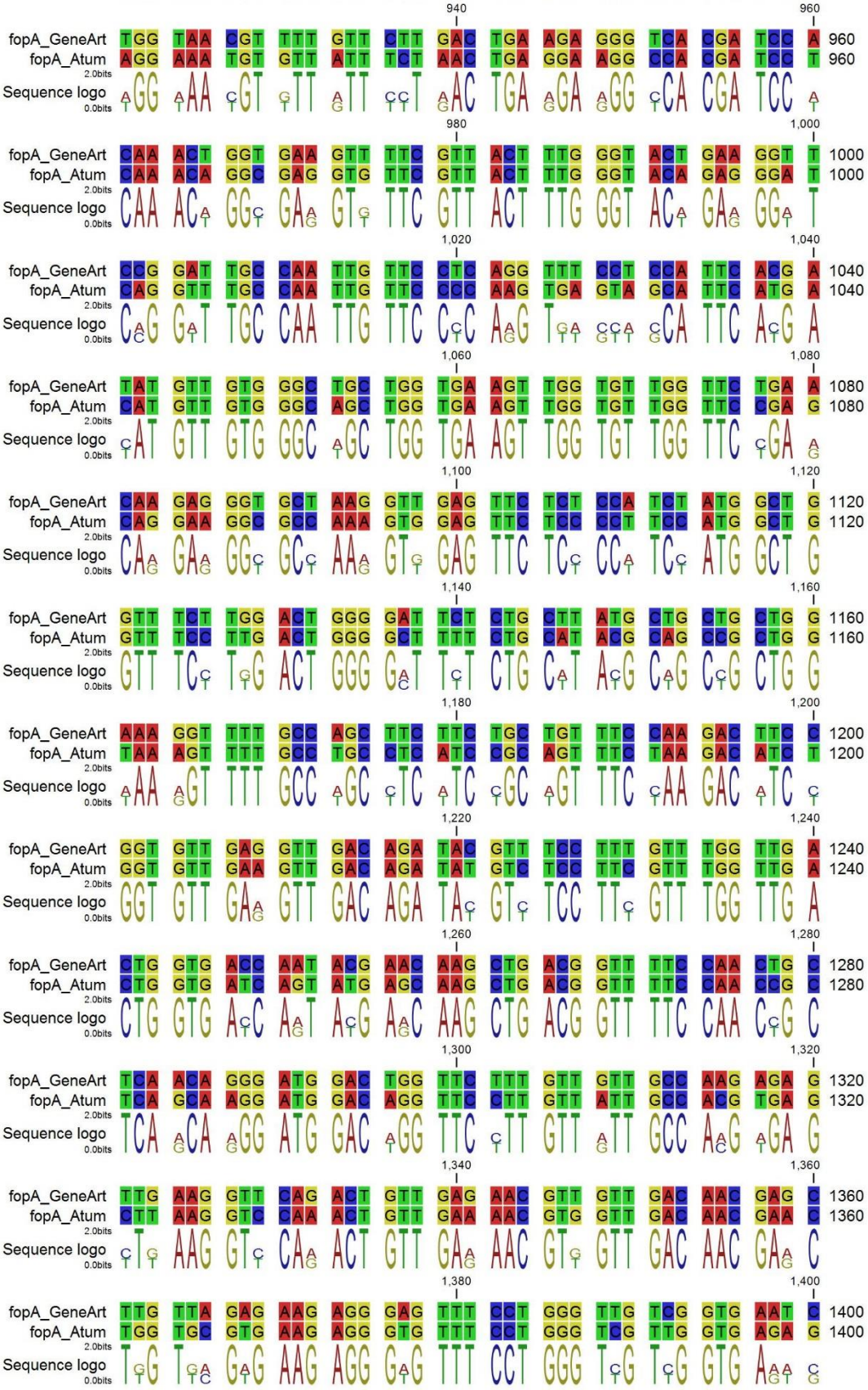
The enzyme dosage ranges evaluated for the response surface methodology (RSM) study did not produce an optimum for enzyme dosage for both enzymes for all the responses. Therefore, further kinetic study and characterization of the enzymes producing scFOS is required as well as techno-economic analysis to determine the economic optimum for enzyme dosage as the cost of biocatalyst can contribute significantly to an industrial process. Industrial sugar streams were demonstrated to be a viable low-cost alternative for producing scFOS. However, the study here was performed at small scale in shake flasks and pilot scale studies are needed to assess the viability of the process. The composition of the impurities in the different molasses substrate should be determined and their impact evaluated.

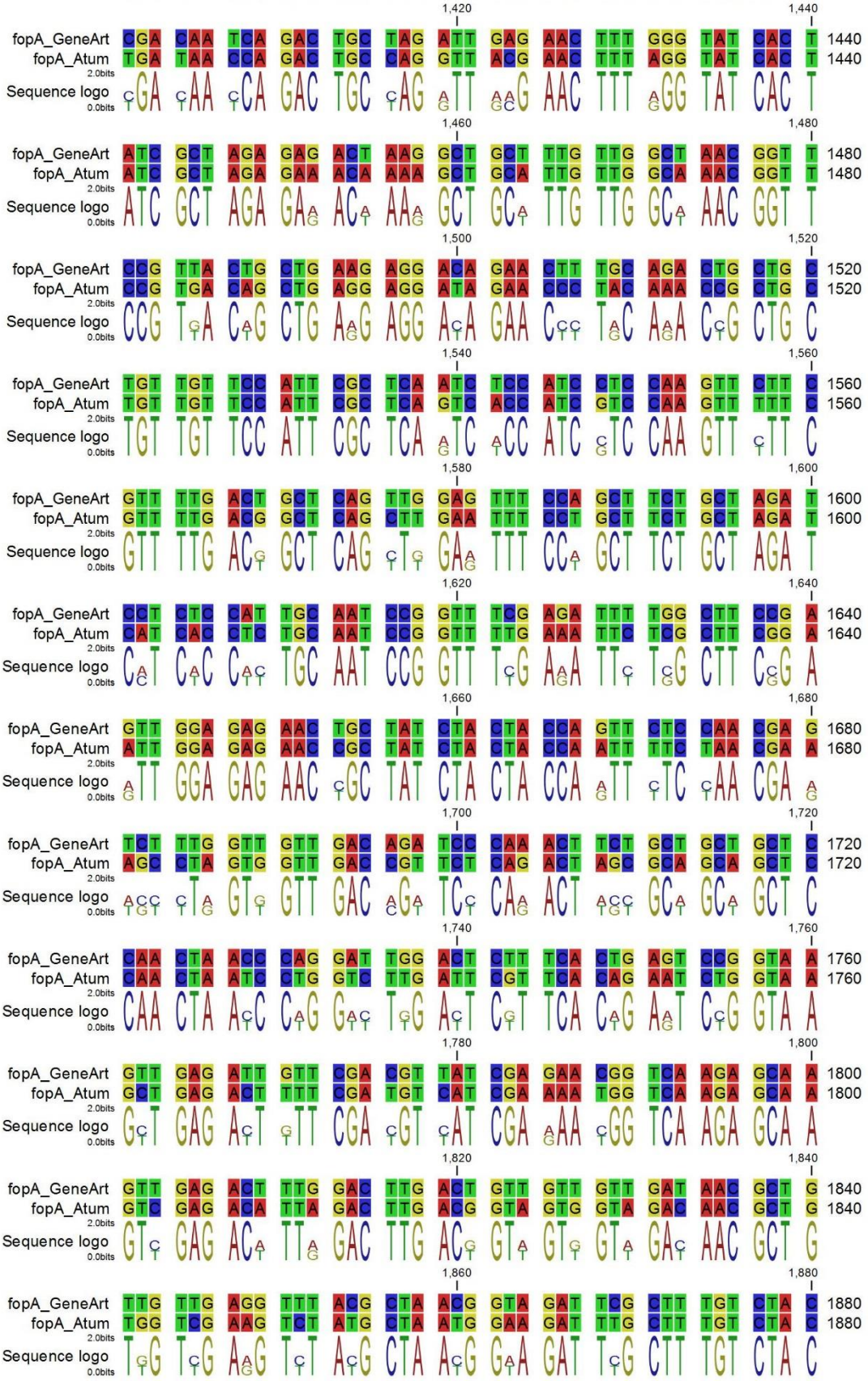
Appendices

Appendix A - Strain construction









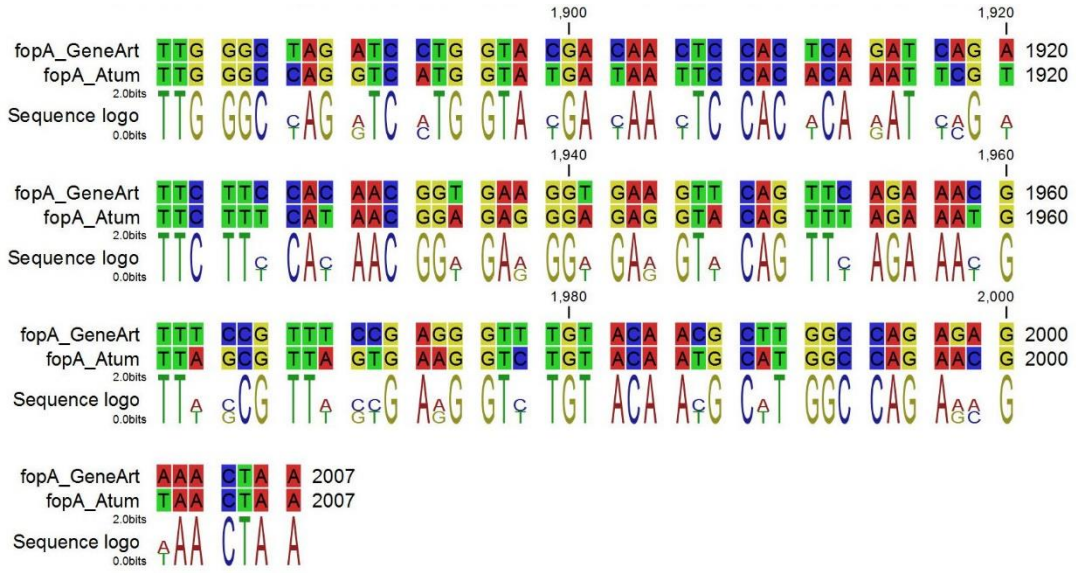


Figure A-1. Gene sequence alignment of the two codon-optimized *fopA* genes by Geneart® and ATUM.

Appendix B - Bioreactor cultivations

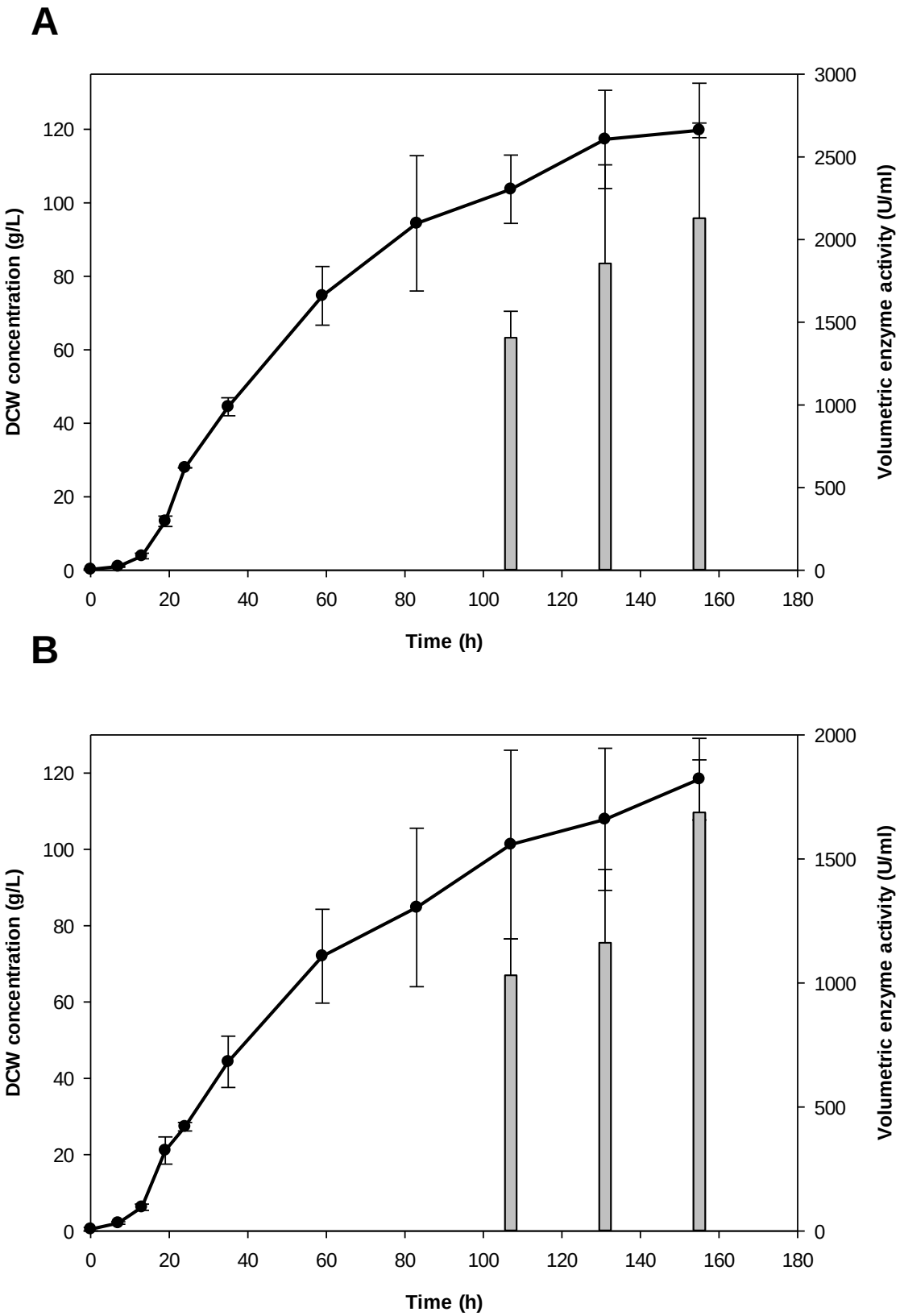


Figure B-1. Dry cell weight (DCW) concentrations (solid line) and volumetric enzyme activities (grey bars) over time for DO-stat cultivations for (A) GAPfopA and (B) GAPfopA_V1. Cultivations performed at 30 °C, pH 5 and dissolved oxygen controlled at 30% and enzyme activities determined at 107, 131 and 155 h. Error bars denote standard errors (n = 2).

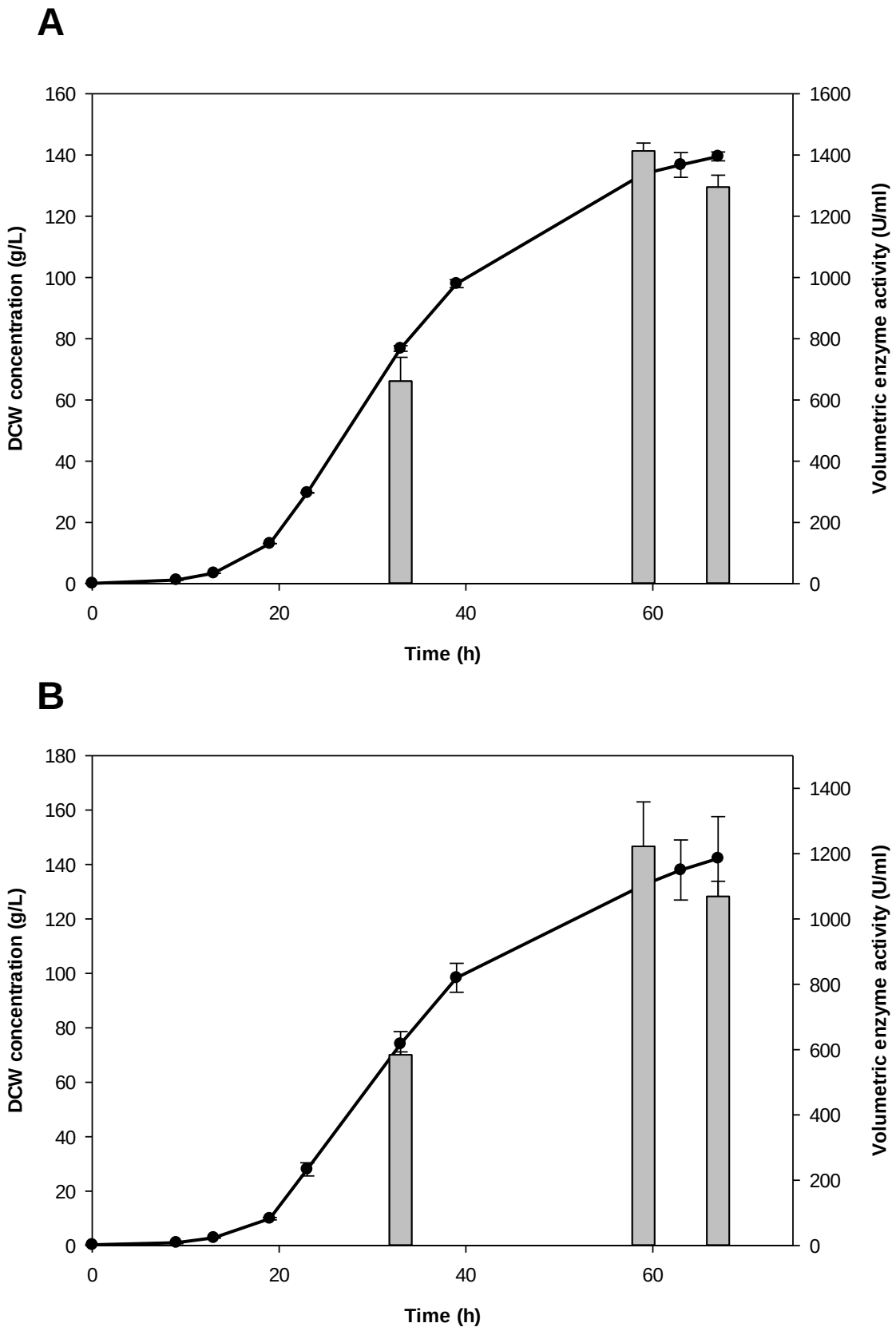


Figure B-2. Dry cell weight (DCW) concentrations (solid line) and volumetric enzyme activities (grey bars) over time for constant feed cultivations for (A) GAPfopA and (B) GAPfopA_V1. Cultivations performed at 30 °C, pH 5 and dissolved oxygen controlled at 30% and enzyme activities determined at 33, 59 and 67 h. Error bars denote standard errors (n = 2).

Appendix C – Mathematical models

The polynomial equations based on actual values obtained from the analysis of multiple regression for the GAPfopA_V1 enzyme for the GF2, GF3 and GF4 responses with (A) representing temperature and (B) the enzyme dosage:

$GF2 = 770.41 - 22.44A + 0.18A^2 – 2.41B + 0.02B^2 – 0.002AB$

$GF3 = -414.21 + 14.47A – 0.11A^2 + 0.93B + 0.05B^2 – 0.02AB$

$GF4 = -256.15 + 7.97A – 0.06A^2 + 1.50B – 0.07B^2 + 0.02AB$

The polynomial equations based on actual values obtained from the analysis of multiple regression for the GAPfopA enzyme for the GF2, GF3 and GF4 responses with (A) representing temperature and (B) the enzyme dosage:

$GF2 = 967.11 – 28.15A + 0.22A^2 – 9.49B + 0.21B^2 – 0.045AB$

$GF3 = -516.51 + 17.19A – 0.14A^2 + 5.96B + 0.13B^2 – 0.03AB$

$GF4 = -350.91 + 10.97A – 0.09A^2 + 3.53B – 0.08B^2 - 0.015AB$