# Development of integrated processes for the co-production of inulin, protein, and ethanol from Jerusalem artichoke tubers in a biorefinery

*by*

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## ABSTRACT

Biorefining is an attractive approach to simultaneously address food supply, energy security and global warming. Furthermore, biorefining offers a sustainable strategy to utilise biomass, for energy production, thereby, reducing overreliance on fossil resources. The principal aim of the study was to evaluate the feasibility of a Jerusalem artichoke (JA) tuber-based biorefinery by integrating protein extraction to the conventional inulin extraction process, and subsequently hydrolysing the tuber residues with an enzyme cocktail of crude inulinases and commercial Cellic® CTec3 and Pectinex, for ethanol fermentation. The tuber mash was pressed for protein extraction from the juice and water extraction from the solid residues. Sequential water-extraction was used for protein and inulin, in the first and second step, respectively. The resulting tuber residues from the sequential extraction was hydrolysed and fermented into ethanol. Fed-batch culture was used to optimise the bioprocess conditions for recombinant endoinulinases production by *Aspergillus niger.*

Comparison of sequential extraction sequences demonstrated that protein extraction in the first and second step, respectively, maximised the selectivity of the extraction and product yields. Both extraction steps utilised water as a solvent, and were optimised with respect to pH, solids loading and temperature for the selective extraction from each dedicated step from tubers. The soluble protein fraction contained a cumulative 71.8% of the protein present in tubers, while 17.1% was present in the inulin extracted in the subsequent step. The inulin yield was 67.6% of the inulin in the tubers, while 11.8% was co-extracted with the protein product. The protein extract was augmented by protein present in the press juice, obtained from tubers prior to the water extraction steps.

High cell density fermentation of *Aspergillus niger* for recombinant endoinulinase production, was achieved through an exponential fed batch method. Endoinulinase production was growth associated at higher growth rates, achieving the highest volumetric activity (670 U/ml) and biomass concentration (33 g/L) at a growth rate (μ) of 0.07 $h^{-1}$. Moreover, the significant decrease in enzyme activity (506 U/ml) and biomass substrate yield (0.043 $g_{biomassDW}/g_{glucose}$) at low μ (0.04 $h^{-1}$) was due to the high maintenance energy requirement. High biomass concentrations resulted in broth viscosity, which necessitated increased agitation for mixing and oxygen transfer. However, this led to pellet disruption and biomass growth in mycelial.

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Moreover, enzyme production profiles, product ($Y_{p/s}$) and biomass ($Y_{x/s}$) yield coefficients were not affected.

High gravity simultaneous saccharification and fermentation (SSF) of the extraction residues, enriched in cellulose and inulin, was achieved with an optimised cocktail of enzymes. A combined inulin and cellulose conversion yield of 74% was achieved during fermentation at 21% w/v solids loading. The optimised enzyme cocktail improved the saccharification and fermentation of the residues, with an ethanol concentration and yield of 38 g/L and 83%, respectively, compared to an unoptimized cocktail with the same protein dosage, with 32 g/L and 59%, respectively, both at the maximum attainable solids loading of 21% w/v.

Therefore, the current data demonstrated the potential of integrating a protein extraction with conventional inulin extraction from JA tubers and fermenting the residues into ethanol with an optimised enzyme cocktail.

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**Opsomming**

Bioraffinering is ’n aantreklike benadering om gelyktydig voedselverskaffing, energiesekuriteit en aardverwarming aan te spreek. Verder bied bioraffinering ’n volhoubare strategie om biomassa te gebruik, wat aanhorigheid op fossielhulpbronne verminder. Die hoofdoel van die studie was om die uitvoerbaarheid van ’n aardartisjok (JA) -knolgebaseerde bioraffinadery te evalueer deur proteïenekstraksie saam met die konvensionele inulienekstraksieproses te integreer, en vervolgens die knolresidu’s met ’n ensiemmengsel van ru-inulienase en kommersiële Cellic® CTec3 en Pectinex te hidroliseer, vir etanolfermentasie. Die knolmengsel is gepers vir proteïenekstraksie vanuit die sap-en-waterekstraksie vanuit die soliede residu’s. Sekwensiële waterekstraksie is gebruik vir proteïen en inulien, in die eerste en tweede stap onderskeidelik. Die resulterende knolresidu’s vanuit die sekwensiële ekstraksie is gehidroliseer en gefermenteer na etanol. Gevoerde-lotkultuur is gebruik om die bioproseskondisies vir rekombinante endo-inulienaseproduksie deur *Aspergillus niger* te optimeer.

Vergelyking van sekwensiële ekstraksiereekse het gedemonstreer dat proteïenekstraksie in die eerste en tweede stap, onderskeidelik, die selektiwiteit van die ekstraksie- en produkopbrengste gemaksimeer het. Beide ekstraksiestappe het water as oplosmiddel gebruik, en is geoptimeer met betrekking tot pH, vastestoflading en temperatuur vir die selektiewe ekstraksie vanuit elke toegewyde stap van knolle. Die oplosbare proteïenfraksie het ’n kumulatiewe 71.8% van die proteïen teenwoordig in knolle, bevat, terwyl 17.1% teenwoordig was in die inulien wat geëkstraheer is in die opvolgende stap. Die inulienopbrengs was 67.6% van die inulien in die knolle, terwyl 11.8% geëkstraheer is saam met die proteïenproduk teenwoordig in die perssap, verkry uit knolle voor die waterekstraksiestappe.

Hoë seldigtheid van *Aspergillus niger* vir rekombinante endo-inulienaseproduksie is bereik deur ’n eksponensiële voerlotmetode. Endo-inulienaseproduksie is groei geassosieer by hoër groeitempo’s, wat die hoogste volumetriese aktiwiteit (670 U/ml) en biomassakonsentrasie (33 U/ml) by ’n groeitempo (μ) van 0.07 $h^{-1}$, bereik het. Verder het die hoë onderhoudenergievereiste ’n beduidende afname in ensiemaktiwiteit (506 U/ml) en biomassa-substraatopbrengs (0.043 $g_{biomassaDW}/g_{glukose}$) by lae μ (0.04 $h^{-1}$) tot gevolg gehad. Hoë biomassakonsentrasies het sopviskositeit tot gevolg gehad, wat verhoogde roering vir

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vermenging en suurstofoordrag genoodsaak het. Hierdie het wel tot korrelversteuring en biomassagroei in miseliaal gelei. Ensiemproduksieprofiele, produk- ($Y_{p/s}$) en biomassa- ($Y_{x/s}$) opbrengskoëffisiënte is verder nie geaffekteer nie.

Hoë gravitasie, gelyktydige sakkarifikasie en fermentasie (SSF) van die ekstraksieresidu's, verryk in sellulose en inulien, is bereik met 'n geoptimeerde mengsel van ensiemes. 'n Kombinasie van inulien en sellulose omsettingsopbrengs van 74% is bereik deur fermentasie by 21% w/v vastestoflading. Die geoptimaliseerde ensiemmengsel het die sakkarifikasie en fermentasie van die residu's verbeter, met 'n etanolkonsentrasie en -opbrengs van 38 g/L en 83%, onderskeidelik, in vergelyking met 'n mengsel wat nie geoptimeer is nie met dieselfde proteïendosis, beide by die maksimum vastestoflading van verkrygbare vastestoflading van 21% w/v.

Daarom het die huidige data die potensiaal gedemonstreer van integrering van 'n proteïenekstraksie met konvensionele inulienekstraksie uit JA-knolle en fermentering van die residu's na etanol met 'n geoptimeerde ensiemmengsel.

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# Contents

ABSTRACT ... iii

Opsomming ... v

Acknowledgements ... vii

Abbreviations ... xv

Nomenclature ... xvii

CHAPTER ONE: INTRODUCTION ... 1

1.1. Contextual background ... 1

1.2. Rationale of study ... 2

1.3. Structure of Thesis ... 4

2. CHAPTER TWO: LITERATURE REVIEW ... 5

2.1. Jerusalem artichoke tubers based biorefinery ... 5

2.1.1. Overview: Origin, distribution and uses ... 5

2.1.2. JA tubers yields and impacts of growing conditions on yields ... 5

2.1.3. Composition of JA tubers and distribution of inulin ... 6

2.2. Strategies for the development of JA tubers as a feedstock for a multiproduct biorefinery ... 8

2.3. Inulin-based nutraceutical ... 10

2.4. Inulin structure and synthesis ... 11

2.5. Inulin extraction ... 12

2.7. JA tuber protein ... 16

2.8. Protein extraction ... 16

2.8.1. Protein solubilisation: An overview ... 17

2.8.2. Alkaline and acid protein solubilisation ... 17

2.8.3. Water extraction ... 18

2.8.4. Salt extraction ... 19

2.8.5. Ultrafiltration ... 19

2.9. Challenges of sequential protein and inulin extraction ... 20

2.10. The importance of inulinases in JA based biorefining ... 22

2.10.1. Overview of inulinases ... 22

2.10.2. Endoinulinases native sources and production ... 23

2.10.3. *A. niger* in the production of recombinant enzymes ... 24

2.10.4. Mechanism of overcoming limitations of high-cell density culture of *Aspergillus* ... 27

2.9. Integration of ethanol production with protein and inulin production using JA tubers ... 28

2.9.1. Overview ... 28

Stellenbosch University https://scholar.sun.ac.za

2.9.2. Fermentation of tubers for ethanol production ........ 29
2.9.3. Enzymatic hydrolysis of JA tubers and synergism of cocktail enzymes ........ 30
2.11. Conclusions from literature ........ 31
3. CHAPTER THREE: STUDY AIM AND OBJECTIVES ........ 34
CHAPTER 4 ........ 38
4. Sequential extraction of protein and inulin from the tubers of Jerusalem artichoke (*Helianthus tuberosus* L.) ........ 38
Sequential extraction of protein and inulin from the tubers of Jerusalem artichoke (Helianthus tuberosus L.) ........ 41
Abstract ........ 41
Highlights ........ 42
4.1. Introduction ........ 43
4.2. Materials and methods ........ 44
4.2.1. Tuber feedstock ........ 44
4.2.2. Analysis ........ 45
4.2.3. Inulin determination ........ 45
4.2.4. Protein solubility ........ 46
4.2.5. Emulsification properties ........ 47
4.2.6. Foaming properties ........ 47
4.2.7. Water retention ........ 47
4.2.8. Preliminary screening ........ 47
4.2.9. Sequential extraction from the pulp ........ 48
4.2.10. Experimental design: Protein and inulin sequential extraction ........ 48
4.2.11. Statistical analysis for protein functional properties ........ 49
4.3. Results and discussion ........ 49
4.3.1. Chemical composition of tubers ........ 49
4.3.2. Protein and inulin recovery from the press-juice ........ 50
4.3.3. Significant process conditions for inulin and protein extraction ........ 50
4.3.4. Optimisation of process parameters for selective extraction of protein and inulin from tubers ........ 51
4.4. Conclusions ........ 62
4.5. Competing interests ........ 63
4.6. Acknowledgements ........ 63
4.7 Supplementary material ........ 64
CHAPTER FIVE ........ 66
5. Bioprocess optimisation for high cell density endoinulinases production from recombinant *Aspergillus niger* ........ 66

Stellenbosch University https://scholar.sun.ac.za

Bioprocess optimisation for high cell density endoinulinase production from recombinant *Aspergillus niger* ........ 69
Abstract ........ 70
5.1. Introduction ........ 71
5.1. Materials and methods ........ 72
5.1.1. Media and cultivation conditions ........ 72
5.1.2. DNA manipulations and gene amplification by PCR ........ 73
5.1.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) ........ 74
5.1.4. Pre-inoculum preparation and cultivation medium for the fermentations ........ 75
5.1.5. Bioreactor operating conditions and growth medium ........ 75
5.1.6. Exponential feeding ........ 75
5.1.7. Enzyme assay ........ 76
5.2. Results and discussion ........ 77
5.2.1. Strain development ........ 77
5.2.2. Effects of glucose feed concentration on DO levels and mixing as well as subsequent impact on biomass and enzyme production ........ 77
5.2.3. Effects of nutrient concentration on biomass yield and enzyme production ........ 83
5.2.4. Effects of growth rate on biomass growth and enzyme production as well as yields and productivities ........ 86
5.3. Conclusions ........ 89
CHAPTER SIX ........ 90
6. Maximising the benefits of enzyme synergy in the simultaneous saccharification and fermentation of Jerusalem artichoke (*Helianthus tuberosus*) tuber residues into ethanol ........ 90
Maximising the benefits of enzyme synergy in the simultaneous saccharification and fermentation of Jerusalem artichoke (*Helianthus tuberosus*) tuber residues into ethanol ........ 93
Abstract ........ 94
Statement of Novelty ........ 95
6.1. Introduction ........ 96
6.2. Materials and methods ........ 98
6.2.1. Raw material handling and preparation ........ 98
6.2.2. Yeast, inulinase strains and inoculum preparation ........ 98
6.2.3. Endoinulinases and exoinulinases production ........ 99
6.2.4. Enzyme cocktails ........ 99
6.2.5. Enzymatic hydrolysis ........ 100
6.2.6. Statistical design of experiments ........ 101
6.2.8. Fed-batch simultaneous saccharification and fermentation ........ 101
6.2.9. Analytical methods ........ 102

Stellenbosch University https://scholar.sun.ac.za

6.3. Results and Discussion ........ 102
6.3.1. Chemical composition of the JA tuber extraction residue ........ 102
6.3.2. Screening for enzymes for hydrolysis of the extraction residue ........ 103
6.3.3. Optimization of the enzyme proportions in the cocktail and synergistic interactions of the enzymes during enzymatic hydrolysis ........ 104
6.3.4. Fed-batch simultaneous saccharification and fermentation ........ 109
6.3.5. Effects of solids loading on enzyme synergy and fermentation productivity in fed-batch SSF ........ 111
6.4. Conclusions ........ 116
6.5. Competing interests ........ 116
6.6. Acknowledgements ........ 116
CHAPTER SEVEN: CONCLUSIONS AND RECCOMENDATION ........ 117
7. Conclusions and recommendations ........ 117
7.1. Conclusions ........ 117
7.2. Recommendations ........ 120
CHAPTER EIGHT: APPENDIX ........ 121
8.1. Exponential feeding equation derivation ........ 121
8.1.1. Overview ........ 121
8.1.2. Biomass balance ........ 121
8.1.3. Glucose substrate balance ........ 122
8.2 Application of the method ........ 124
CHAPTER TEN: REFERENCES ........ 131

Stellenbosch University https://scholar.sun.ac.za

## List of figures

Figure 2.1: Illustration of an inulin polymer with 36 fructose molecules. N is the number of fructose molecules (Redrawn according to Li et al.,2015). ........ 12
Figure 2.2: Differential hydrolysis of inulin by exoinulinase and endoinulinase into fructose and fructooligosacharides, respectively (Redrawn according to Li et al.,2015) ........ 23
Figure 4.1: Standardized Pareto chart to estimate the significant factors for inulin (A) and protein (B) extraction from JA residue after pressing ........ 51
Figure 4.2: Response surface plot showing inulin extraction (a) and protein co-extraction (b) from the pressed tuber and inulin extraction from the protein extraction residue (c), as a function of pH and temperature ........ 53
Figure 4.3: Response surface plot showing protein extraction (a) and inulin co-extraction (b) yields from the pressed tuber as a function of pH and temperate ........ 57
Figure 5.1**:** A schematic representation of the vector used in this study to construct the recombinant A. niger D15[InuA]. ........ 74
Figure 5.2: Silver-stained SDS-PAGE for crude supernatant from recombinant A. niger strain grown in shake flasks in MM media at 30 $^{o}$C for 60 hours. Lane 1 is the molecular marker, lane 2-3 and 5-8 was obtained from A. niger D15 (InuA) strains and lane 4 is the A niger D15 (pGT control). The black arrow indicates the band representing endoinulinases (InuA) ........ 77
Figure 5.3: Effects of feed concentration on biomass production (A), enzyme activity (B) DO (C), and agitation speed (D) during endoinulinases production from recombinant A. niger D15 (InuA) strain. Exponential feeding was used and four feed concentrations (15, 100, 200 and 300 g/L) were evaluated at a growth rates close to µmax. The nitrogen-nutrients concentration was 3.8 g/L. Agitation speed was cascaded between 250-400 rpm. The feeding start and end point (s) are indicated by an arrow pointing upwards and downwards, respectively ........ 82
Figure 5.4: Fungal growth morphology during endoinulinases production from recombinant A. niger D15 (InuA) strain in high-cell density fed-batch culture. The pellet morphology (A) was predominant during the batch phase of the culture while mycelial morphology (B) was predominant during the feeding phase ........ 83
Figure 5.5**:** Biomass concentration (A), enzyme activity (B) and DO (C) using different concentration of the nitrogen sources. A cocktail of three nitrogen sources which are yeast extract, peptone and casamino acids in proportions of 50, 25, and 25 %, respectively. An exponential feeding rate of µ = 0.07 was used with a fixed glucose feed of 300 g/L. Agitation speed was cascaded between 250-400 rpm. The feeding start and end point (s) are indicated by an arrow pointing upwards and downwards, respectively ........ 85
Figure 6.1: Screening enzymes for inulin and fibres hydrolysis to obtain sugars for ethanol fermentation. The data represents mean values of triplicates with standard deviation. The different letters represent differences that are statistically significant ........ 104
Figure 6.2: Pareto chart summarizing the effects of the different cocktail proportions on the sugar recovery ........ 108
Figure 6.3: Ethanol concentrations (g/L) and sugar conversion yields (A and B, respectively) for fed-batch simultaneous saccharification and fermentation of JA tubers solid residues obtained after sequential extraction ........ 110
Figure 7.1: Process flow for the integrated process of sequential protein and inulin extraction as well as SSF of the extraction residue ........ 119
Figure A1: Estimated biomass profile at 1 min interval during the fed batch phase a $\mu_{set}$ = 0.07 ........ 125
Figure A2: Estimated biomass profile every 10s during the fed batch phase a $\mu_{set}$ = 0.07. The figure also includes a summary of the experimental biomass profile ........ 126

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Figure A3: Predicted mass of glucose required during the exponential feeding phase to maintain the desired growth rate (a $\mu_{set}$ = 0.07), pumped at 1 min intervals........................................................ 127
Figure A4: Predicted mass of glucose required during the exponential feeding phase to maintain the desired growth rate (a $\mu_{set}$ = 0.07), pumped every 10 sec................................................................ 127
Figure A5: Estimated feed flow rates required to meet the culture substrate need at 1 min and 10 sec intervals to maintain a $\mu_{set}$ = 0.07 ............................................................................................ 129
Figure A6: Estimated feed flow rates required to meet the culture substrate need at 10 sec intervals $\mu_{set}$ = 0.07 .......................................................................................................................... 129
Figure A7: An illustration of the calibration curve used to set the pump to pump set point............ 130

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## List of tables

Table 2.1: Comparison of the chemical composition of inulin contain plants. Values are presented as % on dry matter (DM) basis ........ 7
Table 2.2: Summary of potential inulin-based biorefinery products and fermentation technologies... 9
Table 2.3: Inulin and protein healthy benefits, functional properties and uses........ 11
Table 2.4: Inulin extraction conditions and yield from dried and wet inulin-containing tubers ........ 14
Table 2.5: Comparison of inulinase activity between native and recombinant host ........ 24
Table 2.6: The impact of *A. niger* growth morphology on recombinant protein production........ 26
Table 4.2: Protein and inulin yields from the press juice, predicted models and confirmatory tests. Inulin and protein recoveries were determined as % yield based on the inulin and protein content, respectively, in tuber. Statistical analysis was done with a 95% confidence level ........ 60
Table 4.3: Functional properties of protein from the press juice and solvent extraction step. Statistical analysis was done with a 95% confidence level ........ 61
Table 5.1: The DNA sequences of the oligodeoxyribonucleotide primers used in this study ........ 74
Table 5.2: Biomass growth and enzyme production productivities at glucose feed concentrations for exponential fed batch cultures of A niger D15 (InuA) strain at a nitrogen concentration of 3.8 g/L... 81
Table 5.3: Biomass growth and enzyme production productivities at different nutrient concentrations for exponential fed batch cultures of the *A niger* D15 (*InuA*) strain ........ 84
Table 5.4: Biomass growth and enzyme production productivities at two specific growth rates for exponential fed batch cultures of *A niger* D15 (*InuA*) strain ........ 87
Table 6.1: Compositional analysis of the raw tuber and tuber residues[a] on dry matter basis........ 103
Table 6.2: Mixture design summarising the cocktail components and proportions as well as sugar recoveries........ 107
Table 3: Summary of the regression analysis for the saccharification and SSF mixture designs illustrating the significant factors for the hydrolysis of JA tubers at different solids loading ........ 108
Table 6.4: Mixture design illustrating the ethanol yields and residual sugars from the fed-batch simultaneous saccharification and fermentation of the tuber residue ........ 115
Table A1: Specified and experimental parameters for batch and fed-batch cultures ........ 124

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**Abbreviations**

| | |
|---|---|
| CAGR | compound annual growth rate |
| FOS | fructooligosaccharides |
| DP | degree of polymerisation |
| JA | Jerusalem artichoke |
| LC | lignocellulosic |
| LCF | lignocellulosic fibres |
| TG | Triglycerides |
| ha | hectare |
| DM | Dry matter |
| IOS | inulooligosaccharides |
| SCO | single cell oil |
| SCP | single cell protein |
| SST | sucrose-sucrose fructosyltransferase |
| FFT | fructan fructosyl transferase |
| SBS | short bowel syndrome |
| GHGs | greenhouse gases |
| SSF | Simultaneous saccharification and fermentation |
| GRAS | Generally Regarded As Safe |
| NREL | National Renewable Energy Laboratory |
| HPLC | high-performance liquid chromatography |
| BCA | bicinchoninic acid |
| FS | forming stability |

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ANOVA analysis of variance

FFD full factorial design

CCD central composite design

RSM response surface methodology

S1 scenario 1

S2 scenario 2

Da daltons

d.f. degrees of freedom

DS degree of synergy

VHG Very high gravity

*pyrG* Orotidine 5-monophosphate decarboxylase encoding gene

*prtT* protease-deficient

*phmA* nonacidifying

*EnInu* endoinulinase

*ExInu* exoinulinase

PDA potato dextrose agar

YPD Yeast potato dextrose

*gpd* glyceraldehyde-3-phosphate dehydrogenase promoter

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

MM minimal medium

U amount of enzyme required to produce 1µmol of reducing sugar per min under assay conditions

kDa kilodaltons

PST pump set-point (%)

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**Nomenclature**

DW dry weight (g)

t time (min or sec)

$X_t$ biomass (g) at time t

$X_0$ biomass ($g_{DW}$) at time 0

μ specific growth rate ($h^{-1}$)

$u_{set}$ set specific growth rate during fed batch phase ($h^{-1}$)

S mass of glucose fed (g)

Sf concentrated glucose feed (g/L)

V Volume of broth (L)

$Y_{xs}$ Biomass yield coefficient, ($g_{DW}.g_{glucose}^{-1}$)

$Y_{ps}$ product yield coefficient

$Y_{px}$ product on biomass yield coefficient

F flowrate (L/h)

D Dilution rate ($h^{-1}$)

$V_f$ Volume of glucose feed (ml)

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# CHAPTER ONE: INTRODUCTION

## 1.1. Contextual background

Rapid population growth continues to exert extensive pressure on the ecosystem due to increased and unsustainable demand for basic resources such as water, food, and energy. The increased demand is in turn a major ecological concern since it translates to an increase in carbon emissions, further complicating the efforts to tackle global warming (United Nations Sustainable Development, 1992). Moreover, the growth of the middle class and changes in wealth distribution have led to a major increase in the demand for dairy products and meat. This further threatens food security through an increased demand that cannot be matched by supply and ultimately results in food inflation (Godfray *et al*., 2010).

Although extreme weather conditions and water availability as well as supply, play an integral role in food production and supply (Hanjra and Quresh, 2010, Schneider, *et al*., 2011), energy supply and cost is also a major threat to food security and socioeconomic stability (Zhang and Vesselinov, 2016). In addition, the agricultural sector is heavily reliant on fossils fuels with an estimated contribution of 60% of the energy needs in primary agriculture and renewable energy making up as little as 6% (FAO, 2011).

The over-reliance of the agriculture sector on fossil fuels is a major concern due to the non-renewable nature of the sources, depletion of reserves and heavy greenhouse gases (GHGs) emissions (Woods *et al*., 2010) and this has severe implications for food and energy security. Food production, processing and distribution is estimated to contribute 20% of greenhouse gases emissions globally. Furthermore, crop and livestock production make up to 28 and 34%, respectively, of this share (FAO, 2011).

Therefore, the complex interaction among population growth, food, water and energy security and climate change necessitate an integrated approach to achieve social and economic security. In addition, alternative and renewable resources as well as efficient ways to exploit them, are imperative for successful and sustainable achievement of these goals.

Biorefineries possess the potential to minimize the overreliance on fossil resources for increasing energy, fuel, and chemicals demand (Apprich *et al.,* 2014; da Silva *et al*., 2014). Furthermore, biorefineries are central to the use of renewable resources and development of technologies with reduced waste production (Cherubin, 2010) and emission of GHGs

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(Herrero and Ibanez, 2015). The biorefinery concept has the potential to contribute to economic growth through job creation and infrastructure development (Wellisch *et al*., 2010). However, biorefineries face several challenges such as seasonal feedstock variation, lack of well-developed processing technology, complex feedstock composition, and competition for land to cultivate biomass feedstock, human settlement, and food production (Maity, 2015).

The competition between land use for food production, human settlement and biorefinery feedstock production is a major concern for sustaining livelihoods as economies face multiple problems that need the same resources for solutions (IEA, 2014; Rasul, 2016; Shanak *et al*., 2018). The US department of energy reported that 1.3 million tonnes of biomass was available in 2005 and this has the potential to meet 40% of the country's petroleum consumption (Maity, 2015). Moreover, 20 million tonnes of the annual production of non-edible oil seeds in India was estimated to have a potential to supply approximately 2.5% of the country's petroleum consumption based on 2011 usage (Maity, 2015). Therefore, the need to balance land use amongst food production, human settlement and biorefinery feedstock production is of utmost importance, particularly when the world is faced with rapid population growth.

### 1.2. Rationale of study

Jerusalem artichoke (*Helianthus tuberosus* L.) tuber is an abundant source of both inulin (Kosaric *et al*., 1984) and protein (Johansson *et al*., 2015; Bekers *et al*., 2008). The plant is fast growing (Long *et al*., 2016), with tuber yields of at least 15 tones $ha^{-1}$ (Monti *et al*., 2005; Baldini *et al*., 2011). The tuber is a rich source of fermentable sugars, inulin which makes up to 80% of the tuber dry matter (Johansson *et al*., 2015). However, the use of land for JA cultivation for ethanol production may not be sustainable due to food security concerns.

The use of JA tuber in a biorefinery based strategy has the potential to improve the feasibility of ethanol production through fermenting the residual sugars, resulting from inulin and protein extraction. Inulin is used as nutraceutical, and the protein may be used as a nutritional supplement, with health benefits. Alternatively, the protein could be utilised as an animal feed. This strategy has the potential to result in synergistic benefits among food, animal feed, and fuel production.

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Currently, there is a paucity of experimental data on the selective and sequential extraction of inulin and protein from JA tubers. Furthermore, there is a lack of data on the functional properties of JA tuber protein, which are important for the protein application as a dietary supplement and in food processing. Therefore, an understanding of the potential impacts of sequential extraction, of inulin and protein, on the yields, quality and functional properties of the products is important for the integration of inulin and protein extraction in the JA tuber biorefinery.

Inulin hydrolysis into monomeric fermentable sugars or a range of products will expand the product portfolio and may enhance the feasibility of a JA based multiproduct biorefinery. Although, inulinases are native from microorganism (Singh and Chauhan 2018), which are also the best source for industrial production (Leelaram *et al*., 2016), the low enzyme productivity from native strains is a disadvantage. Filamentous fungi, *Aspergillus* sp. is a robust organism that is widely used in industrial recombinant enzyme production (Rose and van Zyl, 2008; Krull *et al*, 2008). However, the submerged fermentation is limited by viscosity constraints that impact the achievement of high biomass density and subsequently volumetric enzyme yields (Singh *et al*., 2018). The pellet morphology of the *A. niger* in submerged culture, has been reported to improve mixing efficiency and nutrient transfer in high density cell culture as well as enzyme production in recombinant *Aspergillus* sp. (James *et al*., 2007; Papagianni and Mattey, 2006). Thereby, an assessment of the impact of viscosity, growth rate and fungal morphology on endoinulinase production in recombinant *A. niger* is important in getting insight into the potential use of recombinant *A. niger* strain in enzyme production.

The tuber extraction residues, from the inulin and protein extraction, are rich in residual inulin and lignocellulosic fibres (LCFs) that can be valorised through fermentation into ethanol. Due to the heterogenous nature of the tuber residue, a cocktail of multi-functional hydrolytic enzymes could be important for the efficient saccharification during fermentation of the residue. High-solids fermentation is advantageous for the achievement of high concentration of ethanol of at least 40 g $L^{-1}$, however, this can result in severe viscosity limitations and consequently reduced fermentation efficiency (Srichuwong *et al*., 2009, Lim *et al*., 2013). The use of enzyme cocktails has been reported to reduce viscosity during tuber fermentation (Shrichuwong *et al*., 2009; Yingling *et al*., 2011). There is, however, a lack of experimental data on the co-fermentation of inulin and lignocellulosic fibres from JA tubers. Furthermore, no

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experimental data is available on the use of enzyme cocktails and demonstration of their underlying synergistic mechanism for co-fermentation of the heterogenous tuber residues.

A multi-product strategy involving the production of inulin, protein and ethanol may potentially enhance the feasibility of a JA based biorefinery. This strategy has the potential provide synergistic benefits between food or animal feed and fuel production, for the growing population and demand for health food products, while mitigating the severe effects of global warming.

### 1.3. Structure of Thesis

This thesis is organised in eight chapters which includes a detailed review of literature, objectives, experimental data presentations and conclusions for the development of a JA-tuber based biorefinery. Chapter two is the literature review that gives a critical analysis of existing literature and identify gaps, thereof. The literature analysis paved way for the novelty, aims and objectives of the study which are outlined in chapter three. The experimental work and data addressing each objective are presented in chapters four to six of the thesis. Each chapter is presented in the form of either a published article or manuscript. Chapter seven presents the global findings and conclusions of the study and demonstrates how the different experimental data correlate. Recommendations for future studies are presented in chapter seven as well. The final chapter, eight, is an illustration of how the fed batch method was applied in chapter five (objective 2) of the study.

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# 2. CHAPTER TWO: LITERATURE REVIEW

## 2.1. Jerusalem artichoke tubers based biorefinery

### 2.1.1. Overview: Origin, distribution and uses

Jerusalem artichoke (*Helianthus tuberosus* L.) is a multifunctional crop (Valentine *et al*., 2012) and a potential feedstock for bioenergy, biochemicals, feed and food production. JA is a herbaceous perennial plant composed of the stalk and flower as well as tubers that develop underground (Kosaric *et al*., 1983; Yang *et al*. 2015). The plant belongs to the same family (Compositae) as the sunflower (*Helianthus annus* L.) (Caserta and Cervigni, 1991). The Compositae family also include other inulin containing plants such as chicory (*Cichorium intybus* L.) and dandelion (Apolinário *et al*., 2014).

JA is native to the temperate North America; however, its cultivation was adopted in Europe around the seventeenth century (Bajpai and Bajpai, 1991; Stauffer *et al*., 1981). The plant is widely cultivated in China with uses such as a vegetable for human consumption due the health benefits and soil rehabilitation since it a highly salt tolerant plant (Xiao *et al*., 2011). Protein accumulation in the tubers has resulted in the use of tubers as an animal feed (Alla *et al*., 2014).

Moreover, JA cultivation is widely practised in Europe (CABI, 2018) and used as a vegetable or animal fodder (Xiao *et al*., 2011; Alla *et al*., 2014). Canada was reported to produce approximately 1.3 million tonnes of JA, while Russia has approximately 3000 ha of JA production (Starovoitov *et al*., 2018). Egypt is also a major grower of JA and the tubers were part of the tuber export, in 2017, to the United Kingdom and valued at US$5.88 million (Trading economic 2019).

### 2.1.2. JA tubers yields and impacts of growing conditions on yields

JA has gained interest as a rich source of inulin and biorefinery feedstock due to its good agronomic traits. It can be cultivated under a range of microclimatic conditions (Izsaki and Kadi, 2013), is fast growing (Long *et al*., 2016), requires minimal inputs, such as fertilizer and pesticide, for commercial cultivation, and produces high tuber yields (Yang *et al*., 2015). Climate and rainfall patterns are important factors in determining the tuber yields. (Long *et al*., 2015; Monti *et al.,* 2015).

Monti *et al*., (2005) reported that there was no significant difference in the inulin yield (800 g $m^{-2}$) between tubers cultivated under irrigation and rain-fed conditions, with an average rain

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of 300 mm which was almost 50% of the water used in the former. The ability of Jerusalem artichoke to acclimatise to limited water conditions was also reported by Baldini *et al*., (2011), where the crop was cultivated in Bologna, a hot arid region in Italy with rainfall in the range of 410-516 mm per annum. Tuber yields were reported to be as high as 15 t $ha^{-1}$ on dry mass basis, which is equivalent to ca. 9 t $ha^{-1}$ of sugar yields. Moreover, the yields were obtained with a nitrogen and phosphate fertiliser application in the range of 43-100 kg $ha^{-1}$ (Monti *et al*., 2005; Baldini *et al*., 2011). The sugar yields are higher than reported for sugar beet (5.5 t $ha^{-1}$) cultivated with nitrogen and phosphate application of 124, and 124 kg $ha^{-1}$, respectively (Mubarak et at., 2016).

Liu and co-workers (2012) reported shoot and tuber yields in the range of 5-10 t $ha^{-1}$ on dry mass basis, from Jerusalem artichoke cultivated in the semi-arid region of Gansu province in China. The region has an annual rainfall of 556 mm with 60% occurring during the cultivation season and 156 free-frost days. The JA tuber yields are higher than tuber yields from chicory (8.9 t $ha^{-1}$) (De Mastro *et al*., 2004), a the major feedstock crop for industrial inulin production (Zarroug *et al*., 2016).

### 2.1.3. Composition of JA tubers and distribution of inulin

Jerusalem artichoke tubers make between 20-30 % of the total dry matter of the crop. Table 2.1 is a comparative summary of the composition and yields of Jerusalem artichoke tubers with other commercial sources of inulin. The inulin composition of the tubers is also comparable to chicory root. The tubers are largely composed of inulin, which accounts for approximately 69-80% of the tuber dry matter (DM) (Table 2.1). In addition, the tuber is composed of insoluble fibres that consists of cellulose (2.73%), hemicellulose (2.22%) and lignin (0.52%) (Rubel et al., 2014). A protein content in the range of 7.5-18 % of dry matter has been reported to be present in the tuber (Johansson *et al.,* 2015; Bekers *et al*., 2008).

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Table 2.1: Comparison of the chemical composition of inulin containing plants. Values are presented as % on dry matter (DM) basis

| Source | Inulin | Protein | Ash | Fibre* | Inulin yields (ton/ha) | References |
|---|---|---|---|---|---|---|
| JA tuber | 69-80 | 7.5-18 | 6.1 | 2.7 | 4-12 | Gunnarsson *et al*., (2014); Rube *et. al*., (2014), Johannson *et al*., (2015) |
| Agave leaf | 45 | 35 | 5.9 | 38.4 | 2-4 | Bouaziz *et al*., (2014) |
| Chicory Root | 80 | 5 | 3.8 | n.a. | 3-5 | Jurgonski *et al*., (2011) |
| Dahalia | 86 | 2 | 3.1 | 41 | 5-11 | Nsabinama and Jiang, 2010 |
| Globe artichoke | 30 | 25 | 94 | n.a. | 7-9 | Pandino *et al*., 2011 |
| Agave pina | 71 | n.a. | n.a. | n.a. | 5-6 | Mellado-Mojica and López (2012) |

n.a.-not available

*determined as the total amount of hemicellulose and cellulose

The composition, distribution, and degree of polymerisation (DP) of inulin and other soluble carbohydrates in tuber, vary with season, age of tuber at harvesting and storage conditions after harvesting (Praznik and Beck, 1987; Kocsis *et al*., 2007). The DP and distribution of the inulin is important for the development of optimal process to solubilise and extract inulin from the tubers. The DP also determines the range of uses of inulin in the food or nutraceutical industries (Franck, 2002).

The inulin content of the tuber was reported to decrease from 63.5% of dry mass, at 14 weeks after plantation, to 35.2% at 44 weeks after plantation. Similarly, the average degree of polymerisation ($DP_{av}$) of inulin, from the crude tuber extract, decreased from 11.2 to 6.5 during the same period. In contrast, sucrose concentrations increased from 3.5% to 8.9% (Kocsis *et al*., 2007). The DP is a measure of the degree of polymerisation and is important because it determines the range of uses of inulin in the food or nutraceutical industries (Franck, 2002).

Praznik and Beck, (1987) illustrated that the degradation of inulin polymers was related to external stress such as frost and drought. The data from their studies showed a reduction in

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the content of inulin of DP>30 from 54% during the precipitation period to 34% during a drought period and a further decrease to 14% during the frost period. Moreover, Li *et al*., (2015) reported a maximum inulin content and $DP_{av}$ of 12.2 % of wet tuber mass and 19, respectively from tubers harvested 50 days from flowering.

### 2.2. Strategies for the development of JA tubers as a feedstock for a multiproduct biorefinery

The incorporation of a protein extraction step into the conventional inulin extraction process, will make the tuber a potential feedstock for multiproduct biorefineries. JA tubers have between 7.5-18% DM of protein (Gunnarsson *et al*., 2014) and most of it is co-extracted into the inulin product as an impurity (Yi *et al*., 2009). Furthermore, the tubers are easy to process since they are largely composed of inulin, a non-recalcitrant polymer compared to lignocellulosic fibres (Long *et al*., 2016). Therefore, the high sugar yields of tubers, the potential of inulin use as an ingredient in the food, pharmaceutical as well as the chemical industry, non-recalcitrant nature of inulin and the protein component, which makes up to 18% DM, make the tubers a potential feedstock for a multiproduct biorefinery. The biorefinery will integrate the production of food and nutraceutical products with bioenergy. Furthermore, the availability and incorporation of green extraction processes could make a JA tuber-based biorefinery environment-friendly. Green extraction processes are environment-friendly because they do not use solvents such as chloroform, acetone, ethanol and have minimal energy requirements (Cherubin, 2010; Maity, 2015).

Although, Jerusalem artichoke tubers are an attractive and potential feedstock for biorefining, there is a paucity of experimental data demonstrating the technical feasibility of an integrated approach for the use of JA tubers to co-produce value added products, such as inulin and protein, with bioenergy. Currently, most of the available literature data is on the hydrolysis of inulin to fermentable sugars for ethanol, single cell protein, acetic acid and biodiesel production (Gunnarsson *et al*., 2014; Yang *et al*., 2015).

Table 2.2 is a summary of potential inulin-based biorefinery products and fermentation technologies. The production of ethanol from JA tubers has been done through consolidated bioprocessing (CBP) using recombinant *S. cerevisiae* in batch fermentation mode (Remize *et al*., 1998; Zhang *et al*., 2010).

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Table 2.2: Summary of potential inulin-based biorefinery products and fermentation technologies

| PRODUCT | MICROBE/ENZYME | FERMENTATION | REFERENCE |
|---|---|---|---|
| IOS | Endoinulinase (*Xanthamonas* sp. *and Pseudomonas* sp.) | Batch | Cho *et al*., (2001) |
| IOS | Purified commercial endoinulase from *A. niger* | n.a. | Mutanda *et al*., (2008) |
| Fructose | Inulinase (*Kluyveromyces* sp) | Continuous packed bed column | Wenling *et al*., (1999) |
| SCP | *Cryptococcus aerus G7a* | Batch | Gao *et al*., (2007) |
| Fructose | Partially purified extracellular inulinase (*A. niger* mutant) | Continuous packed bed column | Nakamura *et al*., (1995) |
| FOS | Immobilised commercial endoinulinase from *A niger* | Continuous packed bed column | Nguyen *et al*., (2010) |
| SCO | Co-cultures of *R mucilaginosa* and *P guilliermondii* | Batch | Zhao *et al*., (2011) |
| IOS | Recombinant *E. coli* expressing endoinulinase | Continuous packed bed column | Yun *et al*., (1999) |
| IOS | Recombinant E. coli expressing | Continuous packed bed column | Yun *et al*., (1999) |
| Fructose | Partially purified exoinulinase (*Kluyveromyces marxianus*) | Batch | Sarup *et al*., (2006) |
| IOS | Immobilised endoinulinase *(Pseudomonas* sp) | Continuous packed bed column | Yun *et al*., (1997) |
| Fructose | n.a. | n.a. | Abozed *et al*., (2009) |
| Ethanol | Recombinant *S. cerevisiae* | Batch | Remize *et al*., (1998) |
| Ethanol | Recombinant *S. cerevisiae* | Continuous packed bed column | Remize *et al*., (1998) |
| IOS | Immobilised endoinulinase (*Aspergillus ficuum*) | Batch | Jin *et al*., (2004) |
| IOS | Immobilised endoinulinase (*Aspergillus ficuum*) | Batch | Jin *et al*., (2004) |
| IOS | Recombinant *S. cerevisiae (Inul)* | Batch | Kim *et al*., (2006) |
| IOS | immobilised purified endoinulase (*Xanthomonas sp*) | Batch | Park *et al*., (1999) |
| IOS | Immobilised purified endoinulinase (*Penicillium* sp) | Batch | Nakamura *et al*., (1997) |
| SCO | *Rhodotorula mucilaginosa* TJY15a | Batch | Zhao *et al*., (2010) |
| SCO | *Rhodotorula mucilaginosa* TJY15a | Batch-fed | Zhao *et al*., (2010) |
| SCO | Recombinant *Yarrowia lipolytica* (*INU1*) | Batch | Zhao *et al*., (2010) |
| SCP | Mutant *Cryptococcus aureus* G7a | Batch | Zhao *et al*., (2010) |
| Fructose | Immobilised partially purified inulinase *(Kluyveromyces)* | Continuous packed bed column | Wei *et. al*., (1999) |
| Fructose | Immobilised partially purified exoinulinase *(K. marxianus*) | Batch | Singh *et. al*., (2007) |
| Fructose | Cocktail of *A niger* and *C. guilliermondii* hyrosylates | Batch | Sirisansaneeyaku *et al*., (2007) |
| Ethanol | Recombinant *S. cerevisiae (INU1)* | Batch | Zhang *et al*., (2010) |

*IOS: Inulooligosaccharides

DP: Degree of polymerisation

SCO: Single cell oil

SCP: Single cell protein

n.a.: not applicable

The use of CBP, an energy and cost-efficient method, demonstrates the potential for the use of the tubers as an alternative and sustainable feedstock for ethanol production. However, there is a need to evaluate the potential of an integrated strategy for the extraction of inulin and protein, for use as a nutraceutical and high-value food product or nutritional supplement, respectively. Subsequently the potential to co-ferment the residual inulin and lignocellulosic fibres in tuber residues to ethanol will be investigated.

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The summary from table 2.2 shows that the partial hydrolysis of inulin into inulin oligomers commonly known as fructooligosaccharides (FOS) is a preferred bioprocessing pathway for JA tubers. The data also demonstrates the use of free or immobilised endoinulinase for the partial hydrolysis of inulin into FOS (Yun *et al*., 1997; Jin *et al*., 2004; Nguyen *et al*., 2010). Complete hydrolysis of inulin into fructose is also an alternative processing option for inulin from JA tubers (Singh *et al*., 2007; Zhang *et al*., 2010). It is, therefore, important to develop recombinant expression systems for inulinase expression and bioprocessing systems for high enzyme yields and volumetric activities. Moreover, a cost-effective system for enzyme production is important towards the development of a technically and economically feasible JA tuber biorefinery.

## 2.3. Inulin-based nutraceutical

Consumer attitudes towards nutraceuticals are changing as a result of the products' perceived heath and nutritious benefits (Bech-Larsen and Grunert, 2003; Urala and Lahteenmaki, 2007). Frost and Sullivan, (2011) reported that 35% of consumers globally were aware of the health benefits of nutraceuticals. Moreover, the global nutraceutical market compound annual growth rate (CAGR) was an estimated 14.7% in the period 2002-2010 and worth US$ 140.1 billion (Frost and Sullivan, 2011). The Middle East and African nutraceutical market recorded the highest growth rate during the period 2007-2011; with an estimated CAGR of 8.7%, reaching a market value of US$ 2.5 billion (Research and Markets, 2012).

The global market for fibre-based nutraceuticals is growing rapidly and was estimated to reach 766.9 million Euros in 2015 with an estimated CAGR of 14% in the period 2008-2015 (Frost and Sullivan, 2008). Inulin and inulin-based fructooligosaccharides (FOS) dominate the (prebiotic) market, with an estimated market share of 70% and over 10% annual growth rate (Frost and Sullivan, 2008). FOS had a 15.4% market share (worth 45.8 million Euros) in the prebiotics market (Frost and Sullivan 2008). Furthermore, the inulin market is forecasted to grow with a CAGR of 6.5%, to reach a market value of US$ 2.5 billion by 2024 (Mondor Intelligence, 2018).

Nutraceuticals are food products or extracts from foods that possess health benefits beyond the primary nutritional and dietary benefits (Palthur *et al*., 2010). They are consumed in the form of a conventional food or a dietary supplement (Kalra, 2003). Nutraceutical ingredients

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include soluble fibres (prebiotics), probiotics, vitamins, carotenoids, fatty acids, minerals, and peptides. Vitamins, similar to antioxidants, can be taken as nutraceutical supplements to minimise the risks of hypertension and cardiovascular disease (Houston, 2013). The summary in table 2.3 is an illustration of the functional properties and health benefits of inulin-based nutraceuticals which are used as functional foods ingredients. The summary shows that inulin-based nutraceuticals reduce the risk of cancer, diabetes, obesity and cardiovascular diseases, and have immuno-modulatory functions (Femia *et al*., 2002, Roller *et al*., 2004, Roberfroid, 2007, Singh *et al*., 2008, Kuo *et al*., 2013).). The use of inulin as an ingredient in food processing has no negative impacts on the properties (taste, texture, mouthfeel, heat stability, water retention) and quality of these conventional food products (Franck, 2002; Singh *et al*., 2008).

Table 2.3: Inulin n health benefits, functional properties and uses

| Nutraceutical | Health benefits | Functional properties | Uses | Functional advantages |
|---|---|---|---|---|
| Native inulin | Improves iron (Fe) absorption<br>Reducing risk of cardiovascular diseases and obesity | Good gelling, foaming and emulsion properties | Fat replacement in the processing of frozen desserts, table spreads, dairy products, and salad dressings | Low calorie |
| FOS | Reducing risk of cancer and diabetes | Sweet<br>Highly soluble<br>Good, water activity, emulsion, and heat stability | Sugar replacement in biscuits, chocolates, and cereals | Improve taste, mouthfeel and crispiness |

FOS: Fructooligosaccharides

## 2.4. Inulin structure and synthesis

Inulin is a polyfructan found in a range of plants as a storage carbohydrate (Apolinaro *et al*., 2014). Jerusalem artichoke, chicory, dahlia and agave are common plants that are known to have a significant quantity of inulin ranging between 15-21% w/w of wet tuber mass (Kosaric *et al*., 1984; Kaur and Gupta, 2002; Gupta *et al*., 2003). Inulin is also found in common household fruits and vegetables such as onion, banana, and garlic (Moshfegh *et al*., 1999).

Inulin from JA and chicory is a linear polymer while it is branched in agave. Linear polyfructan is composed of β(2 → 1) linked D-fructose monomers (Figure 2.1) that may be terminated with a D-glucose monomer linked to a fructose through an α-(2 →1) bond (Barclay et al.,

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2010). The degree of polymerisation (DP) refers to the number of fructose monomers in an inulin chain and ranges between 2 and 60 for native inulin. Branched inulin is also composed of β(2 → 1) bonds, with a significant amount of β(2 → 6) linkages (Ravenscroft *et al.,* 2009).

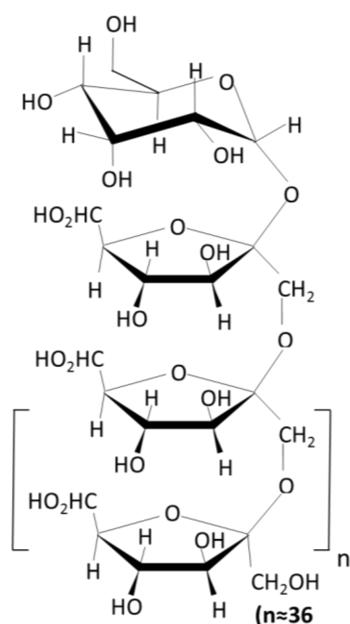


Figure 2.1: Illustration of an inulin polymer with 36 fructose molecules. N is the number of fructose molecules (Redrawn according to Li *et al*.,2015).

Inulin is synthesised in plants by sucrose-sucrose fructosyltransferase (SST) and β(2 → 1) fructan fructosyl transferase (FFT) from sucrose to chains of various lengths (Itaya *et al.*, 1999). SST initiates inulin synthesis through the transfer of a fructose molecule from a sucrose molecule to another (Edelman and Jefford, 1968; Wagner and Wiemken, 1987) and subsequent elongation of the trisaccharide follows through FFT, which transfers more fructose molecules from a sucrose molecule (Edelman and Jefford, 1968).

### 2.5. Inulin extraction

Hot water diffusion is a common method of inulin extraction (Franck 2002) and is currently applied at industrial scale for the extraction of inulin from chicory (Meuser *et al.*, 2009). Hot water extraction, however, requires high extraction temperatures and long duration to achieve economic yields (Lingyun et al., 2007, Yi et al., 2009; Tewari et al., 2015). Microwave (Tewari et al., 2015) and ultrasound-assisted extraction (Lingyun et al., 2007) are alternative methods that are being researched. The former uses microwave energy to rapidly heat the

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solvent and this subsequently results in the rapid disruption of the biomass matrix and mass transfer of the extract into the solution due to the localised temperature and pressure (Zhang, 2007; Zhou, 2008). Ultrasound extraction uses ultrasound to disrupt plant cell wall and this results in improved mass transfer of the extract (Lingyun, 2007).

Inulin extraction is based on the solubility of inulin in water and is temperature as well as pH dependent (Yanovsky and Kingsbury, 1933; Naskar *et al*., 2010). A neutral pH (pH 7) is suitable for inulin extraction to avoid inulin hydrolysis (Matusek *et al*., 2009; Homme et al., 2003). In addition, inulin extraction can be performed from either fresh or dried tubers (Lingyun *et al*., 2007; Li *et al*., 2012; Tewari *et al*., 2015). The former process option is ideal since drying has been shown to lead to inulin degradation, which consequently reduces the DP and limits the application of the derived inulin (Frank, 2002). Moreover, the extraction of inulin from wet tubers will reduce the cost of drying and storage (Lingyun et al., 2007).

Extraction conditions such as temperature (50-80 $^{o}$C), time (20 - 60 min) and solid loading (2.5 - 10%) have been reported (Table 2.4) to affect the inulin yield (Tewari et al., 2015; Bekers *et al.*, 2008; Lingyun *et al*., 2007). Lingyun *et al*., (2007) reported a maximum inulin yield of 78% of the tuber dry mass. The preferred extraction conditions for the maximum inulin yield were a temperature of 76.6 $^{o}$C at a solid loading of ~ ca. 10% w/v for 20 min.

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Table 2.4: Inulin extraction conditions and yield from dried and wet inulin-containing tubers

| **Method** | **Tuber form** | **T (°C)** | **Time (min)** | **Yield (%)** | **Reference** |
|---|---|---|---|---|---|
| Hot water | Fresh | 80 | 120 | 69 | Yi *et al.*, 2009 |
| Hot water | Frozen | 80 | 120 | 83 | Yi *et al.*, 2009 |
| Hot water | Dry | 80 | 120 | 98 | Yi *et al.*, 2009 |
| Hot water | Fresh | 50 | 240 | 21 | Bekers *et al.*, 2008 |
| Hot water | Dry | 50 | 240 | 95 | Bekers *et al.*, 2008 |
| Hot water | Dry | 68 | 40 | 12 | Milan *et al.*, 2011 |
| Ultrasonic | Dry | 37 | 25 | 24 | Milan *et al.*, 2011 |
| Hot water | Frozen | 80 | 60 | 14.42 | Li *et al.*, 2012 |
| Hot water | Frozen | 80 | 45 | 16.3 | Li *et al.*, 2012 |
| Microwave | Dry | 90 | 30 | 63 | Tewari *et al.*, 2015 |
| Hot water | Dry | 90 | 30 | 51 | Tewari *et al.*, 2015 |
| Hot water | Dry | 76 | 20 | 78 | Lingyun *et al.*, 2007 |
| Hot water | Dry | 40 | 20 | 57 | Lingyun *et al.*, 2007 |

Moreover, the yield of inulin was reduced at lower temperatures, for instance, with a yield of 57.7% at 40 °C was observed. Li *et al.*, (2012) reported a yield of 14.42% on wet mass basis at optimal conditions of 80 °C, 10% w/v of wet tubers and 60 min; temperature, solid loading and time, respectively. Bekers *et al.*, (2008) obtained an inulin yield of ca. 95% at 50 °C for 240 min with a solid loading of 11% w/v of dry mass. However, a lower yield of 21%, was obtained when using wet tubers and this could be attributed to the high viscosity of wet tubers which subsequently impacts mass transfer (Lingyun et al., 2007).

The summary from table 2.4 illustrated that microwave and ultrasound assisted extraction resulted in higher inulin yields compared to hot water extraction. Lingyun et al., (2007)

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reported an inulin yield of 83% with ultrasound assisted extraction compared to 76% with the conventional method. Moreover, the extract from the former consisted of 62% of oligomers compared to 64% from the latter. Tewari *et al*., (2015) also reported a higher inulin yield (63%) with microwave assisted extraction, however, the recovered inulin extract was of lower degree of polymerisation. The highly localised temperature and pressure induced by microwave energy was reported to result in inulin degradation to monomers which also affects the yield (Tewari et al., 2015). The degradation of inulin thereby limits the applications of the inulin extract obtained through ultrasound and microwave assisted extraction. In addition, the methods are ideal for the extraction and depolymerisation of inulin required for use in oligomeric form.

### 2.6. Inulin purification

The crude inulin extract from the tuber can either be purified/fractionated for use in powder and syrup form or partially hydrolysed into FOS (Franck, 2002). Fractionation involves the isolation of low DP (<10) fructans (Roberfroid, 2005) using techniques such as simulated moving bed chromatography (Gramblicka and Polakovic, 2007). The resulting hydrolysate from inulin hydrolysis is a mixture of sugars, FOS and long chain inulin (DP>10) and requires purification to obtain a pure mixture of FOS. The purity ranges from 50-95% depending on the intended use of FOS. The resulting liquid fraction from FOS purification is a rich source of sugars that can be used for ethanol production (Gramblicka and Polakovic, 2007).

A range of techniques are available that can be applied for the fractionation and purification of inulin and FOS. Ultrafiltration, nanofiltration, activated charcoal and SMB are some of the available techniques, with the latter having the greatest potential for industrial scale application since it is operated in a continuous mode, has stable resins and does not use organic solvents (Nobre *et al*., 2013). SMB is an ion exchange chromatography-based technique and consists of a series of chromatographic columns that work in a continuous counter-current motion of the solid phase relative to the liquid phase (Bubnik *et al*., 2004).

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## 2.7. JA tuber protein

The protein content of the tubers ranges between 7.5-18% DM (Gunnarsson *et al*., 2014; Rube *et. al*., 2014; Johannson *et al*., 2015), which is predominantly of globulin and albumin family (Johannson *et al*., 2015). Albumin is water soluble while globulin salt-soluble, thus suitable extraction process should be capable of extracting both types of protein. Albumins and globulins are also the primary proteins in soy and pea proteins and have been reported to enhance the nutritional quality of these soy and pea proteins (Boye et al., 2010). Soy and wheat-based protein make up the bulk of the market for consumer-grade plant proteins (Frost and Sullivan, 2013).

The essential amino acid content of the tuber protein is generally comparable to that of pea and soy protein (Table 2.5). However, lysine is relatively lower, at 42 mg/g compared to the range of 71-91 mg/g for the pea and soy protein. Notably, the content of sulphur containing amino acids of the tuber is comparable to the pea and soy protein. Moreover, Cieslik *et al*., (2011) reported a protein and essential amino acid content in the JA tuber that was higher than in potato. Potato protein is a new entrant in the market of plant proteins and currently produced and marketed by Solanic® for applications as an ingredient in the processing of beverages and a range of food products (Solanic, 2013).

Table 2.5: Comparison of relative quantities of essential amino-acids

| Source | Amino acid composition (mg/g) | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | Lys | Met | Cys | Try | Thr | Iso | Leu | Phe | Tyr | Val | |
| Pea | 71 | 6 | 5 | 10 | 38 | 47 | 71 | 30 | 35 | 55 | Overduin et al., (2015) |
| Whey | 111 | 15 | 10 | 30 | 45 | 55 | 111 | 25 | 45 | 55 | Overduin et al., (2015) |
| JA | 42 | 5.9 | 4.3 | 6.8 | 26 | 21 | 30 | 47 | 19 | 26 | Bogucka and Jankowski (2020) |
| Potato | 1.24 | 0.3 | 0.2 | 0.3 | 0.7 | 0.8 | 1.2 | 0.9 | 0.4 | 1.4 | Cieslik et al., 2011 |
| Soy | 91 | 10 | 6 | 5 | 50 | 39 | 67 | 40 | 38 | 34 | Tomoskozi et al., (2001) |
| Pea | 85 | 5 | 6 | 5 | 36 | 38 | 63 | 58 | 42 | 52 | Tomoskozi et al., (2001) |

## 2.8. Protein extraction

The choice of the protein extraction and purification method, and the process conditions are critical to obtain high quality proteins with minimal loss of the physicochemical properties, such as solubility and foaming capacity that make the proteins suitable for applications as food grade products. Moreover, the combination of the methods and process conditions should be carefully investigated to suite the solubility properties of the target protein to be extracted (Manamperi *et al*., 2011; Tan *et al*., 2011). Protein extraction can be achieved

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through dry (classification and pin milling) or wet processing. Dry processing results in lower purity (38-65%) than wet processing with a purity of >70% (Boye *et al*., 2010). Wet processing involves the initial solubilisation of the protein from a solid residue and then protein recovery from the liquid fraction using ultrafiltration or precipitation (Boye *et al*., 2010; Taherian *et al*., 2011). The liquid fraction also contains traces of ash, polysaccharides and fatty acids (Manamperi *et al*., 2011). Alkaline, water, salt and acid extraction are commonly used wet processing methods (Pace *et al*., 2004; Boye *et al*., 2010). Moreover, wet processing strategies are also widely applied in commercial protein extraction from soybean and yellow pea (Mizubuti *et al*., 2000; Pace *et al*., 2004; Barbosa *et al*., 2007).

### 2.8.1. Protein solubilisation: An overview

A range of chemical methods are available for protein solubilisation, however, their use at industrial level is limited either by low yields, high cost, low purity, reduced protein quality, or a combination of the factors (Fabian and Yu, 2011). Furthermore, harsh protein solubilisation methods, such as >pH 9 and temperature above 60$^{o}$C, have been reported to result in anti-nutritional factors that reduce the bioavailability of amino acids, protein quality and digestibility (Gilani *et al*., 2012). Ethanol extraction severely affects protein solubility, and this ultimately reduces their functional properties. Robbins and Ballew, (1982) reported that the extraction of soy proteins at extreme alkali conditions (pH 12.5) reduces the bioavailability of sulphur containing amino acids. Subcritical water extraction is also a potential technique. However, Sereewatthanawut *et al*., (2008) reported a decrease in the content of amino acids as the temperature of extraction increased beyond 180 $^{o}$C.

### 2.8.2. Alkaline and acid protein solubilisation

Alkaline extraction is used for protein solubilisation from plant sources such as peas, soybean and rice-bran, and is also applicable at industrial level. The solubilisation process disrupts protein-protein interactions (disulphide linkages, hydrogen bonds, ionic interactions and hydrophobic interactions) and selectively makes the proteins available in an aqueous form (Tecson *et al*., 1971; Pace *et al*., 2004). The pH, temperature and salt concentration are important process conditions to be considered for alkaline extraction (Lai *et al*., 2013; Baborsa *et al*., 2007). Alkaline conditions in the range of pH 7.5-9 are used for protein extraction from peas and soybeans (Mizubuti *et al*., 2000; Pace *et al*., 2004; Barbosa *et al*., 2007).

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The effects of each of these process conditions vary depending on the protein composition or target protein. Baborsa *et al*., (2007) reported that the suitable extraction conditions for an isoflavone-rich soy protein isolate were pH 9 and a temperature of 55°C, and that the ionic concentration did not have a positive impact on the yield of the protein isolate. Mizubuti *et al.*, (2000) used response surface methodology to demonstrate that only pH (8.5) and liquid:solid (5:1) ratio were the significant factors to maximize the yield of pigeon pea protein extraction. The above studies reported proteins yields of 63% and 74.8%, respectively, at the optimum conditions.

Enzyme assisted alkaline protein extraction has been reported to enhance protein recovery (de Moura, *et al*., 2008; Sari *et al*., 2013). Sari *et al*, (2013) reported that the addition of an alkaline protease enzyme increased the protein yield from soybean meal from 80-90% and 15-50% from rapeseed. de Moura *et al*., (2008) reported a protein yield of 85% with the use of an endo-protease. The use of enzyme assisted extraction should, however, be weighed against the marginal cost of using the enzyme.

Acid extraction involves protein solubilisation under acidic conditions and can be used with ultrafiltration for protein recovery (Boye *et al*., 2010, Lam *et al*., 2016). Acid extraction of grass pea protein resulted in a 92% yield at an optimum pH, time and solids loading of 2.57, 48 min, 10% w/v, respectively. The protein isolates showed a lighter colour and better foaming and emulsification capacity compared to alkaline extracted isolates (Feyzi *et al*., 2018). Thereby, the solubility pH range of proteins is wide and needs to be specifically determined due to its importance in protein recovery and functional properties (Mizubuti *et al*., 2000; Pace *et al*., 2004; Boye *et al*., 2010, Feyzi *et al*., 2018)

### 2.8.3. Water extraction

Water soluble proteins can be extracted directly from the biomass with water at a neutral pH. Albumin type of protein are water soluble and can be extracted from their plant source directly with water (Boye *et al.,* 2010; Johannson *et al*., 2015). Although, there are no literature reports of water protein extraction from JA tubers or any tubers, water extraction has been reported for peas. Water extraction usually requires multiple extraction steps to improve the protein yields (Martin-Cabrejas *et al*., 1995). Protein yields of up to 60% have been reported for chickpeas, with multiple extraction steps employed to enhance the protein recovery (Cai *et al*., 2001).

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### 2.8.4. Salt extraction

Salt extraction is based on the solubility of certain proteins in a salt concentration and the protein salting in and out phenomenon (Can Karaca *et al*., 2011, Stone *et al*., 2015). The salting in phenomenon occurs at low ionic concentration where the salt ions shield the protein molecules from each thereby promoting solubilisation (Duong-Ly and Gabeli, 2014). The effectiveness of the method is dependent on the ionic strength which in turn is a factor of the type of salt used (Marquez *et al*., 1996; Stone *et al*., 2015). Calcium chloride and ammonium sulphate are commonly employed for salt extraction. The salt solubilised protein can be subsequently recovered through precipitation which is achieved by dilution of the solution to lower the ionic strength (Duong-Ly and Gabeli, 2014, Can Karaca *et al*., 2011). The precipitated protein is then recovered through centrifugation or filtration methods. Protein recoveries of 84 and 87% have been reported with salt extraction, for bean protein (Marquez *et al*., 1996) and chickpea (Paderes–Lopez *et al*., 1991), respectively. Moreover, Can Karaca *et al*., (2011) illustrated that salt extracted soy protein isolates had superior functional properties i.e. emulsification and creaming stability than isoelectric precipitated isolates. Stone *et al*., (2015) also demonstrated that salt-dialysis extracted pea protein isolate had superior functional properties compared to alkaline-precipitation extracts.

### 2.8.5. **Ultrafiltration**

The protein recovery process is vital to isolate and concentrate proteins from the liquid fraction to an isolate with minimal (<10%) anti-nutritional components and impurities such as ash, sugars, phytic acids, and fatty acids (Singh *et al*., 2008). Ultrafiltration has been reported to be efficient in protein recovery and reduction of anti-nutritional factors. (Lai *et a*l., 2013; Taherian *et al*., 2011). Boye *et al*., (2010) reported that yellow pea protein recovered through ultrafiltration had minimal phytic acid content and excellent physicochemical properties. Moreover, a yield of 57% was achieved compared to a yield of 55% through isoelectric precipitation. Lai *et al*., (2013) reported a protein recovery yield of 90.3% with ultrafiltration at pH 9. Moreover, this resulted in a lower ash and phosphate content in the recovered protein isolate. Lai *et al*., (2013), reported a protein recovery yield of 97.75% with ultrafiltration at pH 7.5.

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### 2.8.6. Protein extraction from tubers

Protein extraction from tubers is not well published however, the knowledge of the protein-type composition of the tubers may be used to determine the potential extraction process. JA tubers are primarily composed of globulin and albumin type proteins (Johannson *et al*., 2015). Albumin is water soluble while globulin salt soluble. Moreover, albumins and globulins are also the primary proteins in soy and pea proteins (Boye et al., 2010). Therefore, protein extraction processes such as alkaline and salt extraction may be considered as potential methods for protein extraction from JA tubers.

Protein extraction from potato juice is a common waste valorisation option in the potato processing industry. Extraction of starch is the primary processing option, and this is preceded by the rasping of the potato and separation of the fruit pulp into fibres, starch slurry and protein rich juice (Bartova and Barta, 2009). The protein is subsequently precipitated from the juice with ethanol, ferric chloride (Bartova and Barta, 2009), sulphuric acid (Waglay et al., 2014), chromatograph or filtration methods (Lokra et al., 2019).

## 2.9. Challenges of sequential protein and inulin extraction

The co-production and implications of integrating inulin and protein extraction from JA tuber are not well documented in literature; however, the potential reductions in yields, purity and quality of inulin and protein are inevitable during sequential extraction. Therefore, the potential impacts of integrating the extraction processes for inulin and protein on both the yield and quality of the extracts will be discussed.

In summary, the data from literature showed an overlap in the temperature for inulin and protein solubility, consequently demonstrating the potential of poor selectivity in water extraction. Therefore, a non-solvent approach, based on the pressing of the tuber pulp can offer an alternative to selectively isolate one of the products from the press juice and the other from the solid residue. Moreover, the impacts of solids loading on the solubility of protein and inulin from JA tubers are not well documented. Thus, differential solubility and selective extraction based on solids loading is another alternative approach which is applicable to water extraction.

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An insignificant inulin degradation and subsequently yield loss could be experienced during sequential extraction if protein extraction is performed at room temperature prior to inulin, since protein solubilisation is performed at alkaline (pH>7.5) conditions (Lai et al., 2013; Baborsa *et al*., 2007). Inulin and FOS are prone to hydrolysis under acidic conditions (pH <4) and high temperatures (50 $^oC$). Furthermore, the rate and degree of hydrolysis increase as the pH decreases and temperature increases (Matusek *et al*., 2009; Homme *et al*., 2003; Glibowski and Bukowska, 2011). Matusek *et al*., (2009) reported that the hydrolysis of FOS was insignificant at pH 2.7 and 3.3 at a temperature of 60 $^oC$. FOS hydrolysis was, nonetheless, reported to be significant between 70-80 $^oC$ and to rapidly increase as temperature increased beyond 90 $^oC$. The experimental data from both Homme *et al*., (2003) and Glibowski and Bukowska (2011) showed no inulin and FOS hydrolysis at pH 9 and temperature range of 80-120$^oC$.Homme *et al*., (2003) reported that no sucrose and FOS hydrolysis was observed even after 24 hours of exposure at pH 9 and temperatures between 80-120 $^oC$.

Lim *et al*., (1999) reported that increasing the temperature of extraction and molarity of sodium hydroxide (NaOH) improved the efficiency of protein removal from rice starch. The study showed that increasing the extraction temperature from 20 to 40 $^oC$ further reduced the protein content in starch by approximately 1% point (~25% reduction). The change in temperature and concentration of NaOH also resulted in an increase in the amount of soluble starch loss. Increasing the temperature of extraction from 25-45 $^oC$ resulted in an increase in starch loss from 1.42% to 2.94%. The starch loss was 1.38% when the protein extraction was done with 0.2% NaOH at 20 $^oC$ compared to 1.08% with 0.1% NaOH.

The literature data illustrates an uncertainty in the exact effects of sequential inulin and protein extraction on the extent of the yield loss. However, the data shows that some level of co-extraction may be experienced despite the sequence of extraction. Severe inulin co-extraction will be experienced if the protein is extracted first and at temperatures above room temperature. Protein extraction at temperatures around 50 $^oC$ prior to inulin is likely to result in the loss of short chain (DP 2-10) inulin oligomers because of the differential solubility of inulin. Wada *et al*., (2005) reported that the solubility of native inulin ($DP_{av}$ 12, DP 2-60) was higher than that of HP-inulin (DPav 25, DP 10-60) in the temperature range of 25-70 $^oC$ which formed the scope of the study. The solubility of FOS and HP-inulin, at 50 $^oC$ was reported to

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be 22 % and 3 %, respectively. Furthermore, Kim *et al*., (2001) reported a solubility of 1.2 % for HP-inulin at the same temperature.

Water soluble proteins constitute a significant portion of impurities from inulin extraction (Liu *et al.*, 2012; Zhi-fu *et al*., 2009). The proteins have been reported to possess temperature dependent solubility (Gujska and Khan, 1991; Christopher *et al*., 1998; Rashid *et al*., 2014). Moreover, proteins are known to denature as the temperature increases beyond their stability range. Pelegrine and Gasparetto, (2005) reported that the solubility of whey proteins at pH 6.8 decreased with increasing temperature, thus indicating thermal denaturation. The solubility dropped from 87 g/100 g to 67 g/100g protein when the temperature was increased from 40 to 60 $^{o}$C. These results demonstrate that the process of sequential extraction may result in loss of protein yields and physicochemical properties as a results of inulin extraction prior to protein extraction.

## 2.10. The importance of inulinases in JA based biorefining

### 2.10.1. Overview of inulinases

Inulin hydrolysis into a wider range of products, could play a significant role in enhancing the economic value of a JA tuber-based biorefinery (Li *et al*., 2013; Long *et al*., 2016). Although acid hydrolysis of inulin is an efficient and cost-effective method, it is undesirable due to Maillard reactions, between inulin and protein components, that cause pigmentation, difficult to separate and results in microbial inhibitors (Qui *et al*., 2018). Enzyme hydrolysis is thus a better alternative, which fits well within green production technology and biorefining (Herrero and Ibanez, 2015). Furthermore, enzyme hydrolysis can be potentially integrated with extraction or fermentation thereby enhancing the productivity and feasibility of a JA biorefinery (Qui *et al*., 2018).

Inulinases are a furan fructose hydrolase type of enzymes that act on the $\beta(2 \rightarrow 1)$ linkages of the inulin (Franck, 2002). Inulinases are divided into two groups, exoinulinases (E.C.3.8.1.80) and endoinulinases (E.C.3.2.1.7), and distinguished by their differential hydrolysis (Figure 2.2) of the $\beta(2 \rightarrow 1)$ linkages (Singh and Chauhan, 2018). Exoinulinases act by cleaving a fructose monomer from the non-reducing end of inulin, while endoinulinase randomly cleaves the inulin $\beta(2 \rightarrow 1)$ linkages to produce inulooligosaccharides (Singh and Chauhan, 2018).

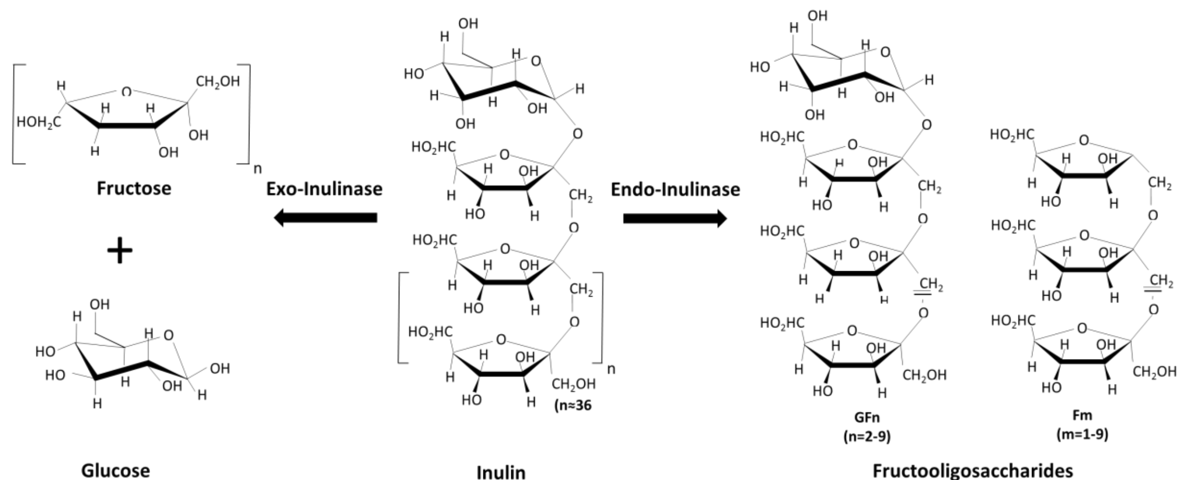


Figure 2.2: Differential hydrolysis of inulin by exoinulinase and endoinulinase into fructose and fructooligosacharides, respectively (Redrawn according to Li *et al*.,2015)

### 2.10.2. Endoinulinases native sources and production

Endoinulinases naturally exist in plants, fungi, yeast and bacteria (Li *et al*., 2012; Singh *et al*., 2013; Sing and Chauhan 2017). Microorganisms are the best sources for commercial endoinulinase production (Leelaram *et al*., 2016), because of their ease for large scale production, however, the low enzyme productivity from native strains is a major disadvantage (Table 5). In addition, the production of non-homogeneous enzymes from native host is a major challenge due to co-expression and secretion of other native enzymes (Rose and van Zyl, 2008). High enzyme volumetric activity and homogeneity are important to enable cost effective and efficient downstream processing in commercial production (Cheng *et al*., 2002).

Singh *et al*., (2013) isolated an endoinulinase producing *Bacillus safensis* from *Asparagus* sp. root tubers, with an activity of 12.56 U/ml after a 20-hour shake flask cultivation at 37$^{o}$C. An endoinulinase producing *Aspergillus tritici* isolated from *Asparagus* sp. root tubers showed an activity of 23.01 U/ml after a 70-hour cultivation in a shake flask (Singh et al. 2016). Li *et al*., (2002) reported an endoinulinase activity of 16.70 U/ml from a native strain of *Yarrowia lipolytica*. In contrast recombinant strains produced enzyme with endoinulinases volumetric activity higher than the native strains (Table 2.6).

Recombinant DNA technology is a commonly used method for heterologous protein expression and yield improvement in enzyme production from microorganisms (Rose and van Zyl, 2008; Alriksson *et al*., 2009; Leelaram *et al*., 2016). Moreover, heterologous protein expression offers a method for enzyme production with improved homogeneity (Rose and van Zyl, 2008). The activity in recombinant strains of 20 to 200-fold higher than the native

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strains (Table 2.6), demonstrates the potential of recombinant strains for high level expression and secretion of industrially important enzymes. Furthermore, the volumetric activities were generally higher for bioreactor cultivation, in comparison to shake flask cultivation, thereby illustrating the potential of upscaling the production of recombinant endoinulinases without compromising the yields.

Table 2.6: Comparison of inulinase activity between native and recombinant host

| **Organism** | **Enzyme** | **Enzyme Activity (U/ml)** | **Mode** | **Reference** |
|---|---|---|---|---|
| *Y. lipolytica* | endo-inulinase | 16 | shake flask | *Li et al*. 2012 |
| *B. safensis* | endo-inulinase | 12 | shake flask | Singh *et al*. 2013 |
| *Pichia pastoris** | inulinase | 286 | bioreactor | Zhang *et al*. 2009 |
| *P. pastoris** | inulinase | 1222 | shake flask | Zhang *et al*. 2012 |
| *K. marxianus* | inulinase | 208 | shake flask | Silva-Santisteban *et al*., 2009 |
| *P. Pastoris** | endo-inulinase | 3860 | bioreactor | Chen *et al*. 2015 |
| *Penicilliun oxalicum* | inulinase | 38 | shake flask | Sing and Chauhan 2017 |
| *B. safensis* | endo-inulinase | 28 | shake flask | Singh and Singh 2014 |
| *P. pastoris** | endo-inulinase | 1650 | bioreactor | Yang *et al*. 2016 |
| *Xanthomonas sp.* | endo-inulinase | 20 | shake flask | Park and Yun 2001 |

*Recombinant host

### 2.10.3. *A. niger* in the production of recombinant enzymes

Filamentous fungi, *Aspergillus* sp., is widely used as a host for industrial production of recombinant protein. *Aspergillus* sp. has rapid growth, high production and secretion capacity, as well the ability to perform post translational modifications (Rose and van Zyl, 2008; Krull et al, 2008). The expression of inulinase from the native host is induced by inulin and sucrose and repressed by glucose (Liu et al., 2013). Thus, expressing endoinulinase from *A. niger* under a glucose induced promoter will ensure sufficient homogenous production of the target protein (Rose and van Zyl, 2008; Alriksson *et al*., 2009).

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Moreover, the use of A. niger for the recombinant production of endoinulase, which has potential application inulin hydrolysis in the food and pharmaceutical industry (Fanck, 2002, Singh and Chauhan, 2018, will be advantageous due to the species' GRAS (Generally Regarded As Safe) status and thereby (Krull *et al*., 2013).

High volumetric enzyme activities can also be achieved through high biomass density cultures in submerged fermentation (Cheng *et al.,* 2002; Xiong *et al*., 2007). In addition, overcoming viscosity challenges in high density fermentation of *Aspergillus* is crucial for high biomass yield and volumetric concentration of the recombinant protein (Lopez *et al*., 2005; Driouch *et al*., 2010).

Poor mixing due to high viscosity consequently results in inefficient mass transfer, nutrient, and oxygen diffusion thereby, limiting the productivity (Papagianni and Mattey, 2006; Karahalil 2017; Singh *et al*., 2018). *A. niger* has been reported to change from pellet to hyphal morphology to adapt to low oxygen conditions associated with high-cell density fermentation (James *et al.,* 2007; Papagianni and Mattey, 2006). In addition, high agitation is necessary for high cell density cultures, to ensure efficient mixing and consequently efficient oxygen and nutrient diffusion. However, this results in severe shearing forces, which may cause fungal fragmentation. Lopez *et al*., (2005) demonstrated that agitation speeds of > 300 rpm caused excessive shearing and fungal fragmentation and subsequently growth as well as enzyme production cessation. Therefore, the morphological change is due to a combination of factors including low dissolved oxygen in the culture, the resulting shear forces from high biomass concentration collision and high agitation speed.

The different culture morphology of *Aspergillus sp* have in some instances shown to have different impacts on both fungal growth and recombinant enzyme production (Table 2.7), thus affecting the enzyme yield and productivity of recombinant systems (Porcel *et al.,* 2007; Haack *et al*., 2007; Haq *et al*., 2015). Moreover, the mechanism through which the impacts are exerted, differ by strain and type of recombinant protein. Sporh and co-workers (1999) reported that the reduction in amylase production by *A. oryzae*, following a morphology change from pellet to mycelial, was due to a significant reduction of actively growing tips when the fungi grew in mycelial form.

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Haack and co-workers (2006) reported that *A. oryzae* growth and lipase production were inhibited by low oxygen availability because of increased biomass concentration, morphology change from pellet to mycelium, during the feeding phase of an exponential fed-batch culture. Lopez and co-workers (2005) reported that *A. terruus* grew in large fluffy pellets morphology at DO levels of 80% saturation and agitation speeds less than 300 rpm. *A terruus* however, grew in small pellet at agitation speeds > 300, and this resulted in significant lovastatin productivity reduction (Lopez *et al*., 2005).

In contrast, phytase production from *A. ficuum* (Coban *et al*., 2015) and fructofuranosidases production from *A. niger* (Driouch *et al*., 2012) were enhanced by fungal growth in small pellets. Driouch *et al*., (2012) further illustrated that the total biomass obtained when the fungi was growing in large pellets was not significantly different to the biomass when fungi was growing in small pellets. Thus, the enhanced enzyme productivity was attributed to efficient aeration and nutrient transfer in small pellets, with larger pellets having limited oxygen and nutrient transfer to the inner parts of the pellet (Driouch *et al*., 2010 and Driouch *et al*., 2012).

Table 2.7: The impact of *A. niger* growth morphology on recombinant protein production

| **Fungal Strain** | **Enzyme/Protein** | **Morphology change** | **Impact on enzyme productivity** | **Reference** |
|---|---|---|---|---|
| A. oryzae | Amylase | Pellet to mycelia | Reduction | Sporh et al., 1999 |
| A. terruus | Lovastatin | Large to small pellets | Reduction | Lopez et al., (2005) |
| A. ficuum | Phytase | Large to small pellets | Enhanced | Coban et al., 2015 |
| A. niger | Fructofuranosidases | Large to small pellets | Enhanced | Driouch et al., 2012 |
| A.oryzae | Lipase | Pellets to mycelia | Inhibited | Haack et al., (2006) |

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### 2.10.4. Mechanism of overcoming limitations of high-cell density culture of *Aspergillus*

Submerged high biomass density culture is an industrially suitable strategy for high enzyme productivity in *Aspergillus* sp. and recombinant protein production. However, the viscosity limitations, associated with high cell density, severely impacts the mixing, oxygen, and nutrient distribution efficiency. Therefore, this results in low productivity than the maximum attainable.

Morphology engineering, through the manipulation of cultivation conditions such as inoculum concentration, pH, dissolved oxygen concentration and agitation, promotes fungal growthin pellet morphology, and thus has the potential to minimise problems associated with high cell density fermentation (Karahalil 2017; Singh *et al*., 2018). A culture pH of 5 has been reported to enhance pellet morphology for *Aspergillus niger* in submerged fermentation (James et al., 2007). Moreover, the use a pre-culture inoculum is better in promoting pellet morphology than the use of a spore inoculum (James *et al*., 2007; Papagianni and Mattey, 2006).

The feed concentration is an important factor in reducing broth viscosity through controlling the biomass concentration during fed batch fermentation (Bhargava *et al*., 2003; Lopez *et al*., 2005). The reduced viscosity is necessary for efficient mixing, oxygen, and nutrients diffusion (Bhargava *et al*., 2004; Driouch *et al*., 2010). The feed concentration should be enough to reduce the viscosity without compromising the volumetric productivity of the culture. Thus, the use of a dilute feed, to control viscosity, should be avoided due the effect of culture dilution and consequently limiting the attainment of high biomass concentration and enzyme yield.

The composition and relative ratios of the carbon and nitrogen source is also an important determinant of the fungal morphology for submerged *A. niger* culture (Kumar *et al.,* 2006). The composition of the nutrients should be selected considering the nature of the host and subsequently optimised to enable the highest possible enzyme yield. Lopez and co-workers (2003) reported the best productivity of lovastatin in *A. terreus* when a slowly metabolised carbon source was used under limiting nitrogen conditions. Furthermore, the pellet growth morphology was maintained under the conditions and this was ideal for optimal lovastatin production. The use of highly metabolizable nutrients results in rapid growth in filamentous

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morphology, which results in oxygen transfer limitations, and subsequently limits the achievement of high biomass yields and protein synthesis (Kumar *et al*., 2003).

## 2.9. Integration of ethanol production with protein and inulin production using JA tubers

### 2.9.1. Overview

The continued reliance on fossil fuels is unsustainable due to the increased emission of GHGs, depletion of resources and price increases (EIA, 2018a; Woods *et al*., 2010). The International Energy Agency reported that the 4% increase in renewable energy production and use (EIA 2018b) played a major role in reducing $CO_2$ emission, which could have been 50% more than currently reported (EIA 2018a). Ethanol is currently the most widely used biofuel, with 90% of its production dedicated for energy consumption (AGMRC, 2017).

A wide range of biomass feedstock are available for ethanol production, however, there still exist sustainability and technical challenges related to their use (Yuan *et al*., 2011; Thompson 2012; Favaro *et al*., 2017). The use of grain crops for bioethanol production is a threat to food security due to competition for arable land (Tenenbaum, 2008; Thompson, 2012). Although agricultural and forestry waste are cheap, abundant, and a potential feedstock for ethanol, LCFs based biomass still have major challenges due their recalcitrant nature. Thus, the exorbitant cost of pre-treatment and large enzyme dosage requirement could ultimately offset the low biomass cost (Favaro *et al*., 2017).

JA tubers on the other hand are composed of inulin, a fructose polymer that is relatively soluble in water (Yanovsky and Kingsbury, 1933; Naskar *et al*., 2010), and therefore do not need expensive pre-treatment before fermentation (Yuan *et a*l., 2011). JA is a drought and salt stress tolerant crop and requires minimal cultivation inputs such as the use of inorganic fertiliser and pesticides, which are environmental-unfriendly (Zhang *et al*., 2011; Gunnarson *et al*., 2014, Long *et al*., 2014). In addition, the limited or no use of synthetic fertilisers and pesticides contributes to minimising the emission of GHGs and contamination of the underground water table (Long *et al*., 2014). Therefore, an integrated approach, involving protein and inulin extraction for use as food products or the protein as animal feed, and subsequently fermenting the extraction residues into ethanol, for the use of JA tubers as biorefinery feedstock may not result in competition for arable land between food and fuel production.

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### 2.9.2. Fermentation of tubers for ethanol production

Simultaneous saccharification and fermentation (SSF) is commonly applied in the industry for ethanol production from starch and LCF-based biomass (Olofsson *et al*., 2008; Yuan *et al*., 2011) and has the potential to make ethanol production from JA tubers economically feasible (Kadar *et al*., 2004). Furthermore, high solid loading fermentation is important in improving the productivity of SFF by increasing the concentration of ethanol, which subsequently reduces the distillation cost during downstream processing (Puligundla *et al*., 2011, Liu *et al*., 2012, Wang *et al*., 2013).

However, high solid loading fermentation of tubers is limited by their high viscosity (Srichuwong *et al.,* 2009). High viscosity results in poor mass transfer and consequently incomplete hydrolysis as well as low ethanol yields (Srichuwong *et al*., 2009, Lim *et al.,* 2013, Zhang *et al*., 2011). Moreover, high solid loading increases the external osmotic pressure thereby reducing cell viability and growth of the yeast and in turn fermentation productivity (Liu *et al*., 2012, Wang *et al*., 2013).

Fed batch fermentation is a process option that helps to alleviate mass transfer limitations associated with high solids loading fermentation (Hoyer *et al*., 2010; Huang *et al* 2011). The gradual addition of the substrate thereby reduces broth viscosity and enables sufficient mixing for optimal fermentation productivity (Hoyer *et al*., 2010). Furthermore, cell wall degrading enzymes such as cellulase, xylanase and pectinase have been reported to reduce the viscosity of tubers such as cassava and potato (Shrichuwong *et al*., 2009; Yingling *et al*., 2011). The enzymes reduce the viscosity through disruption of the cell wall matrix that traps water, thereby decreasing the viscosity of tuber residues during fermentation (Poonsrisawat *et al*., 2011).

Zhang and co-workers (2010) demonstrated that the use of xylanase significantly reduced the viscosity of sweet potato mash, resulting in an ethanol yield and theoretical yield equivalent of 135.1 g/kg and 90.7%, respectively. Lim and co-workers (2013) achieved 14.92% (v/v) ethanol and 91.0% of theoretical yield with the use of Viscozyme® in the high gravity fermentation of potato mash. The use of amylase has also been reported to enhance high solids fermentation of cassava mash at solids loading of 40% (w/v) (Yingling *et al*., 2011).

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### 2.9.3. Enzymatic hydrolysis of JA tubers and synergism of cocktail enzymes

The successful use of JA tubers as a feedstock for ethanol production is dependent on the efficient hydrolysis of the complex polymers into fermentable sugars (Goncalves *et al*., 2015). The primary source of sugar present in JA tubers is in the form of inulin, a fructose polymer that is relatively soluble in water (Yanovsky and Kingsbury, 1933; Naskar *et al*., 2010) and does not require expensive pre-treatment before hydrolysis and fermentation (Yuan *et al*., 2011). The tuber residues from the extraction of inulin and protein, are however, enriched with lignocellulosic fibres. Therefore, the tuber residue will require a cocktail of inulin and lignocellulosic hydrolysing enzymes to effectively hydrolyse the sugar polymers for fermentation.

Enzymatic hydrolysis is also important in the disruption of cell wall components (Yingling *et al.,* 2010) of tubers such as pectin (Lim et al., 2013). Pectin is a well-documented contributor of viscosity during tuber fermentation (Zhang *et al*., 2010; Lim *et al*., 2013). Thereby the inclusion of an accessory pectin degrading enzyme may enhance the hydrolysis efficiency of JA tubers and fermentation productivity of the high solids SSF of the tuber residues.

Enzyme cocktails work in synergy and this synergy can be reciprocal where the effect of one enzyme liberates the substrate for the other and vice versa (Hunag *et al*., 2012; Berlin *et al*., 2006). Accessory enzymes also contribute to the synergy even when they are indirectly involved in the hydrolysis of sugar polymers to fermentable sugars (Sun *et al*., 2015). In addition, synergism depends on the molar ratio, the density and crystallinity of the interacting molecules as well as degree of polymerisation of the sugars (Hu *et al*., 2011; Hu *et al*., 2014).

Liu and co-workers (2016) reported that reciprocal synergy existed between endo- and exoinulinases in the hydrolysis of inulin. The study demonstrated that endoinulinases was important in disruption of the supramolecular structure due the enzyme's ability to randomly cleave inulin into oligomers of low DP. Inulin cleavage into oligomers subsequently makes it more soluble and accessible to exoinulinases hydrolysis into fermentable monomers. Consequently, the cleavage of these oligomers by exoinulinases is important in reversing endoinulinases inhibition by the oligomers.

The addition of pectinase has been reported to enhance the hydrolysis of cellulose, in lignocellulosic biomass, through the hydrolysis and disruption of pectin coating cellulose

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fibres to liberate cellulose for its respective enzyme (Hu *et al*., 2011). The enzymes lytic polysaccharide monooxygenases have been demonstrated to significantly improve the efficiency of cellulase, through the disruption of the cellulose crystalline structure and subsequently improving cellulose solubility (Hu *et al*., 2014; Jung *et al*., 2015).

Therefore, to achieve sufficient biomass hydrolysis with low enzyme dosage, the composition of the biomass and the synergistic mechanisms of the enzymes should be considered in the formulation and optimisation of enzyme cocktails.

### 2.11. Conclusions from literature

The steady growth in the global nutraceutical and functional food market (Frost and Sullivan, 2011) is attributed to increasing consumer health concerns such as diabetes, obesity, and atherosclerosis; nutraceuticals' perceived health as well as nutritious benefits (Bech-Larsen and Grunert, 2003; Urala and Lahteenmaki, 2007). Moreover, there is also a relationship between the demand for plant-derived nutraceuticals and protein supplement, and productive land because these products are derived from crops such as JA, agave, and chicory, potato, and peas. The increase in agricultural activities will consequently increase the energy demand and overreliance on fossil fuels, thereby further complicating measures to deal with global warming. This increase in nutraceuticals, protein and energy demand will ultimately put pressure on resources; therefore, there is a need to prospect for new feedstocks for nutraceutical ingredients, protein and bioenergy and ways to exploit them sustainably while mitigating the dire effects of global warming.

Currently there is a wide range of literature-based data on the use of Jerusalem artichoke tuber as a feedstock for ethanol, fructooligosaccharides and fructose production from inulin (Chi *et al.*, 2011; Yang *et al*., 2015). The tuber also contains protein with a significant proportion of essential amino acids (Cieslik *et al*., 2011). In addition, a biorefinery approach to the utilisation of biomass feedstock has been reported to improve the sustainability of commercial exploitation of crops for food, animal feed, biofuels, and chemicals production (Kachrimanidou *et al*., 2015; Xiang and Runge, 2014). Therefore, the co-production of inulin and protein from the tuber may potentially improve the sustainability of processes involving the former. Moreover, the extraction process of inulin, from the tuber, results in yields less than the theoretical maximum yield from the raw material (Tewari *et al*., 2015; Bekers *et al*., 2008; Lingyun *et al*., 2007). Therefore, the residual inulin and insoluble LCFs are a potential

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source of fermentable sugars for ethanol production. Valorisation of the inulin and protein extraction waste residues may further improve the sustainability of the biorefinery because of the co-production of food, feed, and fuel products.

This multiproduct biorefinery approach has the potential to enhance the viability of a JA-based biorefinery, however, yield and quality loss of the products are inevitable, due to the integration of multistep processes. Furthermore, the process conditions in each processing step will affect the quality of the target product and simultaneously influence the accessibility as well quality of the retained product, which is to be extracted in the subsequent step.

The purpose of this study was to optimize and integrate processes to sequentially extract protein and inulin with potential use as a nutritional supplement and nutraceutical, respectively. The desirability function, which enables multi-factor optimisation, was used to develop an optimized biorefinery scenario with specific attention on the yields and functional properties. Furthermore, the optimal biorefinery scenario was achieved with a minimal number of process steps. The $DP_{av}$ and functional properties were used as the performance indicators to determine the quality of inulin-based products and proteins, respectively.

Inulin hydrolysis has an important role in expanding the economic viability of inulin-based biorefining. Therefore, endoinulinases is an important enzyme in enhancing the feasibility of a Jerusalem artichoke based biorefinery involving inulin extraction and processing.

Filamentous fungi, *Aspergillus* sp. is widely used as recombinant host for high-level and homogenous industrial enzyme production (Alriksson *et al*., 2009; Leelaram *et a*l., 2016), due to their rapid growth, high productivity, efficient secretion, as well post translational modifications (Rose and van Zyl, 2008; Krull *et al*, 2008). Moreover, *Aspergillus* sp. has the GRAS status and thereby has a potential application for enzyme production for use in the food and pharmaceutical industries (Krull *et al*., 2013). The interaction between the strain and recombinant gene expressed is an important factor in determining the biomass growth and protein expression productivity in *Aspergillus sp* (Haack *et al*., 2006). Moreover, overcoming viscosity challenges in high cell density fermentation of *Aspergillus* is crucial in the development of *A. niger* as recombinant host, to enable achievement of a high biomass yield and enzyme volumetric concentration of the recombinant protein (Lopez *et al*., 2005; Driouch *et al*., 2010). Therefore, the potential of a recombinant *A. niger* strain as a host for

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endoinulinase production, with a glucose limited fed batch exponential fermentation strategy, was investigated. The relationship between culture viscosity, growth rate, biomass growth and morphology as well as enzyme production during high cell density fermentation of recombinant *A. niger* was investigated.

Protein and inulin extraction from the tubers result in an increase in the proportion of lignocellulosic fibres (LCFs) relative to the residual inulin in the extraction residue. LCFs result in stearic hindrance and unproductive enzyme bindings. Therefore, the design and use of appropriate enzyme cocktails with the ability to disrupt the complex interactions among the different sugar polymers of the tuber waste residues could enhance the fermentation productivity during ethanol production. Moreover, fed-batch fermentation is an industrially relevant method used to attain high ethanol concentration to reduce downstream distillation cost. However, high viscosity is one of the major challenges for tuber fermentation. Therefore, this study explored the potential of fed-batch fermentation in combination with a cocktail of hydrolytic enzymes in overcoming the viscosity limitation during high solid simultaneous saccharification and fermentation of the tuber extraction residues.

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# 3. CHAPTER THREE: STUDY AIM AND OBJECTIVES

The purpose of the study was to investigate and optimise the technical feasibility of JA-based multiproduct biorefinery for the co-production of protein, inulin, and ethanol. The approach for the development of this conceptual biorefinery involved (I) integration of a protein extraction step into the conventional hot-water extraction of inulin, (II) evaluating the potential of recombinant *A. niger* strain as host for the production of endoinulinases and (III) optimisation of an enzyme cocktail for the hydrolysis and fermentation of the protein and inulin extraction residues. This biorefinery approach has the potential to minimise waste production by incorporating protein extraction to the inulin extraction process and subsequently valorising the extraction residues. The use of non-solvent protein extraction may be an important step towards minimising water usage in the sequential extraction step. Moreover, biorefining enables the development of a multi-product approach with a potential to enhance the feasibility of using JA tubers. This section aims to highlight the novel aspects addressed in each of the three main objectives of this thesis.

## 3.1. Integration of protein extraction into the conventional inulin extraction process from JA tubers

A multi-product approach for the production of protein and inulin from JA tubers has the potential to improve the economic benefits of using JA tubers. Furthermore, it mayminimise the waste production associated with the conventional single product use of either inulin extraction or inulin fermentation. Solvent (water) extraction of inulin and protein is a widely adopted and well-developed technology and applicable to a range of feedstocks. However, the overlap in the solubility temperature of inulin and protein will inevitably compromise the selectivity and quality of the extracts. In contrast, previous studies have shown an inverse relationship between inulin yields and solids loading while the extraction of protein is often done at solids loading twice (ca. 20% w/v) as high compared to those for inulin extraction. Moreover, protein extraction from potato juice is a well-established technology in the starch processing industry. Therefore, current contribution demonstrates the technical feasibility of an integrated approach for the co-production of protein and inulin from JA tubers. A three-step sequential extraction was followed for the sequential extraction of protein and inulin from the tuber juice and resulting solids residues. To achieve selective inulin and protein extraction from the solid

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residues, the extraction conditions were optimised relative to the temperature, pH and solids loading to enable selective extraction in each respective step of the sequential process. In addition, the functional properties of the protein were evaluated to determine the impacts of the process sequence and parameters on these properties. The functional properties investigated were emulsification, solubility, foaming and water retention. Finally, the mass balance of the sequential extraction was important to determine the efficiency of the proposed three-step process and the relative composition of residual inulin and LCFs. The latter was important in determining the potential of valorising the tuber residues into ethanol.

The aim of this study was to explore the technical feasibility of the sequential extraction of inulin and protein from Jerusalem artichoke tubers and understand the interrelationships between process conditions and product functional properties, with the following objectives:

- To determine the optimum parameter values for the sequential extraction of protein and inulin from JA tubers
- To use functional properties analysis to identify as well as understand interrelationships between processing sequence or conditions and product properties
- To perform a mass balance to determine the efficiency of the sequential extraction process

## 3.2. The potential of recombinant *A. niger* strain as host for endoinulinases production

Endoinulinases is an inulinases enzyme used in the partial hydrolysis of inulin into fructooligosaccharides. Thus, this enzyme willplay an important role in the expansion of the product portfolio and potentially maximise the economic value of a JA biorefinery. Although *A. niger* is suitable for high cell densities cultivation and large-scale production of recombinant proteins, most of the previous studies on recombinant endoinulinases production have been on the use of yeast and bacterial systems. Viscosity also remains a challenge for high cell density culture, and the resulting mixing inefficiency have a negative impact on the productivity. Controlling the fungal morphology is one of the mechanisms to minimise the broth viscosity and this can be achieved through bioprocess

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conditions optimisation. Nutrient composition, DO levels, agitation, pH, and the growth rate have an impact on both the morphology and productivity of recombinant *A. niger* systems. Moreover, the impact varies depending on the type of enzyme expressed and control of expression. This contribution was important to gain insight on the factors influencing and challenges related to recombinant endoinulinases production from *A. niger* during high cell density fermentation. The effects of the bioprocess parameters, glucose feed concentration, growth rate and the concentration of the nitrogen sources, on biomass growth, fungal morphology, and enzyme production in fed-batch culture were investigated under high cell density fermentation.

The principal aim was therefore, to gain insight on the factors influencing and challenges related to recombinant endoinulinases (InuA) production from *A. niger* strain during high cell density fermentation with the following objectives:

- To determine the effects of glucose feed concentration on DO levels, and mixing as well as subsequent impact on fungal morphology, biomass growth, and enzyme production
- To determine the effects of nitrogen sources concentration on fungal morphology, biomass yield and enzyme production
- To establish the effects of growth rate on biomass growth and enzyme production as well as yields and productivities

### 3.3. Optimisation of enzyme cocktails for the hydrolysis and fermentation of the protein and inulin extraction residues

A biorefinery approach, involving the extraction of protein and inulin from JA tubers, and subsequently producing ethanol from the extraction residues has the potential to enhance the sustainability of the latter and subsequently improve the economic benefits derived from JA. The presence of pectin in the cell-wall of the tubers is a major contributor to the viscosity challenge associated with tuber fermentation. Moreover, the tuber residues are enriched in LCFs thereby adding another layer of complexity to the fermentation process. Therefore, in this contribution, an optimised cocktail of inulin, LCFs and pectin degrading enzymes was applied to harness the synergistic benefits for enhanced hydrolysis and fermentation of the tuber residues. The study investigated the

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efficiency of using an optimised enzyme cocktail for the high-gravity simultaneous saccharification and fermentation (SSF) of tuber residues and the impacts of changing the solids loading on the synergistic interactions.

The principal aim was to valorise the protein and inulin extraction residue of the tubers, through fed-batch SSF into ethanol, with the use of an optimised enzyme cocktail of endoinulinases, exoinulinases, pectinases and cellulases to improve the fermentation productivity of high solids fermentation, with the following objectives:

- To optimise the proportions of lab produced (endoinulinase, exoinulinase) and commercial (Cellic® CTec3 and Pectinex Ultra-SP) enzymes in a cocktail, to minimise the enzyme dosage and maximise the enzyme synergies as well as the hydrolysis performance
- To determine the ethanol yield and productivity of the optimised cocktail in the fermentation of JA tuber residues
- To determine the effects of changing solids loading on the optimum activities and synergy of the cocktail enzymes

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# CHAPTER 4

## 4. Sequential extraction of protein and inulin from the tubers of Jerusalem artichoke (*Helianthus tuberosus* L.)

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Published research paper

Title: Sequential extraction of protein and inulin from the tubers of Jerusalem artichoke (*Helianthus tuberosus* L.)

Journal: Journal of food science and technology

Summary

The current contribution explored the technical feasibility of the sequential extraction of protein and inulin from the tubers of Jerusalem artichoke. The objective was to determine the best parameter values for the sequential extraction of protein and inulin from JA tubers, and use functional properties analysis to identify as well as understand interrelationships between processing sequence or conditions and product properties. The experimental data demonstrated that the best extraction method and sequence involved the pressing of the tuber mash to obtain a protein rich juice with a 52% protein yield. This was followed by two-step water extraction process involving the extraction of protein at room temperature and hot water extraction of inulin, in the first and second extraction step, respectively. The juice and water extraction resulted in an overall 72% protein yield. An inulin yield of 67% was achieved and this was higher than 57% obtained through the conventional hot-water extraction method. The pressing and water extraction step for protein extraction, preceding inulin extraction, used in this study could have resulted in improving the porosity of the tuber material and improved water diffusion and inulin solubility. Protein extraction was performed at 15% w/v solids loading from which inulin solubility was minimal, thereby illustrating that the mass transfer differentials enhanced protein selectivity in sequential extraction. The solubility, emulsification, foaming and water retention properties of the juice-derived proteins were significantly different ($p$-values > 0.05) from the water extracted protein thereby demonstrating that the slightly acidic (pH 5) conditions used in the water extraction may have affected the protein quality. In addition, the inulin extract displayed a degree of

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polymerisation comparable to the inulin obtained using the conventional method. The preceding protein extraction step therefore did not have an impact on the properties of the inulin. A sequential extraction approach to inulin from the tubers, preceded by a two-step protein extraction, was thereby adequate to extract protein and inulin with acceptable functional properties comparable to those of single product extraction from JA tubers and other biomass substrates. The mass balance data further confirmed the accuracy of the approach and illustrated that the tuber residues were enriched in residual inulin (38%) and LCFs (25%) which were subsequently fermented into ethanol (chapter 6) using an optimised cocktail of lab-produced (chapter 5) and commercial enzymes.

Candidate declaration

The nature and scope of my contributions for chapter 4, pages 41-65, were as follows;

| **Name of contribution** | **Extent of contribution (%)** |
|---|---|
| Experimental Planning | 70 |
| Executing experiments | 100 |
| Interpretation of the data | 80 |
| Chapter compilation | 100 |

The contributions of the co-authors to chapter 4, pages 41-65, are the following;

| **Co-author** | **email address** | **Contribution** | **Extent of contribution (%)** |
|---|---|---|---|
| Eugéne van Rensburg | eugenevrb@sun.ac.za | Experimental planning<br>Data interpretation<br>Chapter revision | 5<br>5<br>15 |
| Annie F. A. Chimphango | achimp@sun.ac.za | Experimental planning<br>Data interpretation<br>Chapter revision | 5<br>5<br>15 |
| Johann F. Görgens | jgorgens@sun.ac.za | Experimental planning<br>Data interpretation | 20<br>10 |

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| | | Chapter revision | 70 |
|---|---|---|---|

Candidate Signature Date

Declaration by the co-authors:

The undersigned hereby confirm that:

- The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to CHAPTER 4, pages 41-65,
- No other authors contributed to CHAPTER 4, pages 41-65 besides those specified above, and
- Potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in CHAPTER 4, pages 41-65 of this dissertation.

| Signature | Institutional affiliation | Date |
|---|---|---|
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**Sequential extraction of protein and inulin from the tubers of Jerusalem artichoke (Helianthus tuberosus L.)**

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**Abstract**

An increase in inulin and plant-protein based nutraceutical demand ultimately puts pressure on available resources. Therefore, there is a need to prospect for supplementary feedstocks and sustainable ways to exploit them. The aim of this study was to explore the technical feasibility of sequential extraction of inulin and protein from Jerusalem artichoke tubers and understand the interrelationships between processes and product functional properties. The response surface method was used to determine the optimal parameters for sequential extraction. Protein functional properties analysis were done to identify the effects of the extraction process. The extraction approach adopted in this study was preceded by mechanical pressing of the tuber to yield a protein-rich juice. However, only 52.1% of the protein was recovered from the juice, therefore a subsequent solvent extraction step followed to extract the residual protein and inulin retained in the solids. Selective extraction was achieved when protein was solubilised in the first step of solvent extraction. The overall protein and inulin yield from pressing and both sequential extraction steps were 71.9 and 67.6%, respectively. The yields were substantially higher than the maximum overall yields when inulin extraction, from the pressed tuber, was performed first thus improving yields from 47.9 and 57.3%, respectively. Consequently, mechanical pressing improved the overall protein yield. Sequential extraction resulted in an inulin extract with minimal protein contamination compared to the conventional method. Therefore, sequential extraction was efficient in yielding extracts with reduced impurities and good functional properties.

**Keywords**: Mechanical pressing, nutraceuticals, protein, inulin, biorefinery

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**Highlights**

- Mechanical pressing enabled selective recovery of 52% protein yield from juice
- Mass transfer differentials enhanced protein selectivity in sequential extraction
- Pressing enabled inulin extraction at high solid loading

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## 4.1. Introduction

Plant proteins and polysaccharides are becoming a more significant part of human nutritional diet due to their perceived health benefits (Singh *et al.,* 2008; Franck 2002). Plant proteins are an alternative to animal protein because crop production is associated with lesser greenhouse gases emissions compared to animal farming. Proteins and polysaccharides consumed for their perceived health benefits beyond primary nutritional and dietary benefits, are known as nutraceuticals (Palthur *et al*., 2010). Nutraceuticals have been reported to reduce the risk of cancer, cardiovascular diseases, and have immuno-modulatory functions (Singh et al. 2008).

Nutraceuticals are consumed as dietary supplements or conventional food product (Palthur *et al*., 2010). Furthermore, nutraceuticals are used as ingredients in the processing of conventional foods, such as salad dressings, desserts, and beverages, due to their low cholesterol and calorie content (Singh et al. 2008). Nutraceuticals also enhance the texture, viscosity, water activity, emulsion, heat stability, and mouthfeel of food products (Singh *et al*., 2008; Franck 2002).

Inulin is a popular polysaccharide-based nutraceutical, composed of β (2 → 1) linked D-fructose and found in a range of plants belonging to the family *Compositae* such as chicory, Jerusalem artichoke (JA) and dandelion (Apolinário *et al*., 2014). Chicory is currently the major feedstock crop for industrial inulin production (Zarroug et al. 2016). JA tubers are also rich in inulin, accounting for approximately 80% w/w of the total tuber dry mass (DM). JA tubers are thus, a potential supplementary source of inulin and fructooligosaccharides (Gunnarsson *et al*., 2014).

JA is a herbaceous perennial, plant composed of the stalk and flower, and tubers that develop underground (Yang *et al*., 2015). Although it is native to the temperate North America, its cultivation was adopted in Europe around the seventeenth century (Gunnarson *et al*., 2014). Currently the plant is widely cultivated in China, with uses such as a vegetable for human consumption due the health benefits and soil rehabilitation since it is a highly salt tolerant plant (Xiao *et al*., 2011). Protein accumulation in the tubers has resulted in the use of JA tubers as animal feed (Alla *et al.,* 2014). The plant has however, gained interest as a rich source of inulin. JA is agronomically attractive since it can be cultivated under a range of microclimatic conditions, exhibits good agronomic traits, requires minimal input cultivation, and produces high yields (Yang *et al*., 2015).

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Hot-water extraction is a commonly used method to extract inulin from tubers, followed by inulin purification for use in powder and syrup form or hydrolysis into fructooligosaccharide (FOS) (Franck 2002). Purification involves the removal of impurities, largely composed of water-soluble proteins (Li *et al*., 2012), that affect the functional properties of inulin. In addition, protein accounts for between 7-15% w/w of the tuber DM (Bekers *et al*., 2008; Gunnarsson *et al*., 2014). Therefore, there is a need to explore the potential of an integrated strategy for inulin and protein co-production to minimise protein contamination, purification cost and waste production.

Although the co-production of inulin and protein from JA tubers has a potential to enhance the sustainability of the crop as a biorefinery feedstock, a potential loss in the yield and quality of the products is inevitable due to co-extraction during sequential extraction. Therefore, sequential extraction will only be valuable if it achieves selective extraction of one component over the other by manipulating the differences in the biochemical structure of protein and inulin that impact on solubility. The overlap in inulin and protein solubility will significantly compromise selectivity during extraction (Li *et al*., 2012). The higher temperatures optimal for inulin extraction will lead to severe protein co-extraction thereby compromising selectivity (Lingyun *et al*., 2007). Moreover, inulin co-extraction may occur under mild temperatures favourable for protein solubility (Mizubuti *et al*., 2000). Short chain inulin oligomers (DP 2-9) have been reported to be relatively soluble at temperatures around 25$^{o}$C compared to long chain polymers (DP 10-60) which are soluble at temperatures above 60$^{o}$C (Wada *et al*., 2005).

Therefore, an integrated strategy for the co-production of inulin and protein will justify the commercial and sustainable cultivation of JA for use as a biorefinery feedstock. Furthermore, an integrated extraction process has the potential to yield superior quality products. Thus, the aim of this study was to use the response surface methodology to determine the best sequential extraction parameter values and functional properties analysis to identify and understand interrelationship between processing sequence or conditions and product properties.

## 4.2. Materials and methods

### 4.2.1. Tuber feedstock

JA tubers were obtained from the Glen Agricultural College, Free State, South Africa. Fresh tubers were harvested in the late winter, subsampled for chemical composition analysis and

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subsequently stored at -18 °C. Fresh tubers (80% moisture content) for chemical composition analysis were sliced and dried, in a Scientific® Series 200 oven at 40°C for 60 hours, to constant dry weight with a moisture content of 15%. The moisture content was determined according to the National Renewable Energy Laboratory (NREL) method (Sluiter *et al*., 2008). Dried tubers were crushed (6 mm sieve) and milled (2 mm sieve) at 2000 rpm using a Retsch® ZM 200 labscale mill (Retsch®, Haan, Germany) and fractionated with a Retsch® shaker sieve at 70 amplitudes for 10 mins. The fraction collected in the 4.25 mm sieve was used for the chemical composition analysis using the proximate analysis. The sample was used since deviation to larger particles size may results in an inaccurate carbohydrate content determination due to incomplete hydrolysis while smaller particle size result in carbohydrate degradation (Sluiter *et al*., 2008).

The tuber mash used in the extraction process was prepared from the frozen-tuber stock in two different methods. The tuber mash used in preliminary screening extraction experiments was prepared by washing the frozen tubers and rasping with a hand-grater. The tubers used in the sequential extraction experiments were prepared by manually pressing the tuber mash, with a kitchen juice pressor, and solid residue subsequently used in the optimization experiments. The press-juice was used to determine the inulin and protein concentration. The inulin and protein yields from the juice were determined as a function of the tuber-mash dry weight, the inulin and protein content as described in the analysis section.

### 4.2.2. Analysis

### 4.2.3. Inulin determination

The inulin content of the tubers and extraction solid residues were determined as a function of free and total sugar (fructose) content. The free sugar content was determined by dispersing 200 mg dry raw-tuber granules or dried extraction residue in 50 ml distilled water and incubating at 25°C for 15 min in a shaking water bath. Total sugars, for inulin content measurement, were determined through acid hydrolysis of the tuber granules or extraction residue in 0.2% v/v $H_2SO_4$ (Sigma®, Saint Lois, USA) for 60 min (Gunnarsson *et al*., 2014). Sugars were analysed using high-performance liquid chromatography (HPLC) with a Dionex (Dionex, California, USA) 3000 System equipped with a Grace® (Hichrom, Berkshire, UK) Prevail Carbohydrate ES Column (250 x 4.6 mm) and a Varian® evaporative light scattering detector. The HPLC operating conditions such as the injection volume, column temperature,

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flow rate and gradient mobile phase were 10 µL, 30$^{o}$C, 0.6 ml/min and acetonitrile, respectively. Inulin was defined as non-monomeric polyfructan composed of at least two fructan monomers ($F_2$). Total inulin content was calculated as follows:

$$Total\ Inulin\ (g)\ = (F_{t} - F_0)\ X\ volume\ of\ extraction\ liquid \quad (1)$$

where, $F_0$ and $F_t$ is the amount (g) of fructose before and after acid hydrolysis, respectively. The total solids, ash and structural sugars content of the non-extracted tubers were determined according to the standard NREL procedures (Sluiter *et al.*, 2008; Sluiter *et al.*, 2005; Sluiter *et al.*, 2011). Structural sugars (xylose, glucose and cellobiose) were analysed with HPLC, after acid hydrolysis in 72% $H_2SO_4$ (Sluiter *et al.*, 2011). HPLC analysis was performed with an injection volume of 30 µl in a Thermo separation (Spectralab Scientific Incorporated, Toronto, Canada) Spectra System, equipped with a Shodex refractive index detector operated at 45°C. The system was also equipped with an Aminex HPX-87H Ion Exclusion Column with a Cation-H cartridge (Biorad®, Johannesburg, RSA) and operated at a flow rate of 0.6 ml/min, column temperature of 65°C, an isocratic mobile phase.

Crude protein from raw tubers and extraction residues was determined through the Dumas method. The DUMAS analysis was done according to the Association of Official Analytical Chemist's Standard Techniques (AOAC 2002) in a LECO (Leco Corporation, Saint Joseph, USA) FP 528 system calibrated with ALFALFA with a Nitrogen content of 3.38%. A conversion factor of 6.25 was used for the determination of the crude protein content (Gunnarsson et al. 2014). The concentration of soluble protein from the extracts was determined using the bicinchoninic acid (BCA) assay (ThermoFisher Scientific, Rockford, USA). The bovine serum albumin standard was used, and the protein concentration determined with the DUMAS method describe above.

#### 4.2.4. Protein solubility

Protein solubility was determined according to the method of Stone *et al.*, (2015). A 0.01% w/v freeze-dried protein extract solution was prepared in 0.1N NaCl pH 7, mixed at 50 $^{o}$C for an hour and then centrifuged. The protein content in the supernatant was determined using the BCA assay. Protein solubility was expressed as the amount of solubilised protein relative to the total amount of protein.

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#### 4.2.5. Emulsification properties

A five ml protein extract solution (1%) in distilled water at pH 7 was mixed with an equal volume of canola oil and vortexed briefly. The mixture was centrifuged, with a benchtop mini centrifuge (Biorad®, South Africa), at 5000 rpm for a minute, and emulsion activity was calculated as the ratio of the height of the emulsion layer to the total height of the liquid. Emulsion stability was determined by transferring the emulsion to another test tube and incubating in water bath at 70°C. Emulsion stability was calculated as described for the emulsion activity (Lin and Zayas, 1987).

#### 4.2.6. Foaming properties

Aqueous solutions of the proteins (0.5% w/v) in a 50 ml measuring cylinder were sparged with compressed air for 10 sec and the height of the foam measured at 10 min interval for 30 min. The height of the foam at $T_0$ was the foaming capacity and the foaming stability (FS) was calculated from the equation below

$$FS = \frac{V_0}{Vt}\ x\ 100$$

(2)

where $V_0$ is the volume of the foam in ml at $T_0$ and V is the change in volume of the foam over time (*t*) (Kato *et al*., 1983).

#### 4.2.7. Water retention

The protein water retention capacity was determined according to the method of Stone *et al*., (2015). Briefly, 0.5 g of the protein was suspended in 5 g of water. The mixture was vortexed for 10 sec at 5 min intervals for 30 min, and subsequently centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded, and weight of the pellet was measured. Water retention was calculated by dividing the weight gained by the original weight of protein.

#### 4.2.8. Preliminary screening

Tuber mash was extracted in a 250 ml Erlenmeyer flask at a temperature of 50°C, pH 7 for 30 min in a shaking water bath at 100 rpm, to evaluate the effect of the solids loading on inulin and protein yield. The extract was centrifuged at 5000 rpm for 15 min and liquid fraction used for inulin and protein content determination through HPLC and the BCA assay, respectively. The inulin content was determined with HPLC after acid hydrolysis in 0.2% v/v $H_2SO_4$ and subsequently determined according to equation 1. The inulin and protein yields were calculated as follows:

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$$Yield\ \% = \frac{X_{\mathrm{L}}}{X_{\mathrm{R}}}\ X\ 100 \qquad (3)$$

where, $X_{\mathrm{L}}$ and $X_{\mathrm{R}}$ was the total inulin or protein content (g) in the liquid fraction and in the raw material, respectively.

**4.2.9. Sequential extraction from the pulp**

A two-step approach was used for sequential protein and inulin extraction from the pressed tuber. The two extraction sequences were compared to each other to determine the sequence that achieved an acceptable level of selectivity by minimizing co-extraction. Inulin and protein extractions were performed in 250 ml Erlenmeyer flasks by dispersing 5 g and 7.5 g (DM basis) of the raw material, respectively, in distilled water to a final volume of 50 ml. The pH of the mixture was adjusted with 1M $H_2SO_4$ or 0.01M NaOH (Sigma®) and water bath temperature set, according to the central composite design (CCD). Extraction was performed for 60 min at 100 rpm. The extracts were centrifuged at 5000 rpm for 15 min, and the liquid fraction was used for inulin and protein content determination. The extraction and co-extraction yields were subsequently determined according to equation 3. The molecular weight (MW) and degree of polymerisation (DP) of the inulin extracts were determined through size exclusion chromatography with a Dionex® (Dionex, California, USA) Ultimate 3000 HPLC System equipped with a PSS (GmbH, Mainz, Germany) Suprema 10 µm column set (2 X 3000 Å and 1 x 30 Å) and PSS Pullon standards (315 Daltons- 805 kDa). The operating conditions such as injection volume, column temperature, flow rate and mobile phase were 10 µL, 70$^{o}$C, 1 ml/min and 0.125M ammonium acetate, respectively.

**4.2.10. Experimental design: Protein and inulin sequential extraction**

A $2^3$ full factorial design with 10 runs (two centre points) was used to determine significant factors for protein and inulin extraction from the pressed tuber. The independent variables were pH (3, 5, 7), temperature (25, 42.5, 60$^{o}$C) and solids loading (3, 4.5, 6% w/v on DM basis) whereas the response variables were inulin and protein yield. A five-coded levels CCD was subsequently used to optimize the parameters selective extraction at each step of the sequential extraction process. The first scenario (S1) involved protein and inulin extraction in the first and second step, respectively, while the sequence of extraction was reversed in Scenario 2 (S2). Therefore, the solid residue obtained from the first extraction step was subsequently used as the raw material in the second step to extract the retained product. Each experiment had 13 runs (five center runs). The independent variables for this experiment

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were pH and temperature. The temperature (56, 60, 70, 80, 84$^0$C) and pH (6.3, 6.5, 7, 7.5, 7.7) range for the inulin extraction step were used since several studies have reported an optimum temperature of approximately 70$^o$C and pH 7 (Lingyun *et al.*, 2007). The temperature range (14, 25, 52.5, 80, 92$^o$C) for the protein extraction step was chosen to achieve selectivity since specific proteins have been reported to be soluble at room temperature (Lai *et al.*, 2013) while inulin oligomers soluble at temperatures below 50$^o$C (Wada *et al.*, 2005).

Multiple responses optimization was used to simultaneously optimize the extraction and co-extraction yields in each extraction step. The general formula for the response is shown in the equation below:

$$y_i = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j + \epsilon \quad (5)$$

where $y_i$ is the i$^{th}$ response variable, $x_i$ is the i$^{th}$ input parameter, n is the number of input parameters and $\beta_0$, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are the fixed response, linear, quadratic and cross products coefficients, respectively. The general function optimization method of the Derringer's desirability function was used to search a set of optimal conditions that improve the overall desirability and simultaneously maximize the extraction yield and minimize co-extraction yield. Statistica® (Statsoft Inc., Tulsa, USA) was used for the experimental design and statistical analysis, and all the analysis were performed at 5% level of significance.

#### 4.2.11. Statistical analysis for protein functional properties

Protein functional properties experiments were done in triplicates and analysis of variance (ANOVA) was used to determine the differences in the properties between the juice and solvent extracted protein. ANOVA was performed at 5% level of significance.

### 4.3. Results and discussion

#### 4.3.1. Chemical composition of tubers

The inulin and protein content of the tubers was 71.0% and 7.5% w/w (ESM_2: Table S1) of the tuber DM, respectively. The inulin content is comparable to chicory, currently the major feedstock for industrial inulin production (Zarroug *et al.*, 2016), and those of JA tubers reported in literature (Gunnarsson *et al.*, 2014; Rube *et. al.*, 2014, Johannson *et al.*, 2015). Thus, JA is a potential, additional feedstock for inulin production and biorefinery development. The content of the structural sugars was relatively low, making up to 12% of the tuber composition (ESM_2: Table S1), compared to inulin. Structural sugars are highly

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recalcitrant and not readily soluble in water, thus a bulk of this will be retained in the solid residue. Therefore, the presence of structural sugars in the tuber was unlikely to compromise selective extraction as well as the quality of inulin and protein extracts.

#### 4.3.2. Protein and inulin recovery from the press-juice

Preliminary screening data demonstrated that protein was readily available in the tuber juice, which could be obtained through pulping the tubers and mechanical pressing, similar to solvent-free protein separation from potato pulp (Bartova and Barta, 2009). The protein yield from the juice obtained through pressing, represented 40.8% of the tuber protein, while the inulin yield was relatively low at 2.3% (ESM_2: Fig. S1), demonstrating that pressing was selective for protein extraction. The low inulin co-extraction yield from the juice was expected since inulin extraction is commonly done through hot-water method (Lingyun *et al*., 2006). A subsequent solvent-based (water) extraction step was, therefore, necessary for selectively recovering the residual protein and inulin from the pressed tuber.

#### 4.3.3. Significant process conditions for inulin and protein extraction

A full factorial design (ESM_2: Table S2) was used to determine the significant factors for inulin and protein extraction from the pressed tuber. Inulin and protein yields were both significantly affected by temperature and pH, while the solids loading affected the inulin yields alone (Fig. 4.1). Therefore, a face-centred CCD was selected for the optimization of temperature and pH as key process parameters for selective and sequential extraction of protein and inulin. The solids loading for the protein extraction step was selected as 15% w/v, higher than the 10% w/v for the inulin extraction step, to maximise selectivity for protein extraction, rather than inulin, since inulin yields were inversely related to the solids loading (Fig. 4.1). A solids loading of 15% w/v was utilised for protein extraction step since screening experiments showed a significant decrease in the protein yield when the solids loading was increased beyond 15% w/v. The solids loading for the inulin extraction step was fixed at 10% w/v since high viscosity at high solids loading causes insufficient mixing (Fan *et al*., 2003).

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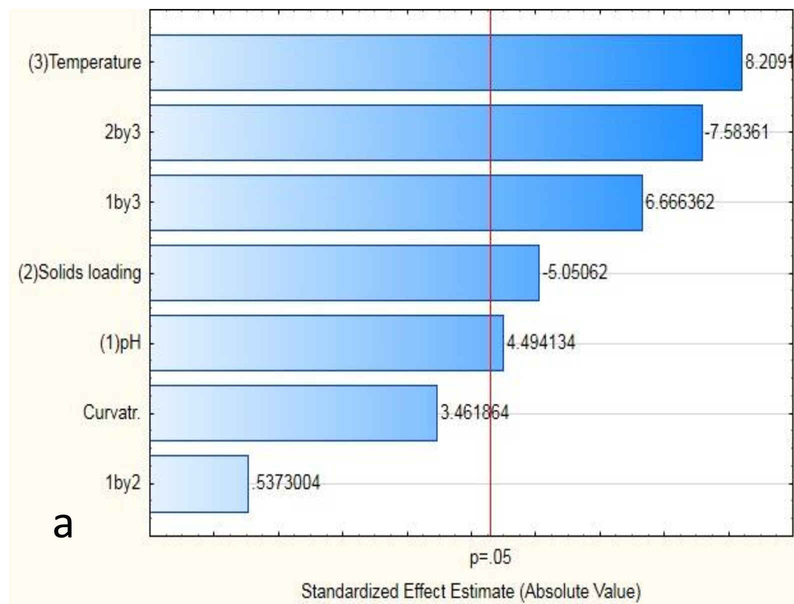


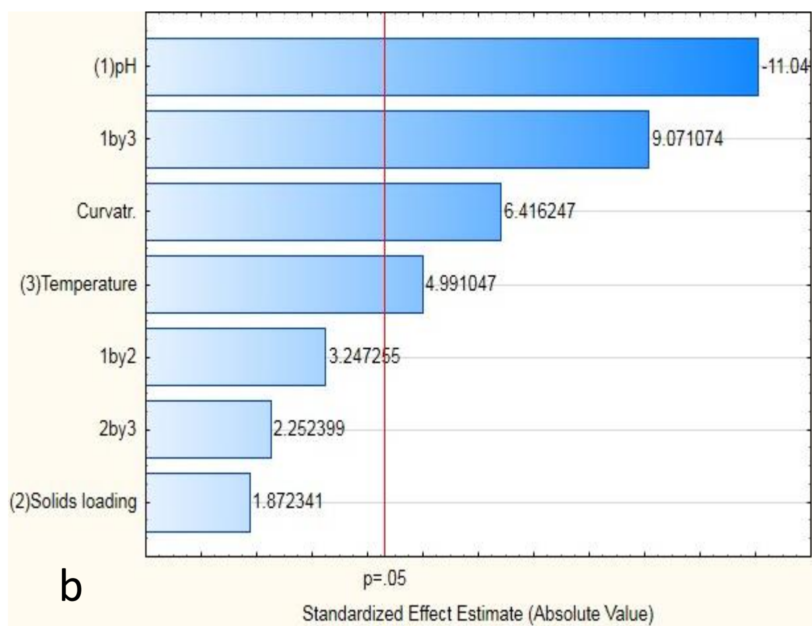


Figure 4.1: Standardized Pareto chart to estimate the significant factors for inulin (A) and protein (B) extraction from JA residue after pressing

### 4.3.4. Optimisation of process parameters for selective extraction of protein and inulin from tubers

#### *4.3.4.1. Model fitting*

The adequacy of the response surface methodology (RSM) model coefficients, for inulin and protein extraction and co-extraction, was evaluated using ANOVA of the response variables,

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and is summarized in Table 1. The model validity was assessed by calculating the coefficient of determination ($R^2$), adjusted $R^2$ and the lack of fit (Table 4.1). The adjusted $R^2$ values for the models were in the range of 0.7-0.9 (Table 4.1), thereby showing that at least 70% of the variation in the extraction and co-extraction yields could be explained by second-order polynomial models in relation to pH and temperature.

*4.3.4.2. Inulin extraction*

Inulin extraction from the protein extraction residues (S1) was compared to inulin extraction from pressed tuber (S2) to determine the process with better selectivity. The observed inulin yields from S1 and S2 were in the range of 55-68.5% (Fig. 4.2c) and 46-62% (Fig. 4.2a), respectively. The yields are superior compared to those reported in literature (14.42-21.69 g/100g of tuber) for JA (Li *et al*., 2012; Bekers *et al*., 2008). Furthermore, the yields were obtained at solids loading four times higher (10% w/v of dry matter) than reported in literature (Li *et al*., 2012; Bekers *et al*., 2008). Interestingly, the maximum inulin yield in this study (67.6%) was more than the inulin yield (51.2%) from chicory roots, at a solids loading of 2.5% w/v DM using the conventional hot-water extraction method (Tewari *et al*., 2015).

In contrast the high inulin yields (27.6-38.7 g/100g of pressed tuber) demonstrated at a higher solids loading, could be attributed to mechanical pressing before sequential extraction, which reduced the moisture content of the pressed tuber, enabling sufficient mixing and mass transfer (Lingyun *et al*., 2007). The removal of proteins before solvent extraction could have significantly contributed to enhanced mass transfer by minimising the viscosity of the solution. Severe protein co-extraction in the range of 47.1-65.8% (corresponding to 2.2-3.6 g/L), occurred when inulin was extracted from the pressed tuber (Fig. 4.2b). The severity of the co-extraction increased with an increase in temperature (Fig. 4.2b) and this could be attributed to enhanced protein solubility (Karazhiyan *et al*., 2011)

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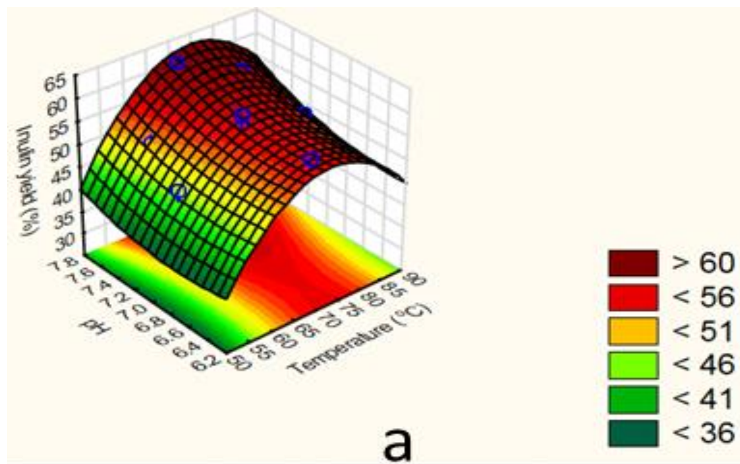


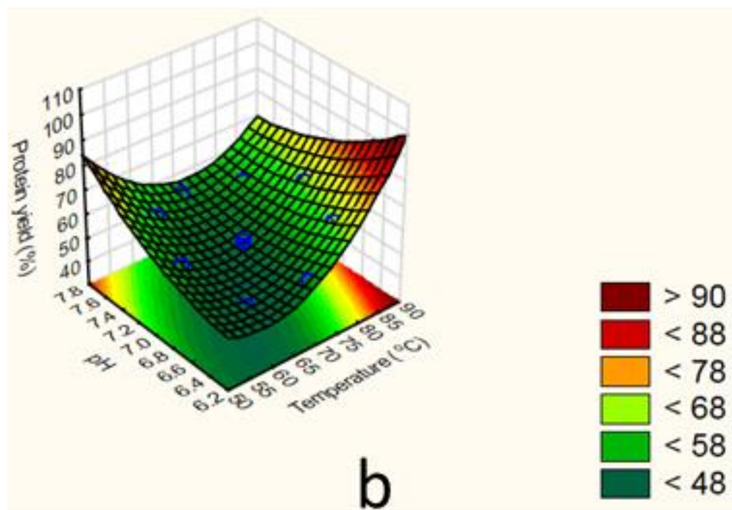


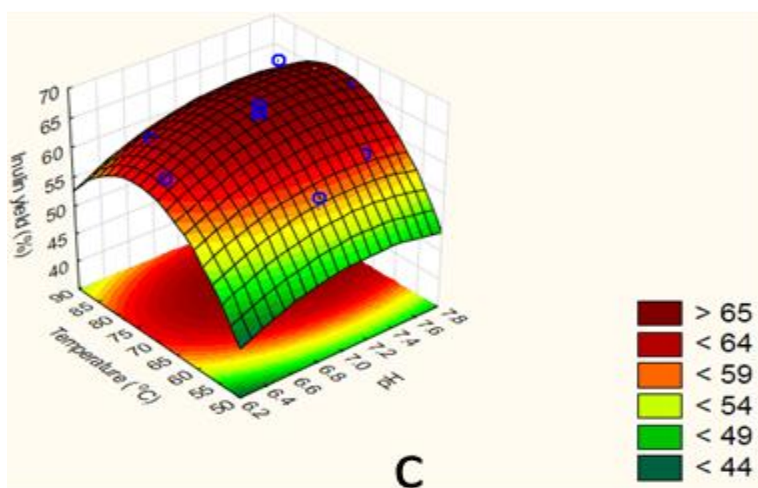


Figure 4.2: Response surface plot showing inulin extraction (a) and protein co-extraction (b) from the pressed tuber and inulin extraction from the protein extraction residue (c), as a function of pH and temperature

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In contrast, protein co-extraction from S2 was substantially lower than in S1, with protein concentrations of 0.3-0.6 g/L, primarily due to low protein contents of these residues. Protein extraction in the first step of sequential extraction was also important in minimising the amount of protein impurities associated with inulin extraction (Li *et al*., 2012). Although inulin extraction yields in S2 were comparable to S1, the unacceptable high protein co-extraction yield (Fig. 4.2b) from the latter, indicated that inulin extraction conditions were not sufficiently selective for inulin. The low protein co-extraction yield paves way for a cost-effective method for inulin downstream processing since there is a potential to eliminate the deprotonation step prior to chromatograph purification (Apolinaro *et al*., 2014).

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**Table 4.1:** Analysis of variance for the CCD models for sequential extraction and coefficients for the predictive models of the inulin and protein extraction/co-extraction yields. ANOVA was determined with a 95% confidence level

| | | Protein extraction (S1[a]) | | | | | | Inulin extraction(S1[a]) | | | Inulin extraction(S2[a]) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Protein yield** | | | **Inulin co-extraction yield** | | | **Inulin yield** | | | **Inulin yield** | | | **Protein co-extraction yield** | | |
| **Source** | **d.f.[b]** | **Co-efficient** | **Sum of squares** | **p-value** | **Co-efficient** | **Sum of squares** | **p-value** | **Co-efficient** | **Sum of squares** | **p-value** | **Co-efficient** | **Sum of squares** | **p-value** | **Co-efficient** | **Sum of squares** | **p-value** |
| **Linear** | | | | | | | | | | | | | | | | |
| Temp (T) | 1 | 0.01 | 31.14 | 0.019 | 0.35 | 4.24 | 0.127 | 4.44 | 50.96 | 0.009 | 5.11 | 63.25 | 0.004 | 5.35 | 132.41 | 0.005 |
| pH (P) | 1 | 10.34 | 16.51 | 0.051 | -2 | 6.67 | 0.086 | 3.65 | 25.08 | 0.03 | 3.25 | 33.95 | 0.014 | -24 | 2.38 | 0.51 |
| **Quadratic** | | | | | | | | | | | | | | | | |
| $T^2$ | 1 | 0. 9 | 3.56 | 0.271 | -0.002 | 22.35 | 0.014 | -0.33 | 63.66 | 0.006 | -0.04 | 87.05 | 0.002 | 0.05 | 172.09 | 0.003 |
| $P^2$ | 1 | -0.94 | 98.64 | 0.002 | -0.33 | 11.89 | 0.039 | 3.99 | 12.66 | 0.077 | 4.05 | 7.315 | 0.13 | 0.599 | 40.03 | 0.04 |
| **Interaction** | | | | | | | | | | | | | | | | |
| TP | 1 | -0.004 | 0.19 | 0.78 | -0.02 | 2.89 | 0.21 | 0.04 | 0.125 | 0.82 | 0.1 | 1 | 0.517 | -0.96 | 92.16 | 0.01 |
| **Regression** | 1 | | 150.06 | 0.001 | | 48.59 | 0.01 | | 151.3 | 0.01 | | 439.06 | 0.01 | | 192.39 | 0.01 |
| **Lack of Fit** | 3 | | 24.05 | 0.12 | | 6.61 | 0.3 | | 16.32 | 0.208 | | 27.97 | 0.084 | | 24.05 | 0.09 |
| **Pure Error** | 4 | | 8.73 | | | 5.21 | | | 9.06 | | | 7.96 | | | 8.73 | |
| **Model** | 12 | | 189.59 | | | 65.33 | | | 171.28 | | | 236.56 | | | 440.7 | |
| **$R^2$** | | 0.82 | | | 0.81 | | | 0.85 | | | 0.95 | | | 0.82 | | |
| **Adjusted $R^2$** | | 0.7 | | | 0.69 | | | 0.74 | | | 0.92 | | | 0.7 | | |

[a]S1 and S2 is scenario 1 (protein extraction and inulin extraction first and second, respectively) and scenario 2 (inulin extraction first) for the sequential extraction, respectively., [b]d.f. is the degrees of freedom

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Inulin extraction data was fitted using quadratic models to describe the effects of temperature (T) and pH (P) on inulin extraction and protein co-extraction yields. The estimated regression models summarising the significant factors are represented below:

$$Inulin\ yield\ (Scenario\ 1) = -109.19 + 3.65P + 4.44T - 0.33T^2 \quad (5)$$

$$Inulin\ yield\ (Scenario\ 2) = -127.29 + 5.11T - 0.04T^2 + 3.25P \quad (6)$$

$$Protein\ coextraction\ yield\ (Scenario\ 2) = 194.60 + 5.35T + 0.05T^2 + 0.599P^2 - 0.24 \quad (7)$$

Regression analysis showed that temperature had the most impact on inulin extraction yields as demonstrated by the size of the temperature (p-value<0.009) relative to pH (p<0.03). The experimental data illustrate that an initial increase in the temperature from 56-74°C results in an increase in the inulin yield and that a further increase in temperature resulted in a decline in the inulin yield. The inulin yield increased as the temperature increased from 56°C reaching a maximum at ca. 74°C, after which the yield decreased. The surface plots (Fig. 4.2a and c) shows the relationship between inulin extraction yields and the temperature. The increase in the yield could be attributed to improved mass transfer and inulin solubility as the temperature increased (Koutinas *et al.*, 2013). A reduction in the inulin yield when the temperature exceeded a certain level (ca. 80°C) has been reported in literature and is due to inulin degradation into monomers (Lingyun *et al*. 2007; Lai *et al*. 2013).

#### ***4.3.4.3. Protein extraction***

The protein extraction step was performed from the pressed tuber and not performed from the solid residues after inulin extraction, due to the severe protein co-extraction from S2 (Fig. 4.2b). The latter process scenario therefore did not meet the requirements of selective inulin and protein extraction. Selective protein extraction was achieved in S1 by performing the extraction at the maximum-feasible solids loadings, (15% w/v; higher than 10% w/v used in the inulin extraction steps), to minimize inulin co-extraction. The optimum temperature and pH for S1 was determined, to simultaneously maximize the protein yield while minimizing the inulin co-extraction yield. The results showed that protein extraction yields were substantially higher compared to the inulin co-extraction yields, ranging between 40-52.5% (Fig. 4.3a) and

4-11.8% (Fig. 4.3b), respectively. Residual inulin in the solid extraction residue was relatively high, ranging between 47.1-52.4 g/100g, corresponding to 74.7-83.2% of inulin from the raw material, thereby confirming that inulin co-extraction was minimal. Protein extraction from the tuber pulp at high solids loading, therefore, demonstrated acceptable selectivity for protein, while retaining inulin in the insoluble form in the solids, for subsequent extraction.

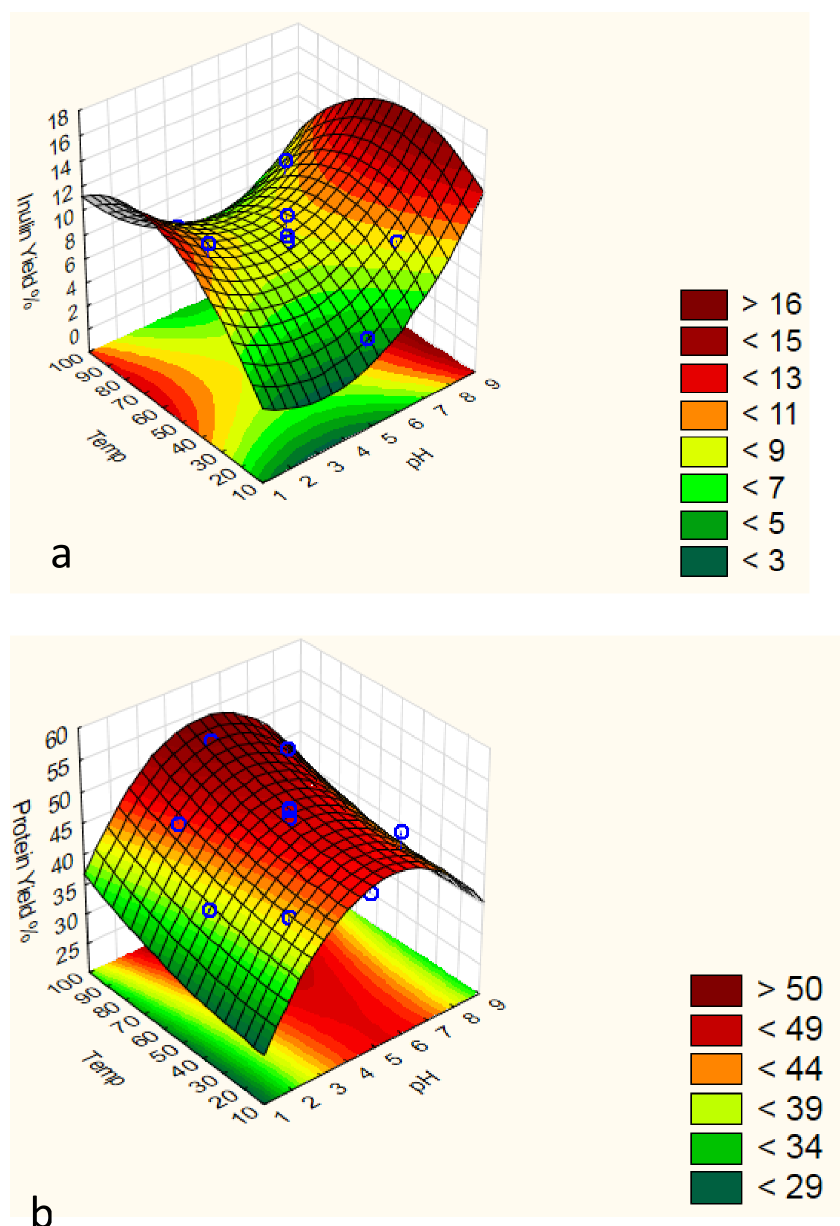


Figure 4.3: Response surface plot showing protein extraction (a) and inulin co-extraction (b) yields from the pressed tuber as a function of pH and temperate

Lower inulin co-extraction yields (4-11.8%) from the protein extraction step (Fig. 4.3b), were demonstrated in comparison to the higher inulin yields (46-67.6%) from the inulin extraction

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steps (Fig 4.2a and c). This was consequently due to differing solids loading between the two process steps. The negative effects of higher solids loading on the inulin extraction yield was observed with the tuber mash and pressed tuber (Fig. 4.1) and could be attributed to mass transfer limitations as the solids loading increases (Koutinas *et al*., 2013). Zarroug *et al*., (2016) reported that a lower water to raw-material ratio decreases the concentration gradient between the plant cell interior and surrounding solvent, thereby reducing inulin diffusion and solubility.

The effects of temperature and pH on the protein extraction and inulin co-extraction yields from the pressed tuber were illustrated by fitting regression models shown below:

$$Protein\ yield\ = 18.89\ +\ 10.34P\ -\ 0.94P^2 + 0.01T + 0.94T^2 \qquad (7)$$

$$Inulin\ Yield\ (coextraction) = 2.68 -\ 0.33P^2 + 0.35T - 0.002T^2 \qquad (8)$$

Equation 7 shows that pH (p-value = 0.003) had a greater effect on protein yields than temperature (0.009) and this was further confirmed by the steeper slope for pH against protein yield (Fig. 4.3a). The negative sign on the coefficient of the quadratic term of pH illustrated that a change of pH from acidic to mild-acidic (pH 3-5) increased the protein extraction yield. Moreover, a change towards pH 7 resulted in a decrease in the yield as illustrated by the response surface plot (Fig. 4.3a). The plot shows that the protein yield reached a maximum at mild acidic conditions (pH 5). Therefore, mild acidic conditions provided sufficient charged ions to change the protein's net charge, resulting in repulsion of the protein molecules and consequently promoting solubility. However, approximately 46% of the protein was recovered with this method. Thus, increasing the ionic strength using a salt (Stone *et al*., 2015) could be considered to enhance the solubility of the residual protein which could be the globulin type found in JA tuber (Johannson *et al*., 2015). Although protein extraction above room temperature was shown to increase the protein extraction yield, it also resulted in a simultaneous increase in the inulin co-extraction yields (Fig. 4.3b). Furthermore, temperature (p-value = 0.005) had the most impact on inulin co-extraction during protein extraction from the pressed tuber, this observation is similar to that observed in the inulin extraction steps (Fig. 4.2a and c). The simultaneous increase in the protein extraction and inulin co-extraction yields relative to temperature were a result of improved mass transfer due to enhanced solvent diffusion, improved inulin and enhanced protein

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solubility (Koutinas *et al*., 2013; Karazhiyan *et al*., 2011). Therefore, a compromise had to be achieved to simultaneously maximise protein extraction yield and minimise inulin co-extraction.

*4.3.4.4. Simultaneous optimisation and model validation*

The Derringer's desirability function was used to simultaneously optimize extraction and co-extraction yields from the sequential extraction steps. The optimal conditions to simultaneously maximize protein extraction and minimize inulin co-extraction in the first extraction step of S1 were pH 5 and temperature of 25$^{o}$C, with protein extraction and inulin co-extraction yields of 46.98 and 6.3%, respectively (Table 4.2). The maximum inulin extraction yield in the second step was predicted to be 67.6% at a temperature of 74.4 $^{o}$C and pH 6.4. It is noteworthy to state that the concentration of the co-extracted protein from the inulin extraction step of S1 was only 0.5 g/L. The optimal inulin extraction yield from the pressed tuber (S2) was 57.3% at a temperature of 70.0 $^{o}$C and pH 7.3. Moreover, the concentration of protein co-extracted under these conditions was 1.5 g/L, equivalent to a protein co-extraction yield of 50.45%. Proteins are surface active molecules and exist in tertiary structures that are stabilised by electrostatic and hydrophobic interactions which are easily disrupted when temperature increases. Proteins have been reported to unfold rapidly above 60$^{o}$C, exposing hydrophilic amino acids which ultimately form hydrogen bonds with water and thus making proteins highly soluble (Mitra *et al*. 2007). Polysaccharides have been reported to form soluble complexes with proteins at pH above protein's isoelectric point where the protein assumes a cationic charge (Cooper *et al*. 2005). On the other hand, inulin possesses differential solubility, relative to temperature, since it is composed of oligomers of different degrees of polymerisation (Wada *et al.,* 2005). For instance, FOS, which is composed of low molecular weight fructans with a degree of polymerization that is less than 10 fructose monomers, is soluble at room temperature (Wada *et al.,* 2005). Thus, overcoming the effects of these complex interactions between proteins and polysaccharides on solubility, was critical in achieving selective extraction. Selective protein extraction in this study was best achieved at room temperature (25$^{o}$C) and pH 5 while severe protein co-extraction was observed during inulin extraction at pH 7 and temperatures above 60$^{o}$C.

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**Table 4.2:** Protein and inulin yields from the press juice, predicted models and confirmatory tests. Inulin and protein recoveries were determined as % yield based on the inulin and protein content, respectively, in tuber. Statistical analysis was done with a 95% confidence level

| | Optimum conditions | | Protein recoveries %[b] (g/100g$_{biomass}$) | | Inulin recoveries % (g/100g$_{biomass}$) | |
|---|---|---|---|---|---|---|
| | Temp °C | pH | Predicted | Confirmatory[a] | Predicted | Confirmatory[a] |
| Protein extraction S1 | 25 | 5 | 46.98 (2.23) | 47.26 ± 2.30 | 6.29 (4.47) | 9.02 ± 0.78 |
| Inulin extraction S1 | 74.4 | 6.4 | 47.65 (0.92) | 52.39 ± 3.15 | 67.6 (43.81) | 69.44 ± 4.12 |
| Inulin extraction S2 | 70 | 7.2 | 50.45 (2.06) | 54.15 ± 2.89 | 57.29 (38.12) | 56.84 3.21 |

Experimental tests were performed to verify the validity and adequacy of the predicted models for inulin and protein extraction for the sequential extraction process. The tests were performed under optimal conditions (Table 4.2) as predicted by the simplex method of the Derringer's desirability function. Three experimental replicates were used, and the experimental values were compared to the predicted values. The observed inulin and protein recoveries from the confirmatory tests were within the 95% confidence interval of the predicted inulin and protein recoveries from the various steps of sequential extraction. These results proved that the models can be used to determine process conditions for the selective and sequential extraction of protein and inulin from the JA tuber pulp obtained after pressing the tuber mash. The molecular weight distribution of the inulin extracts from S1 and S2 was in the range of 397-15103 Daltons (equivalent to DP 2-80) and the average molecular weight was 2521 Daltons ($DP_{av}$ of ~16). Therefore, the inulin extract will have a wider range of applications, such as fat replacements, sweeteners and prebiotics, with or without hydrolysis into fructose monomers or oligomers (Franck, 2002). The freeze-dried inulin powder from S1 contained 63.0% inulin, 8.0% free-sugars and 3.0% protein. Moreover, the protein powder from the juice contained 67.0% protein and 28.0% inulin while the solvent extracted protein contained 56% protein and 39.0% inulin.

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*4.3.5. Protein functional properties*

Protein functional properties are crucial for their application as ingredients in food processing thus, making the extraction process an important step to preserve the properties for subsequent uses. Comparison of the functional properties between proteins from the tuber juice and solvent extraction demonstrated that the extraction method significantly impacted the functional properties (Table 4.3). Protein recovered from the juice had superior solubility than solvent extracted protein. The higher solubility of the former is potentially a result of the native nature of the protein, since no chemicals were applied to extract the protein from the tuber. Alternatively, the differences could be attributed to the primary type of protein found in the juice and water extracts since tuber protein is dominantly composed of albumins and globulins (Johannson *et al*., 2015), which have different solubility characteristics (Boye *et al*., 2010). The solubility of the tuber protein was comparable to that of water and salt extracted pea protein which was in the range of 60-80% (Taherian *et al*., 2011)

**Table 4.3:** Functional properties of protein from the press juice and solvent extraction step. Statistical analysis was done with a 95% confidence level

| Property | Juice[a] | Solvent extracted[a] | p-value |
|---|---|---|---|
| Solubility (%) | 68.2 ± 2.15 | 61.9± 3.12 | <0.05 |
| Water retention (g/g) | 3.18 ± 0.08 | 6.79 ± 1.01 | <0.05 |
| Emulsion capacity (%) | 83.2 ± 5.21 | 91.2 ± 4.02 | <0.05 |
| Emulsion stability (%) | 99.5 ± 4.51 | 98.4 ± 3.86 | <0.05 |
| Foaming capacity (%) | 151 ± 7.71 | 149 ± 7.02 | >.0.05 |
| Foaming stability (%) | 99.2 ± 6.23 | 99.4 ± 5.71 | >0.05 |

[a]Data represent the mean value ± standard deviation of 3 measurements

The water holding capacity of solvent extracted proteins (6.8 g/g) was significantly higher than juice derived protein (3.2 g/g). The water holding capacity was higher than reported for alkaline extracted pea protein which were in the range of 0.6-2.7 g/g (Boye *et al*., 2010). Protein solubility has been reported to be related to the water absorption capacity with less

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soluble protein having the capacity to bind to water due to the abundance of hydrophobic groups on the surface (Stone et al. 2015). Although the emulsion capacity and stability of both protein forms were high (Table 4.3), the properties for solvent extracted proteins were significantly higher than juice obtained proteins. Conversely, the method of extraction did not have an impact on the foaming capacity and stability (Table 4.3). The foaming stability reported for tuber protein was superior to salt and water extracted pea proteins which was in the range of 30-50% (Taherian *et al*., 2011).

The excellent functional properties demonstrated by JA tuber proteins makes them a potential ingredient for application in the food and beverage industries within a wide range of products. The proteins showed high emulsification and solubility properties that are essential for application as emulsifiers or stabilisers in meats, burgers and sausages. The good foaming properties are essential for potential application in ice-cream, spread and salad dressing.

## 4.4. Conclusions

An integrated strategy for protein and inulin extraction was proven to yield products with minimal co-extraction. The preferred extraction process started with pressing of the tuber mash to obtain a protein-rich juice resulting in a protein yield of 52.1% and an inulin loss of 2.3%. Selective inulin and protein extraction during the subsequent solvent extraction step was achieved through manipulation of the process parameters to maximize the yield of one product while minimizing the co-extraction of the product to be extracted at a later stage. Sequential extraction resulted in a further protein yield of 47.6% and inulin yield of 67.6%, from their respective extraction steps. An overall protein balance indicated that 71.8% of protein was extracted into the soluble product streams, while 17.1% was found as a co-extracted product in an inulin-stream. On the other hand, 58.4% of inulin was extracted into soluble product stream, while 11.8% was found as co-extracted product in a protein-stream. An inulin extract with a lower protein contamination was obtained, compared to inulin obtained through conventional method. The low protein content thus eliminates the need for pre-processing steps during downstream purification and therefore has the potential to lower production costs. Moreover, mechanical pressing was an important pre-extraction step that enabled selective protein isolation from the juice and improved the overall yield of protein. Pressing also enabled inulin extraction at high solids loading compare to the conventional

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method. Interestingly, the proteins showed an overall good functional properties with potential applications in the food and/or nutraceutical industry. This study successfully generated data that is important in designing downstream processes for the inulin and protein extracts as well as for techno-economic studies to assess the economic viability of a Jerusalem artichoke-based biorefinery.

### 4.5. Competing interests

The authors declare that there have no competing interests.

### 4.6. Acknowledgements

The authors are grateful to the National Research Foundation for the financial support and the Glen Agricultural College, Bloemfontein, Free State Province, for kindly donating the JA tubers.

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## 4.7 Supplementary material

ESM1: Frozen Jerusalem artichoke tubers

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**Table S1** Factorial design to determine significant factors for protein and inulin extraction

| Run | pH | Solid loading w/v | Temperature °C | Inulin Yield % | Protein Yield % |
|---|---|---|---|---|---|
| 1 | 3 | 2.5 | 25 | 16.73 | 6.61 |
| 2 | 7 | 2.5 | 25 | 15.25 | 4.83 |
| 3 | 3 | 4.5 | 25 | 19.12 | 8.67 |
| 4 | 7 | 4.5 | 25 | 17.03 | 5.64 |
| 5 | 3 | 2.5 | 60 | 24.97 | 6.38 |
| 6 | 7 | 2.5 | 60 | 32.94 | 5.85 |
| 7 | 3 | 4.5 | 60 | 13.41 | 6.41 |
| 8 | 7 | 4.5 | 60 | 23.76 | 4.94 |
| 9 | 5 | 3.5 | 42.5 | 22.74 | 7.71 |
| 10 | 5 | 3.5 | 42.5 | 24.47 | 7.32 |

* The yields (%) are expressed on dry matter basis

**Table S2** Chemical composition of JA tubers

| Component | | % DM |
|---|---|---|
| Extractives | Free Sugars | 2.77±0.1 |
| | Inulin | 71.33±4.34 |
| | Protein | 7.50±0.04 |
| Fibres | Xylose | 3.48±0.24 |
| | Arabinose | 3.00±0.32 |
| | Glucose | 6.23±0.73 |

*Numbers are mean values ± standard deviations for triplicates

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## CHAPTER FIVE

## 5. Bioprocess optimisation for high cell density endoinulinases production from recombinant *Aspergillus niger*

Manuscript

Title: Bioprocess optimisation for high cell density endoinulinases production from recombinant *Aspergillus niger*

Target journal: Applied microbiology and biotechnology

Summary

The principal aim of this chapter was to gain insight into the bioprocessing parameters influencing and challenges related to recombinant endoinulinases (InuA) production from *A. niger* strain during high cell density fermentation. The effects of the bioprocess parameters, glucose feed concentration, nitrogen sources concentration and growth rate (μ), on biomass growth and enzyme production in fed-batch culture were investigated. Although viscosity was prominent during the high cell density culture, the low dissolved oxygen level and the subsequent morphology change, from pellet to mycelial, did not affect the endoinulinases productivity. The experimental data illustrated the effect of a co-operative mechanism, among different factors, in pellet disintegration. The high biomass concentrations resulted in increased viscosity and poor mixing as well as low DO levels which impacted the integrity of the pellets. The high biomass concentration also increases the probability of pellet colliding and friction and consequently pellet disruption from the shear forces of collision. The high agitation speed, to improve the mixing efficiency and oxygen diffusion in the system, is associated with shear forces that disrupt fungal pellets. Increasing the feed concentration showed an advantage in increasing the volumetric productivity, however, it necessitated the supplementation of organic nitrogen sources of up to 12 g/L to prevent nitrogen limitation. This nitrogen was composed of a cocktail of yeast extract, peptone and casamino acids and the inclusion of these in the medium showed no impact on the fungal morphology of *A. niger* and thereby did not have negative impact on the viscosity. Endoinulinases production was not strictly growth associated, and only growth associated at $\mu > 0.04\ h^{-1}$, which had a low maintenance energy requirement. The data demonstrated that, strategies to improve the specific yields of *A. niger* would significantly improve the endoinulinases productivity of the

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system, which was limited in the maximum attainable biomass due to viscosity limitations and had a growth-associated endoinulinases production. The endoinulinases produced in this contribution was an important cocktail component for the optimised cocktail used in the hydrolysis and fermentation of the tuber residues (chapter 6) from the sequential extraction of protein and inulin (chapter 4).

Candidate declaration

The nature and scope of my contributions for chapter 5, pages 69-89, were as follows;

| Name of contribution | Extent of contribution (%) |
|---|---|
| Experimental Planning | 60 |
| Executing experiments | 80 |
| Interpretation of the data | 80 |
| Chapter compilation | 90 |

The contributions of the co-authors to chapter 4, pages 69-89, are the following;

| Co-author | email address | Contribution | Extent of contribution (%) |
|---|---|---|---|
| Shaunita H. Rose | shrose@sun.ac.za | Experimental planning<br>Executing experiments<br>Data interpretation<br>Modification of A. niger and chapter compilation | 5<br>20<br>10<br>10 |
| Eugéne van Rensburg | eugenevrb@sun.ac.za | Experimental planning<br>Data interpretation<br>Chapter revision | 10<br>5<br>25 |
| Annie F. A. Chimphango | achimp@sun.ac.za | Experimental planning<br>Data interpretation<br>Chapter revision | 5<br>5<br>10 |
| Johann F. Görgens | jgorgens@sun.ac.za | Experimental planning | 20 |

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| | | Data interpretation<br>Chapter revision | 10<br>65 |
|---|---|---|---|

Candidate Signature Date

Declaration by the co-authors:

The undersigned hereby confirm that:

- The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to CHAPTER 5, pages 69-89,
- No other authors contributed to CHAPTER 5, pages 69-89 besides those specified above, and

| Signature | Institutional affiliation | Date |
|---|---|---|
| | | |
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| | | |

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**Bioprocess optimisation for high cell density endoinulinase production from recombinant *Aspergillus niger***

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**Abstract**

Endoinulinases gene was expressed in recombinant *Aspergillus niger*, which was modified by the co-author from the Microbiology department of Stellenbosch University, for selective and high-level expression using an exponential fed-batch fermentation. The effects of the growth rate (μ), glucose feed concentration, nitrogen concentration and fungal morphology, on enzyme production were evaluated. A recombinant endoinulinases with a molecular weight of 66 KDa was secreted. Endoinulinases production was growth associated at μ> 0.04 $h^{-1}$, which is characteristic of the constitutive *gpd* promoter used for the enzyme production. The highest volumetric activity (670 U/ml) was achieved at a growth rate of 93% of $\mu_{max}$ (0.07 $h^{-1}$), while enzyme activity (506 U/ml) and biomass substrate yield (0.043 $g_{biomassDW}/g_{glucose}$) significantly decreased at low μ (0.04 $h^{-1}$). Increasing the feed concentration resulted in high biomass concentrations and viscosity, which necessitated high agitation for improved mixing and oxygen. However, the high agitation and low DO levels (ca. 8% of saturation) led to pellet disruption and growth in mycelial morphology. Enzyme production profiles, product ($Y_{p/s}$) and biomass ($Y_{x/s}$) yield coefficients were not affected by feed concentration and morphological change. The gradual increase in the concentration of nitrogen sources showed that, a nitrogen limited culture was not suitable for endoinulinases production in recombinant *A. niger*. Moreover, the increase in enzyme volumetric activity was still directly related to an increase in biomass concentration. An increase in nitrogen concentration, from 3.8 to 12 g/L, resulted in volumetric activity increase from 393 to 670 U/ml, but the $Y_{p/s}$ (10053 $U/g_{glucose}$) and $Y_{x/s}$ (0.049 $g_{biomasDWs}/g_{glucose}$) did not significantly change. The data demonstrated the potential of recombinant *A. niger* and high cell density fermentation for the development of largescale endoinulinases production system.


*Keywords*: Recombinant, A. niger, fed-batch, endoinulinases, morphology, pellet

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### 5.1. Introduction

Endoinulinases is an important polyfructose hydrolysing enzyme used for the production of fructooligosaccharides from inulin, and thus has a wide range of application in the food and pharmaceutical industries (Fanck, 2002, Singh and Chauhan, 2018). Endoinulinases is also important in the fermentation of inulin into ethanol, single cell protein, biodiesel and platform chemicals through enzymatic conversion into simple sugars (Yang *et al*., 2015, Johansson *et al*., 2015, Long *et al*., 2015).

Endoinulinases naturally exist in plants, fungi, yeast and, bacteria, and is produced as an inulinases cocktail composed of endo- and exoinulinases (Li *et al.,* 2012, Singh *et al.,* 2013, Singh and Chauhan 2018). Microorganisms are the best sources for commercial endoinulinases production (Leelaram *et al.,* 2016), because of their ease for large scale production; however, the low enzyme productivity from native strains is a major disadvantage. Moreover, the production of heterogeneous enzymes from native host is a major challenge due to co-expression and secretion of other native enzymes (Rose and van Zyl 2008), and this necessitates costly downstream processing in commercial production (Cheng *et al*., 2002).

High-level and homogenous endoinulinases expression can be achieved through heterologous protein expression using recombinant DNA technology (Alriksson *et al.,* 2009, Leelaram *et al.,* 2016). The filamentous fungi, *Aspergillus* sp., is widely used as host for industrial production of recombinant protein. *Aspergillus* sp. has rapid growth, high production and secretion capacity, as well the ability to perform post translational modifications (Rose and van Zyl 2008, Krull *et al.,* 2013). Moreover, *Aspergillus* sp. has the GRAS (Generally Regarded As Safe) status and thereby has a potential for application in recombinant enzyme production in the food and pharmaceutical industries (Krull *et al.,* 2013).

Fed batch fermentation is an industrially relevant production method, used to achieve high enzyme volumetric activity (Driouch *et al.,* 2012, Chen *et al*., 2015). However, the high viscosity associated with high cell density fermentation severely affects productivity (Casas-Lopez et al., 2005, James et al., 2007). The high broth viscosity impacts the mixing efficiency, oxygen and nutrients diffusion (Bhargava *et al.,* 2004, Driouch *et al*., 2010). Therefore, overcoming viscosity challenges in high density fermentation of *Aspergillus* is crucial to achieve high biomass yield and volumetric concentration of the recombinant protein (Casas-

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Lopez *et al.,* 2005, Driouch *et al.,* 2010). The feed concentration is also an important factor in reducing broth viscosity through controlling the biomass concentration during fed batch fermentation (Bhargava *et al.,* 2003, Casas-Lopez *et al.,* 2005). The use of highly metabolizable nutrients results in rapid growth in filamentous morphology which results in oxygen transfer limitations and reduced protein synthesis (Kumar *et al.,* 2003).

Currently, most of the available literature studies on recombinant endoinulinases production have been in bacterial or yeast hosts, and not filamentous fungi, despite the application of the latter for industrial enzyme production. Therefore, there is a need for the development and optimisation of bioprocess parameters for the use of *A. niger* in the production of endoinulinases.

The aim of the study was to develop and assess the potential of a recombinant *A. niger* strain for endoinulinases production with a glucose limited fed batch exponential fermentation strategy. *A. niger* D15 uridine auxotrophic (*pyrG*), protease-deficient (*prtT*), nonacidifying (*phmA*) mutant was transformed with pGT (*bla* $gpd_p$-$gla_r$) vector containing *Inu A* gene encoding endoinulinases. The transcription was under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter ($gpd_P$) of *A. niger* and glucoamylase terminator ($glaA_T$) of *A. awamori.* Enzyme secretion was directed by the native gene's secretion signal. The effects of the bioprocess parameters, glucose feed concentration, nitrogen sources concentration and growth rate, on biomass growth and enzyme production in fed-batch culture were investigated. The study aimed to gain insight on the factors influencing and challenges related to recombinant endoinulinases production from *A. niger* during high cell density fermentation.

## 5.1. Materials and methods

### 5.1.1. Media and cultivation conditions

All chemicals were of analytical grade and unless stated otherwise, sourced from Merck (Darmstadt, Germany). The *E. coli* DH5α strains were cultivated at 37°C in Terrific Broth and on Luria Bertani agar containing 100 µg ampicillin/ml for selective pressure (Sambrook *et al.,* 1989).

The *A. niger* D15 parental strain was cultivated at 30°C in minimal media (5 g/L yeast extract, 0.4 g/L $MgSO_4 \bullet 7H_2O$, 2 g/L casamino acids, 20 ml 50×AspA (300 g/l $NaNO_3$, 26 g/L KCl, 76 g/L $KH_2PO_4$, pH 6), 0.01 M uridine and 1 ml/L 1000×trace elements) (Rose and van Zyl 2002).

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Transformants were selected for on minimal medium lacking uridine. Media were inoculated to a concentration of $1\times10^6$ spores per ml unless stated otherwise. The *A. niger* D15 transformants were initially cultivated in 20 ml double-strength minimal media (2×MM) containing 10% glucose for screening purposes (enzyme activity determination). Cultivation took place in 125 ml Erlenmeyer flasks on a rotary shaker at 200 rpm at 30°C for three days. Supernatants were obtained by centrifugation at 12 000 *g* for 10 min at room temperature and stored at 4°C for further analysis.

### 5.1.2. DNA manipulations and gene amplification by PCR

Standard protocols were followed for all DNA manipulations and *E. coli* transformations (Sambrook *et al.,* 1989). The *A. niger* ATCC10864 strain was cultivated in minimal media for 72 h. Mycelia were harvested, frozen under liquid nitrogen and the DNA isolated (Rose and van Zyl 2002). The *InuA* was amplified from the genome using the polymerase chain reaction (PCR) with oligonucleotide primers listed in Table 5.1. TaKaRa Extaq Polymerase (TaKaRa Bio Inc. Otso Japan) was used for amplification of the genes with the reaction set up in accordance with the supplier's specifications in the Perkin Elmer GeneAmp® PCR system 2400 (Perkin Elmer, USA). The *InuA* was cloned into the *Not*I site of plasmid pGT (Rose and van Zyl 2002) to obtain pGT-InuA under the transcriptional control of the glyceraldehyde-3-phosphate dehydrogenase promoter ($gpd_P$) of *A. niger* and glucoamylase terminator ($glaA_T$) of *A. awamori.* The *pyrG* marker gene had been retrieved from pBS-pyrGamdS (Plüddemann and Van Zyl 2003) via PCR and was cloned into the *Eco*RI site on plasmid pUC18, generating pUC-pyrG. Spheroplasts were prepared from the *A. niger* D15 (*cspA1*, *pyrG1*, *prtT13*, *phmA*, a non-acidifying mutant of AB1.13, ATCC 9029) strain using lyzing enzymes (Sigma-Aldrich, Steinheim, Germany) in accordance with Punt and van den Hondel (1992). The pGT and pGT-InuA vectors (Figure 5.1) were respectively co-transformed with pUC-pyrG to *A. niger* D15 to generate the *A. niger* D15[pGT-control] and D15[InuA] strains.

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Table 5.1: The DNA sequences of the oligodeoxyribonucleotide primers used in this study

| Primer name | Oligodeoxyribonucleotide primer sequence | Restriction site* |
|---|---|---|
| InuA-left | 5'-TAGCGGCCGCGAATTCATGTTGAATCCGAAGGTT-3' | *Not*I |
| InuA-right | 5'-TAGCGGCCGCGTCGACTTAATCCCCTTCCCC-3' | *Not*I |

* relevant restriction sites are underlined

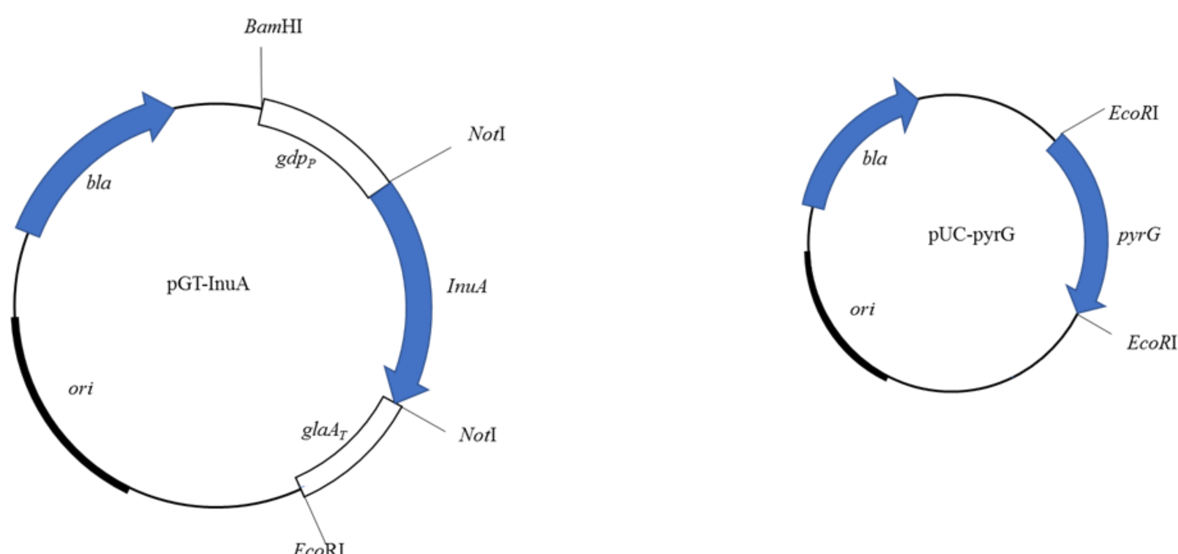


Figure 5.1: A schematic representation of the vector used in this study to construct the recombinant *A. niger* D15[InuA].

### 5.1.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

The proteins in the supernatant samples (20 µL) were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (12% SDS-PAGE) as described by Sambrook et al. (1989). Electrophoresis was carried out at 100 V at ambient temperature and the proteins visualised using the silver staining method (O'Connell and Stults, 1997). The broad-range Page Ruler Prestained SM0671 Protein Ladder (Fermentas, Shenzhen, China) was used as a molecular mass marker.

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### 5.1.4. Pre-inoculum preparation and cultivation medium for the fermentations

Stock cultures of the strains were stored at -80°C in 30% (v/v) glycerol as cryoprotectant. *A. niger* spore production was performed in spore plates containing 18 g/L agar, 2 g/L peptone, 1 g/L yeast extract, 10 g/L glucose and 2 g/L casamino acids with nitrates, at 30°C. The densely conidiated culture was harvested with saline solution (9 g/L NaCl) after 5 days. The minimal medium (MM) without uridine (Plüddemann and van Zyl, 2003) was used for the batch phase and was composed of 20 g/L glucose, 1 g/L casamino acids, 1 g/L peptone, yeast extract and 1.8 g/L MgSO4•7H2O. A pre-inoculum was prepared in an 1L Erlenmeyer flask containing 400 ml of the medium by inoculating with a spore concentration of *Aspergillus* of 1 X $10^6$ spores $mL^{-1}$. The flask was incubated at 30°C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 180 rpm for 12 hours.

### 5.1.5. Bioreactor operating conditions and growth medium

Enzyme production was carried out in batch cultures in 14 L BioFlo 110 bioreactors (New Brunswick Scientific company, Inc, USA) with a 10 L working volume and equipped with a polarographic DOT probe and a glass pH electrode (Mettler, Toledo, Sandton, South Africa). A batch culture with a total volume of 4 L of minimal medium (MM) without uridine (Plüddemann and van Zyl, 2003) was used for the enzyme production. Batch culture fermentation were carried out with an initial total nitrogen concentration in the range of 3.8-18 g/L. The pre-inoculum was subsequently transferred directly from the flask into the bioreactor. The cultivation temperature and pH were 30°C and pH 5.5, respectively. The pH was maintained through the cultivation period using 25 % w/v $NH_4OH$. Furthermore, a constant aeration rate of 0.8 v/v/m was maintained in the bioreactor during the cultivation. The dissolved oxygen was maintained above 30% saturation through a control loop that linked the agitation to the dissolve oxygen. The agitation speed was cascaded between 250 and 400 rpm, with 400 rpm set as a maximum to limit biomass degradation (Casas Lopez *et al.,* 2005).

Foaming was controlled by addition of 0.1% (v/v) of 30% antifoam (Sigma-Aldrich, Kempton Park, South Africa). At the end of the cultivation, the fermentation broth was vacuum filtered with a Buchner funnel and the enzyme activity in the broth determined before storage at 4°C.

### 5.1.6. Exponential feeding

An exponential feeding strategy was employed to evaluate the effects of growth rate on biomass and enzyme concentrations. Feeding was initiated at the end of the batch phase

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following depletion of the carbon source (glucose). Residual glucose was tested with glucose test strips (Accu-Chek®). The prediction of biomass ($X_t$) produced at a time t, using an exponential growth equation 1 (Kim et al., 2004), enabled estimation of the mass of glucose ($S_t$), the primary growth-limiting factor, fed during the fed-batch phase. This subsequently enabled the regulation of a pre-determined growth rate, where the glucose concentration in the reactor is assumed to be zero during the feeding phase (Cheng et al. 2002). Furthermore, the amount of glucose ($S_t$)to maintain a specific cell biomass ($X_t$) was determined according to equation 2 (James *et al.,* 2012; Li *et al*., 2016):

$$X_t = X_0 \, e^{\mu t} \tag{1}$$

where, $X_t$, $X_0$ and µ is biomass is time t, biomass at the end of the batch culture and growth rate.

$$S_t = \frac{V_0 X_0 \, (e^{\mu_{set} t} - 1)}{Y_{X/s}} + S_0 \tag{2}$$

where, $S_t$, $V_0$, $X_0$, $\mu_{set}$, $S_0$ and $Y_{x/s}$ is mass of glucose at time t, volume of broth at the end of the batch culture, biomass at the end of batch culture, the desired growth rate, mass of glucose at time 0 and biomass substrate yield coefficient, respectively.

### 5.1.7. Enzyme assay

Endoinulinases activity was determined based on the method of Chen *et al.,* (2015). Fermentation broth was vacuum filtered with a buchner funnel, crude enzyme subsequently filter-sterilised and diluted with acetate buffer for assaying. A 100 µL solution of 25% w/v inulin (Novozyme), 750 µL of 0.1M sodium acetate buffer (pH 5) and 100 µL of the crude enzyme were mixed and incubated at 50 $^{o}$C for an hour. The reaction was terminated by placing in boiling water for 5 min. The solution was centrifuged and analysed for reducing sugars with the DNS assay. One unit of endoinulinases activity was defined as the amount of enzyme required to produce 1µmol of reducing sugar per min under assay conditions. The positive control assay was performed with a commercial inulinase (Megazyme). The negative control was performed with broth culture from the *A. niger* host strain that had not been transformed with inulinase genes.

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## 5.2. Results and discussion

### 5.2.1. Strain development

Figure 5.2 is an illustration of an SDS PAGE for the selection of the positive transformant strains used in the fed-batch fermentation for endoinulinases (InuA) production. Endoinulinases with a molecular weight of ca. 66 kDa (Xu *et al.,* 2016) was identified relative to the molecular weight marker. The presence of the enzyme was further confirmed by the absence of a corresponding band of 66 kDa from the negative control lane 4 (Figure 2). Therefore, the recombinant *A. niger* overexpressed and secreted the endoinulinasess extracellularly.

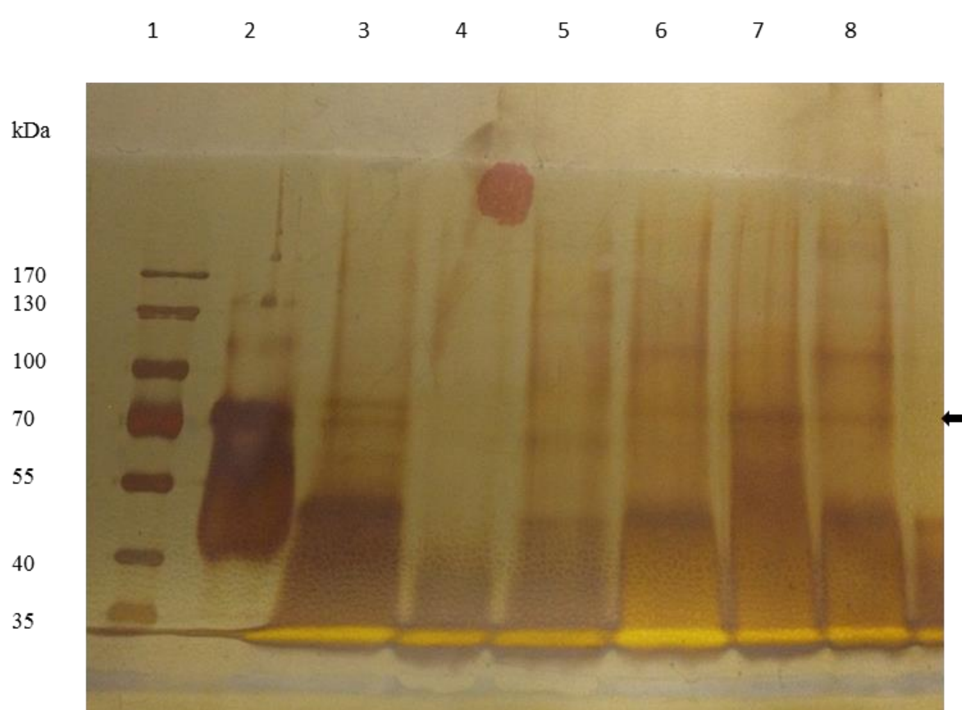


**Figure 5.2:** Silver-stained SDS-PAGE for crude supernatant from recombinant *A. niger* strain grown in shake flasks in MM media at 30 °C for 60 hours. Lane 1 is the molecular marker, lane 2-3 and 5-8 was obtained from *A. niger* D15 (*InuA*) strains and lane 4 is the *A niger* D15 (pGT control). The black arrow indicates the band representing endoinulinases (*InuA*)

### 5.2.2. Effects of glucose feed concentration on DO levels and mixing as well as subsequent impact on biomass and enzyme production

Biomass production, enzyme activity, DO and agitation speed profiles, during endoinulinases production at four different glucose feed concentrations are demonstrated in Fig. 5.3. The maximum specific growth rate ($\mu_{max}$) and biomass yield on substrate ($Y_{x/s}$) estimated in the batch culture prior to exponential feeding were 0.075 $h^{-1}$ and 0.49 $g_{biomassDW}/g_{glucose}$ (data not shown), respectively. Fed batch fermentations were carried out at a constant exponential

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growth rate (μ) of 0.06 $h^{-1}$ for the glucose feed concentration of 15 g/L and 100 g/L and 0.07 $h^{-1}$ for the glucose feed concentration of 200 and 300 g/L.

Biomass concentration and volumetric enzyme concentrations increased significantly ($p<0.05$) when the feed concentration was increased from 15 to 100, 200 and 300 g/L (Fig. 5.3a). Biomass concentration (Fig. 5.3a) and enzyme volumetric activity (Figure 5.3b) increased by almost 2-fold when the glucose feed concentration was increased from 15 to 200 and 300 g/L. The morphology of the recombinant *A. niger* strain used in this study remained in pellet form during culture (Figure 5.4a) and this coincided with DO levels of ca. 30% of saturation (Fig. 5.3c) maintained in the culture broth during fed batch fermentation with a dilute glucose feed (15 g/L).

In contrast, fed batch fermentation with a concentrated feed (100-300 g/L) resulted in a drastic change in the fungal morphology from pellet to mycelia form. The DO levels and agitation remained constant at ca. 30% of saturation (Figure 5.3c) and 250 rpm (Figure 5.3d), respectively, during the first 12 hours in the stationary phase of the culture. However, these changed drastically (Fig. 5.3c and d, respectively) when the culture entered the exponential growth phase. The DO levels dropped rapidly reaching a low of 8% of saturation and remained at this level throughout the fermentation period (Fig. 5.3c), due to the exponential increase in biomass concentration (Fig. 5.3a) and apparent increase in oxygen demand from the increased biomass concentration (Haack *et al.,* 2006, Porcel *et al.,* 2007, Haq *et al.,* 2015). Accordingly, the agitation speed increased, reaching the maximum set-speed limit of 400 rpm, because of the system attempting to maintain the DO setpoint of 30% of saturation (Figure 5.3d). Consequently, gradual pellet fragmentation (Figure 5.4), to mycelial form, was observed following the continued low DO levels of 8% of saturation (Fig. 5.3c) and agitation at 400 rpm (Fig. 5.3d). The experimental data illustrated the importance of bioprocess parameters on fungal morphology and the complex interdependence of the former in controlling the morphology (Walisko *et al.,* 2015). Therefore, the culture conditions can be controlled to induce fungal growth in pellet morphology and subsequently minimise the viscosity limitations associated with fungal growth in mycelial (Casas Lopez *et al.,* 2005, Coban *et al*., 2015; Driouch *et al.,* 2012). High cell densities are responsible for both increased broth viscosity (Driouch *et al.,* 2010 and Driouch *et al.,* 2012) and low DO levels in the culture, due to rapid oxygen uptake (James *et al*., 2007). The internal resistance, because of the former,

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results in inefficient mixing, oxygen as well as nutrient diffusion and subsequent pellet disintegration (Haq *et al*., 2015; Walisko *et al*., 2015). Change in the culture conditions, associated with inefficient mixing, such as pH has been hypothesised to contribute to pellet disintegration due the negative impact on electrostatic forces that contribute to the pellet integrity (Zhang and Zhang, 2016). Broth viscosity, at high cell densities, also increases the probability of collision and friction between the pellets which in turn weaken the hydrophobic and electrostatic interactions that keep the pellet structure intact (Wargenau *et al.,* 2011; Zhang and Zhang, 2016). Although, increasing agitation is necessary to improve mixing efficiency, oxygen and nutrient diffusion, at high biomass concentrations (Lopez et al., 2005; Haack et al., 2006), high agitation speeds results in shear forces that cause pellet disintegration and growth in mycelial which further affect broth viscosity (Driouch *et al.,* 2012; Haq *et al*., 2015). Sporh and co-workers (1998) illustrated that agitation speeds of at least 400 rpm resulted in fungal pellet degradation.

The change in fungal morphology, however, did not affect both biomass growth and enzyme production which contrasts with what has been reported for recombinant *A. niger*. In addition, the feed concentration did not have a significant ($p<0.05$) impact on the yields, biomass yield on substrate ($Y_{x/s}$) or enzyme yield on substrate ($Y_{p/s}$), and the specific enzyme productivity ($Y_{p/s}$) (Table 5.2). However, increasing feed concentration did significantly ($p<0.05$) increase the volumetric productivity ($Q_p$) of the system. For instance, an increase from 15 to 100 g/L resulted in a $Q_p$ increase from 7980 to 9831 U/L/h. The control of bioprocess conditions to support a specific fungal morphology, in recombinant enzyme production using recombinant *Aspergillus* sp., may also contribute to productivity improvements (Lopez et al., 2005; Haack et al., 2006). However, the experimental data for the recombinant endoinulinases production in *A. niger* is contrary to what has been reported previously, regarding enzyme production under critical dissolved oxygen levels and different fungal morphology.

Lopez and co-workers (2005) reported that *A. terruus* grew in large fluffy pellet morphology at DO levels of 80% saturation and agitation speeds less than 300 rpm and that agitation speeds > 300 resulted in growth in small pellet and a significant reduction in lovastatin productivity. Haack and co-workers (2006) reported that *A. oryzae* growth and lipase production were inhibited by low oxygen availability as result of increased biomass

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concentration, morphology change from pellets to mycelium, during the feeding phase of an exponential fed batch culture. The insignificant impact of the fungal morphology on the endoinulinases productivity in recombinant *A. niger* could be attributed to the point of enzyme synthesis. Haack and co-workers reported that lipase production was localised from the hyphal tips of *A. oryzae*, and morphology change from pellets to mycelial reduced the density of active hyphal tips thereby reducing lipase productivity.

In contrast, phytase production from *A. ficuum* (Coban *et al.,* 2015) and fructofuranosidase production from *A. niger* (Driouch *et al*., 2012) were enhanced by fungal growth in small pellets. Driouch *et al*., (2012) further illustrated that the total biomass obtained when the fungi was growing in large pellets was not significantly different to the biomass when fungi was growing in small pellets. High DO levels were not a necessity for recombinant endoinulinases production which was growth-associated (Driouch *et al*., 2010 and Driouch *et al*., 2012), and therefore productivity was not significantly different between pellet and hyphal growth despite the poor aeration and oxygen transfer of the latter.

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Table 5.2: Biomass growth and enzyme production productivities at glucose feed concentrations for exponential fed batch cultures of A niger D15 (InuA) strain at a nitrogen concentration of 3.8 g/L. The data is reported as a mean of three runs with the standard deviations.

| **Parameter** | **15 g/L** | **100 g/L** | **200 g/L** | **300 g/L** |
|---|---|---|---|---|
| $\mu_{set}$ ($h^{-1}$) | 0.06 | 0.06 | 0.07 | 0.07 |
| $\mu_{measured}$ ($h^{-1}$) | 0.06 (±.005) | 0.06 (±0.008) | 0.07 (0.003) | 0.07 (± 0.006) |
| Biomass (g/L) | 3.93 (±0.01) | 15.99 (±0.83) | 18.19 (±0.97) | 18.36 (± 1.92) |
| Biomass (g) | 37 (±0.14) | 86 (±4.14) | 79 (±3.87) | 78 (±6.31) |
| Activity (U/ml) | 83 (±3) | 360 (±24) | 401 (±16) | 393 (±37) |
| $Y_{x/s}$ ($g_{biomassDW}/g_{glucose}$) | 0.048 (±0.001) | 0.048 (0.001) | 0.048 (±0.002) | 0.049 (±0.001) |
| $Y_{p/s}$ ($U/g_{glucose}$) | 9786 (±836) | 9550 (±681) | 10555 (±823) | 10392 (±987) |
| $Y_{p/x}$ ($U/g_{biomassDW}$) | 19988 (±1305) | 19780 (±1417) | 20082 (±965) | 20436 (±1745) |

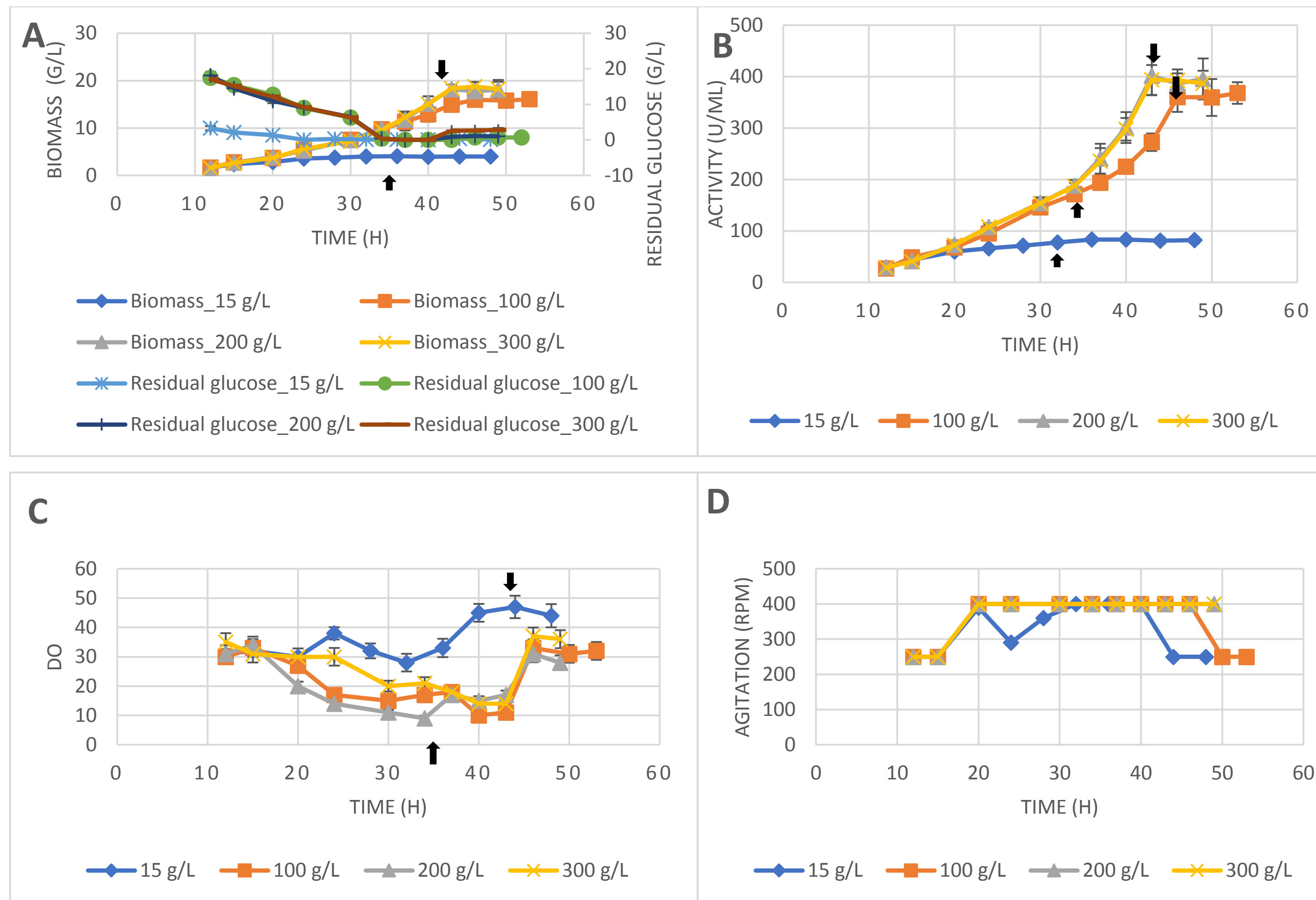


Figure 5.3: Effects of feed concentration on biomass production (A), enzyme activity (B) DO (C), and agitation speed (D) during endoinulinases production from recombinant A. niger D15 (InuA) strain. Exponential feeding was used and four feed concentrations (15, 100, 200 and 300 g/L) were evaluated at a growth rates close to µmax. The nitrogen-nutrients concentration was 3.8 g/L. Agitation speed was cascaded between 250-400 rpm. The feeding start and end point (s) are indicated by an arrow pointing upwards and downwards, respectively

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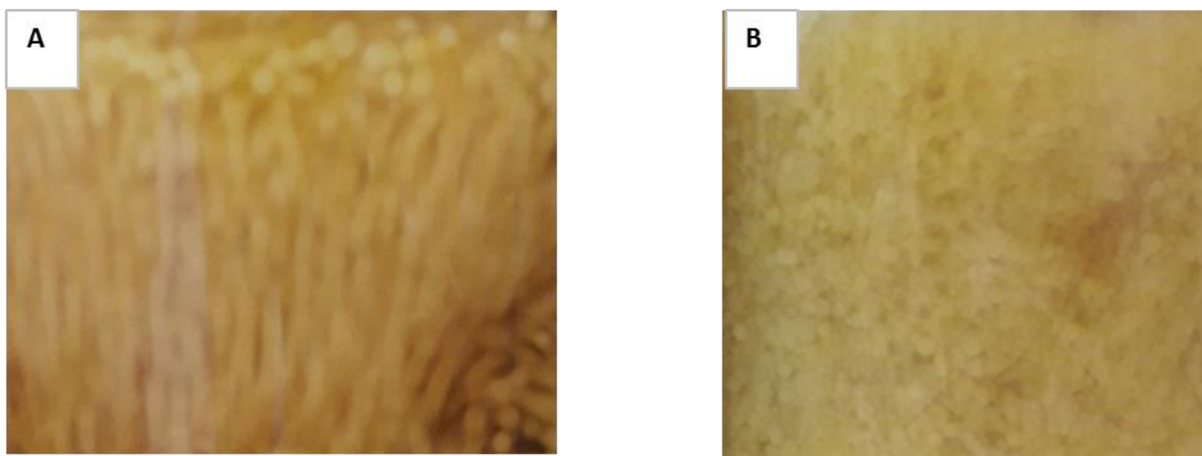


Figure 5.4: Fungal growth morphology during endoinulinases production from recombinant A. niger D15 (InuA) strain in high-cell density fed-batch culture. The pellet morphology (A) was predominant during the batch phase of the culture while mycelial morphology (B) was predominant during the feeding phase

### 5.2.3. Effects of nutrient concentration on biomass yield and enzyme production

Nitrogen sources are vital nutritional components for biomass growth and biosynthesis pathways (Sun *et al.,* 2004, Elisashvili *et al.,* 2009). Moreover, nutrient composition is an important factor in bioprocess optimisation and plays a critical role in maintaining optimal bioprocess conditions for the maximum productivity (Walisko *et al*., 2015). Therefore, fed batch fermentation were performed to evaluate the effect of nitrogen concentration, on fungal morphology, biomass yields, and enzyme productivity. The nutrients were comprised of a cocktail of three organic nitrogen sources, which are yeast extract, peptone and casamino acids in proportions of 50, 25 and 25 %, respectively (Plüddemann and van Zyl, 2003). Exponential fed batch cultures were carried out at a µ of 0.07 $h^{-1}$, glucose feed concentration of 300 g/L and organic nitrogen cocktail concentrations of 3.8, 12 and 18 g/L in the batch culture medium. An increase of approximately 3-fold in the nitrogen cocktail, from 3.8-12 g/L, resulted in a significant ($p<0.05$) increase in the final biomass concentration (Figure 5.5a) and volumetric enzyme activity (Fig. 5.5b), from 18.36 to 34.4 g/L and 393 U/L to 670 U/L, respectively. A further increase of the nutrients to 18 g/L did not result in a further increase in the biomass concentration and enzyme activity (Fig. 5.5a and b, respectively). The data thereby, demonstrated that an increase in the feed concentration should be supplemented with a corresponding amount of nitrogen to ensure the culture has excess nitrogen and carbon limited. Although, the increment in the nutrient concentration resulted in an increase in the final biomass concentration and enzyme volumetric activity, the $Y_{x/s}$ $Y_{p/x}$ and $Y_{p/s}$ did not

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significantly ($p>0.05$) differ (Table 5.3) at the different nutrient concentrations, demonstrating that the enzyme yield on biomass and carbon source (glucose) did not change.

Table 5.3: Biomass growth and enzyme production productivities at different nutrient concentrations for exponential fed batch cultures of the *A niger* D15 (*InuA*) strain. The data is reported as a mean of three runs with the standard deviations.

| Parameter | 3.8 g/L | 12 g/L | 18 g/L | p-value |
|---|---|---|---|---|
| $\mu_{set}$ ($h^{-1}$) | 0.07 | 0.07 | 0.07 | n.a. |
| $\mu_{measured}$ ($h^{-1}$) | 0.07 (±0.006) | 0.07 (±0.003) | 0.07 (±0.002) | n.a. |
| Biomass (g/L) | 18.36 (±1.92) | 34.40 (±0.87) | 33.94 (±1.46) | n.a. |
| Biomass (g) | 78.21 (6.31) | 184.42 (±15.13) | 182.68 (17.14) | n.a. |
| Activity (U/ml) | 393 (±37) | 670 (±28) | 667 (±35) | n.a. |
| $Y_{x/s}$ ($g_{biomassDW}/g_{glucose}$) | 0.049 (±0.001) | 0.049 (±0.001) | 0.049 (±0.001) | p>0.05 |
| $Y_{p/s}$ ($U/g_{glucose}$) | 10391 (±987) | 10053 (±845) | 10068 (±932) | p>0.05 |
| $Y_{p/x\ (U/gbiomassDW)}$ | 21435 (±1745) | 19187 (±1543) | 19581 (±1134) | p>0.05 |

n.a.: not applicable

Therefore, the organic nitrogen sources did not have a direct induction effect on recombinant endoinulinases production, confirming that enzyme production was growth related. The data demonstrated that at a feed concentration of at least 100 g/L glucose, the biomass growth and enzyme production were nitrogen limited at a concentration of 3.8 g/L nitrogen cocktail and conversely carbon limited at an excess nitrogen concentration of 12 g/L. The supplementation of the culture media with organic nitrogen sources of up to 12 g/L was sufficient to prevent nitrogen limitation without impacting the fungal morphology of *A. niger* in a negative way. Fu and co-workers (2014) reported that peptone had a positive impact on pellet formation. However, excess nitrogen components in the medium can result in fungal growth in mycelial form and consequently cause viscosity limitations.

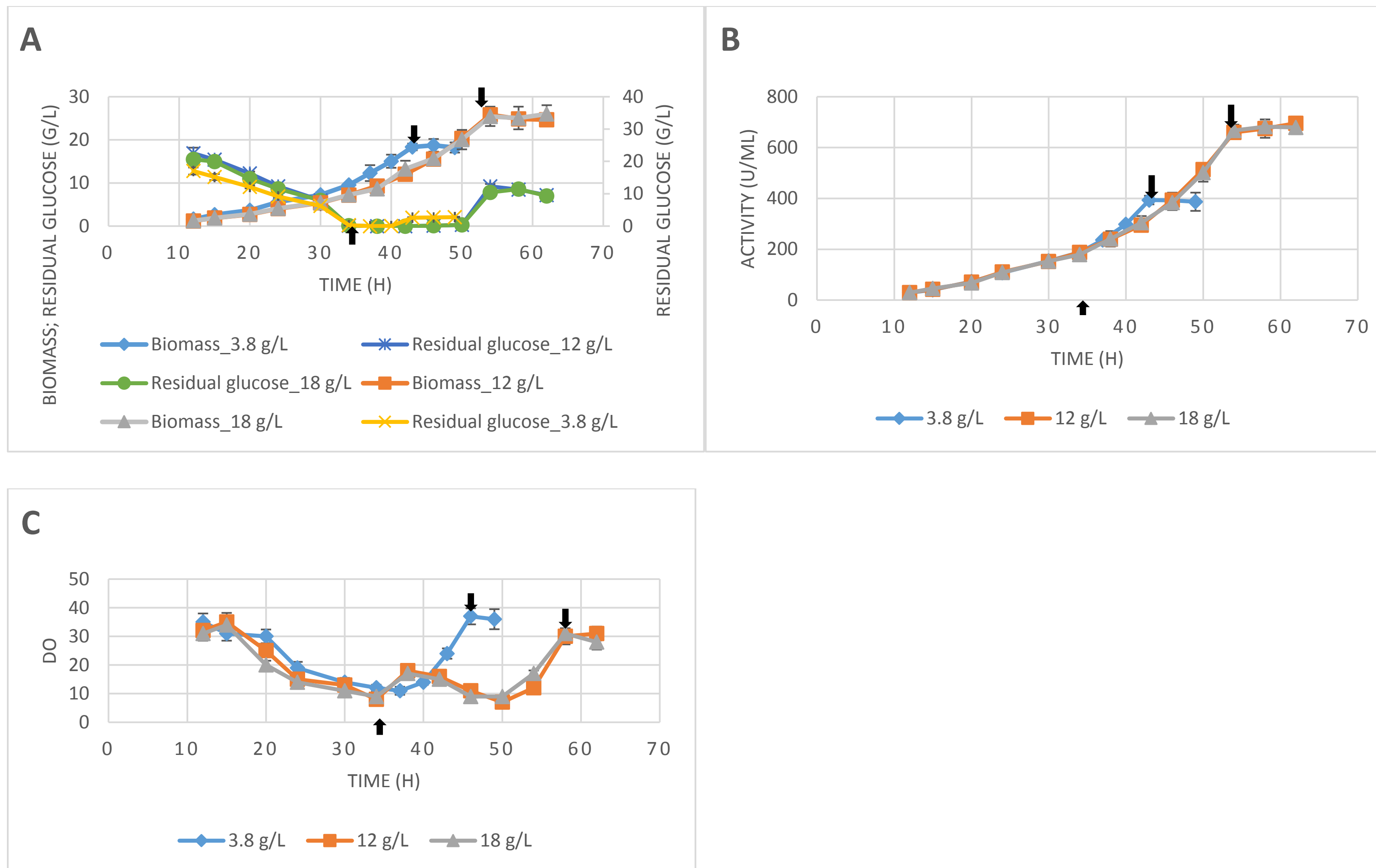


Figure 5.5: Biomass concentration (A), enzyme activity (B) and DO (C) using different concentration of the nitrogen sources. A cocktail of three nitrogen sources which are yeast extract, peptone and casamino acids in proportions of 50, 25, and 25 %, respectively. An exponential feeding rate of μ = 0.07 was used with a fixed glucose feed of 300 g/L. Agitation speed was cascaded between 250-400 rpm. The feeding start and end point (s) are indicated by an arrow pointing upwards and downwards, respectively

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### 5.2.4. Effects of growth rate on biomass growth and enzyme production as well as yields and productivities

Exponential fed batch fermentation at 93% and 53% of $\mu_{max}$, μ of 0.07 $h^{-1}$ and 0.04 $h^{-1}$, respectively, were performed to determine the effects of the growth rate on biomass growth and enzyme production, with a high glucose concentration feed (300 g/L) and excess nitrogen (18 g/L) supplementation. The agitation was cascaded between 250 and 400 rpm to minimize biomass degradation. Exponential feeding resumed after depletion of 20 g/L glucose from the batch culture and was terminated when residual glucose started to accumulate and DO levels spiked from the 8%, maintained during the exponential growth phase, to ca. 20% level of saturation (Fig. 5.6d). The highest biomass concentration was obtained at the higher growth rate of 0.07 $h^{-1}$ and was equivalent to 33.94 g/L, compared to 28.03 g/L obtained at the lower growth rate of 0.04 $h^{-1}$ (Fig. 5.6a). In addition, the highest volumetric activity of 680 U /ml was obtained at the higher growth of 0.07 $h^{-1}$ compared to 507 U/ml obtained at a growth rate of 0.04 $h^{-1}$ (Fig. 5.6b). The highest volumetric activity (680 U/ml) reported in this study was significantly lower than reported in a *Pichia pastoris*, a yeast host, which reported a volumetric activity of 4000 U/ml (Chen et al., 2014). The enzyme yield on this host was 36 U/$g_{biomass}$ in comparison to 20 U/$g_{biomass}$ reported for *A. niger*. The plots for the natural log of the total biomass (Fig. 5.6c) show that an exponential growth rate was maintained during the fed batch and that the measured growth rate deviated by approximately 5% and 10% from $\mu_{set}$ for μ of 0.07 $h^{-1}$ and 0.04 $h^{-1}$, respectively. Moreover, the plots had $R^2$ values of 0.99, further showing that a constant exponential growth rate was maintained during the fed batch fermentations. The end of the fermentation was accompanied by glucose accumulation and subsequent biomass growth cessation (Fig. 5.6a) as well as enzyme production cessation (Fig. 5.6b) and an increase in the DO levels to ca. 30% of saturation (Fig. 5.6d). In addition, visual inspection indicated that the culture was characterised by highly fragmented fungal mycelia components (Fig. 5.4b). Fungal fragmentation (Fig. 5.4b) was preceded by drastic drop in DO level to 8% (Fig. 5.6d), as result of an exponential increase in biomass concentration (Fig. 5.6a), and a subsequent increase in agitation speed, to maximum speed of 400 rpm (data not shown), to improve the mixing efficiency. Fragmentation started (Fig. 5.4) with gradual pellet disruption into mycelia and eventually degradation of the mycelia, at the end of fermentation (T 54 and 66 h for μ of 0.07 $h^{-1}$ and 0.04 $h^{-1}$, respectively), and this coincided with growth

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cessation. Furthermore, the transition from pellet morphology to mycelial, during the exponential growth phase, did not impact endoinulinases production (Fig. 5.6b). Comparisons of the yields and productivities (Table 5.4) between enzyme production at high growth rates (0.07 $h^{-1}$) and low growth rates (0.04 h-1) showed that the biomass and enzyme yield coefficients ($Y_{x/s}$ and $Y_{p/s}$ was 0.049 and 10068, respectively) were significantly ($p<0.05$) higher at the high growth rates (0.07 $h^{-1}$). The substrate specific consumption ($Q_s$ = 0.101) was also significantly higher at high growth rates (0.07 $h^{-1}$) compared to a $Q_s$ of 0.072 at μ of 0.07 $h^{-1}$ and 0.04 $h^{-1}$. The lower enzyme productivities and biomass yield at low specific growth rate could be attributed to a high maintenance energy requirement at low growth rates (Low and Chase 1999, Vos *et al*. 2016). Slow growing biomass has been reported to have high metabolic energy requirements for maintenance of cellular integrity and viability (Tannler *et al*. 2008, Vos *et al*. 2016) at the expense of biomass growth. The growth associated nature of endoinulinases production at higher growth rates, where maintenance energy did not have a significant impact, was attributed to the constitutive *gpd* promoter controlling endoinulinases expression from recombinant *A. niger* (Pal *et al.,* 2006, Rose and van Zyl, 2008).

Table 5.4: Biomass growth and enzyme production productivities at two specific growth rates for exponential fed batch cultures of *A niger* D15 (*InuA*) strain. The data is reported as a mean of three runs with the standard deviations.

| Parameter | 0.04 $h^{-1}$ | 0.07 $h^{-1}$ | p-value |
|---|---|---|---|
| μ ($h^{-1}$) | 0.046 (0.006) | 0.07 (±0.002) | n.a. |
| Biomass (g/L) | 28.03 (2.01) | 33.94 (±1.46) | n.a. |
| Biomass (g) | 150 (±8.41) | 182 (±17.14) | n.a. |
| Activity (U/ml) | 506 (±39) | 667 (±35) | n.a. |
| $Y_{x/s}$ ($g_{biomassDW}/g_{glucose}$) | 0.043[a] (0.001) | 0.049 (±0.001) | $p<0.05$ |
| $Y_{p/s}$ ($U/g_{glucose}$) | 7414 (±642) | 10068 (±932) | $p<0.05$ |
| $Y_{p/x}$ ($U/g_{biomassDW}$) | 17185 (±1048) | 19581 (±1134) | $p<0.05$ |

n.a.: not applicable

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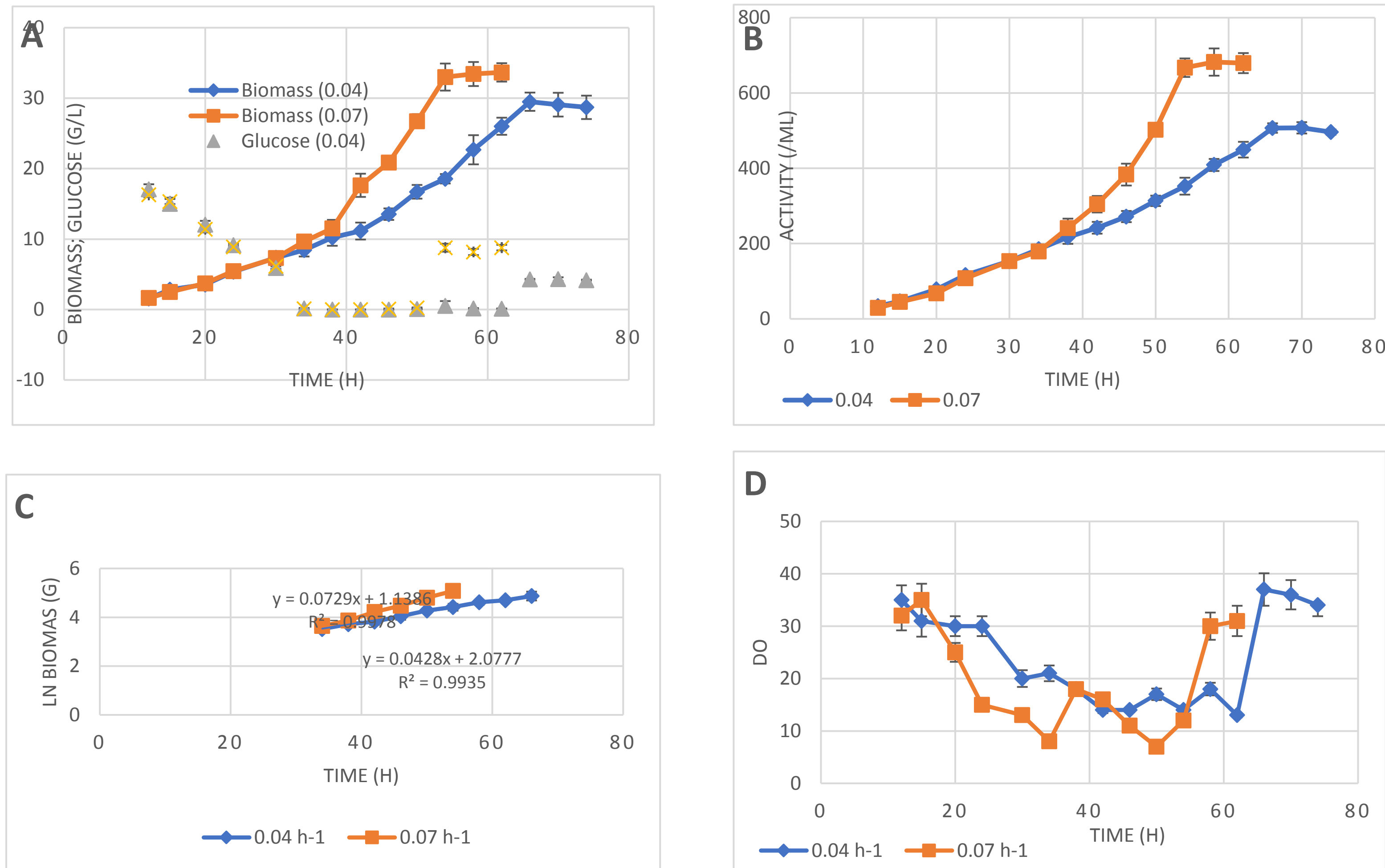


**Figure 5.6:** Biomass concentration (A), volumetric enzyme activity (B), specific growth rate profiles (C) and DO (D) during fed batch fermentation at different growth rates. Agitation speed was cascaded between 250-400 rpm. The feeding start and end point (s) are indicated by an arrow pointing upwards and downwards, respectively

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### 5.3. Conclusions

Viscosity challenges, associated with high cell densities during the fed batch phase, limited the ability of system to achieve enzyme volumetric activity similar to yeast systems. Therefore, strategies that improve the specific productivity of the *A. niger* system will enable attainment of improved volumetric activity, considering the culture has limitations in the maximum attainable biomass concentration. In summary, recombinant endoinulinases production from *A. niger* was not strictly growth associated and only growth associated at high growth rates. Moreover, the high maintenance requirements at low specific growth rate could have resulted in reduced enzyme production. High biomass concentrations combined with increased agitation rates during the fed-batch feeding, resulted in lower DO levels, and a combination of these factors triggered a change from pellet to hyphae morphology. *A. niger* continued to grow and produce the growth-associated enzyme at high growth rates, with low maintenance requirements, even when the DO levels reached critical level of 8% of saturation. Moreover, enzyme production continued with no changes in productivity at this critical level which was characterised by high viscosity and pellet disintegration.

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# CHAPTER SIX

## 6. Maximising the benefits of enzyme synergy in the simultaneous saccharification and fermentation of Jerusalem artichoke (*Helianthus tuberosus*) tuber residues into ethanol

Manuscript

Title: Maximising the benefits of enzyme synergy in the simultaneous saccharification and fermentation of Jerusalem artichoke (*Helianthus tuberosus*) tuber residues into ethanol

Target Journal: Waste and Biomass valorisation

Summary

In this chapter, the protein and inulin extraction residue of the tubers (chapter 4), were valorised through fed-batch simultaneous saccharification and fermentation (SSF) into ethanol and with the use of an optimised enzyme cocktail of endoinulinases (produced from chapter 5), exoinulinases (batch culture produced), pectinases and cellulases, to improve the hydrolysis and fermentation productivity. This contribution was important in demonstrating the technical feasibility of a biorefinery approach to utilising JA tubers, through the co-production of food, feed, and biofuel products, with synergistic benefits to food and energy security. The use of an optimised enzyme cocktail of lab produced (exo- and endoinulinases), and commercial (Cellic® CTec3 and Pectinex Ultra-SP) enzyme significantly improved the fermentation performance. Xylose and glucan recovery yields of 62.5 and 66.7%, respectively, were recorded with the optimised cocktail. The optimised cocktail thereby resulted in extensive LCF conversion with no pre-treatment to the tuber residues. This could be attributed to either the synergistic benefits of using accessory enzymes (pectinases) in the cocktail, protein, and inulin extraction process prior to fermentation or both. The synergistic interactions among the enzyme resulted in ethanol yield increases from as low as 37% to 83% of the theoretical maximum. Furthermore, the use of an optimized cocktail significantly improved the fermentation performance without changing the total protein loading. The ethanol concentration and yield were 32g/L and 59% with cocktail 1 compared to 38g/L and 83% with cocktail 2 which had higher activities of cellulases and endoinulinases. The change in the activities of the cocktail enzymes was necessary to alleviate the poor enzyme accessibility, associated with the high viscosity, and non-productive enzyme and lignin binding

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as the amount of non-fermentable and recalcitrant material increased significantly at high solids loading.

Candidate declaration

The nature and scope of my contributions for chapter 4, pages 93-116, were as follows;

| **Name of contribution** | **Extent of contribution (%)** |
|---|---|
| Experimental Planning | 70 |
| Executing experiments | 100 |
| Interpretation of the data | 80 |
| Chapter compilation | 100 |

The contributions of the co-authors to chapter 4, pages 93-116, are the following;

| **Co-author** | **email address** | **Contribution** | **Extent of contribution (%)** |
|---|---|---|---|
| Eugéne van Rensburg | eugenevrb@sun.ac.za | Experimental planning<br>Data interpretation<br>Chapter revision | 10<br>5<br>20 |
| Annie F. A. Chimphango | achimp@sun.ac.za | Data interpretation<br>Chapter revision | 5<br>10 |
| Johann F. Görgens | jgorgens@sun.ac.za | Experimental planning<br>Data interpretation | 20<br>10 |

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| | | | |
|---|---|---|---|
| | | Chapter revision | 70 |

Candidate Signature                                         Date

Declaration by the co-authors:

The undersigned hereby confirm that:

- The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to CHAPTER 6, pages 93-116
- No other authors contributed to CHAPTER 6, pages 93-116 besides those specified above, and

| Signature | Institutional affiliation | Date |
|---|---|---|
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**Maximising the benefits of enzyme synergy in the simultaneous saccharification and fermentation of Jerusalem artichoke (*Helianthus tuberosus*) tuber residues into ethanol**

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**Abstract**

**Purpose**

An integrated approach for the co-production of food, feed and biofuel products has the potential to improve the sustainability and maximise the economic value derived from Jerusalem artichoke (JA) tubers, with synergistic benefits to food and energy security. This study followed a biorefinery approach, involving the extraction of protein and inulin from JA tubers, and subsequently ethanol fermentation from the extraction residues using an optimised cocktail of hydrolytic enzymes.

**Method**

Inulin and protein products were pre-extracted from JA tubers, resulting in tuber residues enriched in unextracted inulin (38%) and lignocellulosic fibres (25%), both on a dry matter basis. A fed batch fermentation approach was used for ethanol production from JA tuber residues, using a mixture design, to optimize the cocktail of exo- and endoinulinases, Cellic® CTec3 and Pectinex Ultra-SP, to minimise the total enzyme dosage and maximise the sugar conversion and ethanol yields during simultaneous saccharification and fermentation of the tuber residues, by exploiting synergistic action between enzyme components in the cocktail.

**Results**

High gravity fermentation of the tuber residues with 21% w/v maximum solids loading, resulted in ethanol concentration and yield of 38g/L and 83% of the theoretical max, respectively, and a combined inulin and cellulose conversion yield of 74%. Synergistic co-operation among the enzymes significantly improved the hydrolysis of inulin and LCFs into fermentable sugars. The enzyme cocktail demonstrated a degree of synergy that resulted in the highest sugar yield of 390 $mg/g_{biomass}$ compared to yields in the range of 30-195 $mg/g_{biomass}$ for individual enzymes. The xylose and glucan recovery yield for this optimised cocktail was 62 and 66%, respectively. Moreover, ethanol yields were improved from 37%, when using individual enzymes, to 83% of the theoretical max, when using the enzyme cocktail. Cellic® CTec3 and Pectinex Ultra-SP played a major role in the disruption of hemicellulose and pectin, which potentially impedes the accessibility of inulin and cellulose for saccharification.

**Conclusions**

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The high solids fermentation of the JA residues after protein-inulin extraction demonstrated the technical feasibility of a multiproduct JA biorefinery. Synergistic co-operation among the hydrolytic enzymes significantly improved the saccharification and fermentation efficiency of the tuber residues, in part due to optimisation of the composition of the enzyme cocktail preparation. In addition, the optimised enzyme cocktail resulted in substantial lignocellulosic fibres conversion without the pre-treatment of the JA tuber residues. The resulting ethanol yield and concentration was similar to previous reports on ethanol production from unprocessed JA tubers.

**Statement of Novelty**

This manuscript is the first to demonstrate the production of ethanol from Jerusalem artichoke tubers residues, after the sequential extraction of high-value protein and inulin. The use of a unique and optimized cocktail of hydrolytic enzymes enabled high solids loading fermentation of the complex tuber-extraction waste residues. Moreover, the cocktail optimisation approach consequently enabled achievement of high ethanol yields and concentration as well as carbohydrates conversions thereby, making the simultaneous saccharification and fermentation of the tuber residues to be industrially relevant.

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## 6.1. Introduction

The integration of ethanol production from Jerusalem artichoke (JA) tuber residues, after the extraction of high-value protein and inulin products, has the potential to enhance the viability of this biorefinery strategy (Johansson *et al*., 2015; Yang *et al*., 2015). Protein and inulin find application in the food and pharmaceutical industries (Franck, 2002, Singh *et al*., 2008), while ethanol is globally the predominant biofuel. Currently 80% of the ethanol global consumption is for fuel purpose (OECD/FAO, 2016). Thus, this biorefinery approach offers a tool to simultaneously address food and energy security as well as global warming.

JA tubers are rich in inulin, accounting for up to 80% of the dry matter (DM) (Gunnarsson *et al*., 2014) and an attractive biorefining feedstock due to the good agronomic traits of the plant such as high yields, drought resistance and minimal input requirements (Yang *et al*., 2015). Although JA is an herbaceous plant native from the North America, it is widely cultivated in China and Europe for consumption as a vegetable (Bajpai and Bajpai, 1991), and used as animal fodder or soil rehabilitation, due to its ability to adapt to a range of microclimatic conditions (Izsaki and Kadi, 2013). Unlike grain-based feedstock, JA can be cultivated in marginal lands without a threat to and competition with food production (Long *et al*., 2016). In addition, the minimal usage of organic fertiliser and pesticides also contributes to the reduction of greenhouse gases emissions (Gunnarsson *et al*., 2014; Aguilera *et al*., 2015).

Inulin is a non-recalcitrant polyfructan that is relatively soluble in water (Naskar *et al*., 2010), with water-extraction of inulin from JA tubers resulting in yields between 60–80% (Gunnarsson *et al.,* 2014). Although, sequential extraction of protein and inulin has been demonstrated to be a potential biorefinery approach for the use of JA tubers, value-added applications remain to be identified for the extraction residues, which contain residual inulin and LC fibres. Thus, the residual inulin and LCFs can be valorised into ethanol directly by saccharification and fermentation, with no requirement for additional prior pre-treatment to enhance the enzymatic accessibility of inulin and LCF (Yuan *et al.,* 2011). In contrast, agricultural and forestry waste residues, require expensive pre-treatment and high enzyme dosage due to their recalcitrant nature (Hu *et al*., 2011; Liu *et al*., 2018).

Simultaneous saccharification and fermentation (SSF) is commonly applied in the industry for ethanol production from starch and LC-based biomass (Yuan *et al*., 2011; Olofsson, 2008) and has the potential to also make ethanol production from JA tubers economically feasible

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(Kádár *et al*., 2004). Moreover, SSF fermentation productivity can be enhanced by using fed-batch method to achieve high solids fermentation (Liu *et al*., 2010; Zhang *et al.,* 2010). The gradual addition of the substrate during fed-batch is ideal for tuber fermentation which have high viscosity limitations. The resulting high solids SSF is an attractive technology, with water and energy consumption savings. Furthermore, the high ethanol concentration yield results in low distillation cost during downstream processing (Wang *et al*., 2007). High solids fermentation has been widely and successfully used for grain-based ethanol production (Srichuwong *et al*., 2009; Lim *et al*., 2013; Poonsrisawat *et al*., 2011), however, high viscosity remains a substantial limitation in tuber fermentation (Srichuwong *et al.,* 2009; Yingling *et al*., 2011). High viscosity severely affects mixing efficiency thereby, resulting in poor mass transfer and fermentation productivity due to incomplete polysaccharide hydrolysis (Zhang *et al*., 2010, Andersen *et al*., 2008).

Acceptable viscosity levels in high-solids SSF cultures can be maintained through efficient hydrolysis of biomass by enzyme cocktails present in the culture. Shrichuwong *et al*., (2009) demonstrated that the use of enzyme cocktail of pectinases, cellulases and hemicellulases reduced the viscosity of potato mash and resulted in improved starch hydrolysis and fermentation (Srichuwong *et al*., 2009). Cellulases and pectinases cocktails have also been demonstrated to reduce viscosity during sweet potato (Zhang *et al*., 2010) and cassava (Yingling *et al*., 2011) fermentation. The use of additional accessory enzymes to improve fermentation efficiency can increase production costs since high enzyme cost is one of the major challenges in biorefining (Merino *et al*., 2006). Thus, the design and use of optimised proportions of enzyme cocktails with synergistic interactions has the potential to reduce the overall enzyme loading while improving the hydrolysis productivity and ethanol yields (Berlin *et al.,* 2007; Liu *et al*., 2010).

The aim of this study was to convert the residues of the JA tubers, after protein and inulin extraction, into ethanol, through high-solids, fed-batch SSF. A mixture design was used to optimise the proportions of endoinulinases, exoinulinases, pectinases and cellulases enzymes in a cocktail, to improve the fermentation productivity of high solids fermentation. The principal aim was to determine the effects of partially substituting inulinases (exo- and endoinulinases) with pectinases and cellulases, on the saccharification efficiency of the tuber residues. A mixture-design was applied to maximise the benefits derived from the degree of

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synergy (DS) between different cocktail enzyme proportions, so as the minimise enzyme dosages and maximise hydrolysis and fermentation performance.

## 6.2. Materials and methods

### 6.2.1. Raw material handling and preparation

Jerusalem artichoke tubers were donated by the Glen Agricultural College, Mangaung Metropolitan Municipality, Free State, South Africa. Tubers were harvested in the late winter and stored at -18 °C. The frozen tubers were rasped with a hand-grater and pressed with a kitchen juice presser, and the pressed tuber used for sequential protein and inulin extraction according to the method of Maumela *et al*., (2019). The extraction residue was vacuum packaged and frozen at -18 $^{o}$C for use in hydrolysis-fermentation experiments.

### 6.2.2. Yeast, inulinase strains and inoculum preparation

For production of inulinases, *Aspergillus niger* D15 (uridine auxotrophic (*pyrG*), protease-deficient (*prtT*), nonacidifying (*phmA*) mutant was separately transformed with pGT (*bla* $gpd_p$-$gla_r$) vectors containing *EnInu* and *ExInu* genes encoding endoinulinase (chapter 5) and exoinulinase, respectively. Enzyme secretion was under the control of the native gene's secretion signal. Stock cultures of the strains were stored at -80$^{o}$C in 30% (v/v) glycerol as cryoprotectant. *A. niger* spore production was performed on potato dextrose agar (PDA) plates containing 1.8% w/v agar, 0.2%w/v peptone, 0.1% w/v yeast extract, 1% w/v glucose and 0.2% casamino acids with nitrates (0.05% w/v KCL, 0.15% w/v $KH_2PO_4$ and 0.6% $NaNO_3$) and 1mL/L of trace elements, at 30$^{o}$C. The densely conidiated culture was harvested with saline solution (0.9% w/v NaCl) after 5 days. Trace elements contained 2.2% w/v $ZnSO_4.7H_2O$, 1.1% w/v $H_3BO_3$, 0.5% w/v $MnCl_2.4H_2O$, 0.5% w/v $FeSO_4.7H_2O$, 0.17% w/v $CoCl_2.6H_20$, 0.16% w/v $CuSO_4.5H_2O$, 0.15% w/v $Na_2MoO_4.2H_20$ and 5% w/v EDTA (no Na salt).

*Saccharomyces cerevisiae* MH1000 (van Zyl *et al*., 2011) used in the simultaneous saccharification and fermentation (SSF), was stored at -80$^{o}$C in 30% (v/v) glycerol as cryoprotectant. The seed culture was prepared in yeast potato dextrose (YPD) media (7.5% w/v glucose, 10% w/v yeast extract and 2% w/v tryptone). The seed culture was prepared by plating the yeast from the glycerol stock on YPD agar plates and incubating at 30$^{o}$C for at least 3 days. A single colony was subsequently inoculated in a 50 mL YPD media in a 250 mL Erlenmeyer flask and incubated at 30$^{o}$C for 24 h. A 1 mL aliquot was drawn and inoculated into a fresh 50 mL YPD media in a 250 mL Erlenmeyer flask and incubated at 30$^{o}$C for 18 h to

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reach an $OD_{600}$ of ca. 5. The seed culture was then centrifuged at 4000 rpm for 15 min and pellet used for the fermentation and culture was inoculated to a concentration of 1 g/L dry weight yeast.

### 6.2.3. Endoinulinases and exoinulinases production

Enzyme production from *Aspergillus niger* (chapter 5) was carried out in batch cultures in 14 L BioFlo 110 bioreactors (New Brunswick Scientific company, Inc, USA) with a 10 L working volume an equipped with a polarographic DOT probe and a glass pH electrode (Mettler, Toledo, Sandton, South Africa). The enzymes were produced in separate strains which were *A. niger* D15 (*InuA*) and *A. niger* D15 (*InuE*) for endoinulinases (Chapter 5) and exoinulinases, respectively. A batch culture with a total volume of 4 L of minimal medium (MM) without uridine (Plüddemann and van Zyl, W. 2003), supplemented with 1% v/v of trace elements, was used for the enzyme production. The medium was composed of 2% glucose and supplemented with 0.1% w/v casamino acids, 0.8 w/v % peptone, 0.8% w/v yeast extract and 0.08% w/v $MgSO_4.7H_2O$. A pre-inoculum was prepared in 1L Erlenmeyer flask (*un-baffled*) containing 400 ml of the medium by inoculating with a spore concentration of $1x10^6$ spores $mL^{-1}$. The flask was incubated at 30°C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 180 rpm for 12 h. The pre-inoculum was aseptically transferred into the bioreactor from the flask through a silicon tube connecting the flask and reactor. The temperature of the cultivation was maintained at 30°C and pH 5.5 was maintained through the cultivation using $NH_4OH$ (25 % w/v). The dissolved oxygen was maintained above 30% saturation through a control loop that linked the agitation to the dissolve oxygen and a constant aeration rate of 0.8vvm was maintained during the cultivation. Foaming was controlled by addition of 0.1% (v/v) of 30% antifoam (Sigma-Aldrich, Kempton Park, South Africa). The culture broth was vacuum filtered with a Buchner funnel and broth was assayed for enzyme activity before storage at 4°C.

### 6.2.4. Enzyme cocktails

Crude *A. niger* preparations of endo- and exoinulinases, as well as commercial fungal preparations of Cellic® CTec3, Cellic® HTec3 and Pectinex Ultra-SP were used in the saccharification and fermentation experiments. The protein content of the enzyme preparations was determined using the Kjeldahl nitrogen analysis (AOAC Method 11, 2011)

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### 6.2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was used to screen for enzymes to be used in the cocktail for the saccharification of the tuber residues. Endo- and exoinulinases, hemicellulases and cellulases enzymes were selected for screening based on the composition of the substrate solid residues (Maumela *et al.*, 2019). The enzyme dosages were based on a literature survey on the minimum possible enzyme dosage used in saccharification and fermentation studies that resulted in the highest ethanol yields. For the initial screening a dosage of 30 $U/g_{sugars}$ was selected for inulinases (Ge *et al.*, 2005; Zhang *et al.*, 2010), which was equivalent to $12.45mg_{protein}/g_{inulin\ sugars}$, and $15mg_{protein}/g_{glucans}$ was used for cellulases and hemicellulases (Mokomele *et al.,* 2018). The hydrolysis was performed in 50 mL Erlenmeyer flasks with a working volume of 10 mL solution containing 0.1M acetate butter and the enzyme(s) and a solids loading of 3% w/v of the tuber residue dry matter, at pH 5 and 50 $^{o}$C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 100 rpm. The reaction was stopped after 48 h and the sample centrifuged, and supernatant analysed for sugars using HPLC.

Optimisation of the cocktail enzymes proportions was carried out according to the simplex design (Montgomery, 2013). The total enzyme dosage was fixed at $12.45mg_{protein}/g_{inulin\ sugars}$, and proportions of endo- and exoinulinases, cellulases and pectinases varied according to the simplex design (Table 6.2). The enzyme dosage was selected from a literature survey and based on the minimal inulinases dosage that produced the highest ethanol yields and concentration for Jerusalem artichoke tuber fermentation. The dosage was also based on inulin since it was the primary and abundant polymer in the tuber (Maumela *et al.*, 2019) residues. The experiments were performed in batch mode. The degree of synergy (DS) was calculated according to the method of Andersen et al 2008 as follows in equation 1:

$$DS = \frac{Y_T}{\sum \beta_i Y_I} \tag{1}$$

where $Y_T$, $Yi$ and $\beta i$ is the sugar yield achieved by enzymes in combination, sugar yield of enzyme *i* working individually and proportion of enzyme *i* in the cocktail. The DS measures relative degree of the effect of at least two combined enzymes, to the sum of the individual enzymes (Andersen *et al.*, 2008).

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### 6.2.6. Statistical design of experiments

A {2, 4} simplex lattice design augmented with interior points and centroid (Montgomery, 2013) was used to optimise the proportions of blending endo- and exoinulinases, pectinases and cellulases for the saccharification of tuber residue. Hemicellulases was excluded from the cocktail due to the background hemicellulases activity in cellulase (Cellic® CTec3). The goal for the optimisation was to determine the effects of partially substituting inulinases (exo- and endoinulinase) with pectinase and cellulases, on the saccharification and fermentation efficiency of the tuber residues. The ultimate optimisation goal was to determine the cocktail composition that maximises the saccharification of the tuber residues, without exceeding an acceptable total enzyme dosage. The experimental design had a total of 15 runs for both the hydrolysis and SSF experiments. The enzyme dosages were fixed at 20, 40 and 60 $mg_{protein}/g_{sugars}$ with the enzyme ratios varying between 0 and 1.

### 6.2.7. Statistical analysis

The total fermentable sugars and ethanol final concentration was used to determine the effects of the various enzyme mixtures, for the hydrolysis and SSF experiment, respectively. Regression analysis with Statistica® 13.1 was used to determines the effects of the different enzyme mixtures on total sugar yields and final ethanol concentrations as well determining the specific interactions (degree of synergy) among the different enzyme components. Regression analysis was also used to predict the optimum proportion of the enzymes for the maximum ethanol concentration yield.

### 6.2.8. Fed-batch simultaneous saccharification and fermentation

SSF was performed in 50 mL capped flasks with a working volume of 10 mL at an initial solid loading of 6% w/v DM. The tuber residues were autoclaved at 121°C for 15 min in the flask and cooled. Filter sterilised enzymes, sterile corn steep liquor (5% v/v) and 50 g/L of chloramphenicol (0.0001% v/v) were aseptically added to the flask. The yeast inoculum was added to give a concentration of 1 g/L of dry cells and the fermentation was carried out at 37°C, pH 5 and 150 rpm for 96 h. For fed-batch, the additional substrate (5% w/v DM) was fed at 8 hours intervals to achieve a final solids loading of 21% w/v, which was limited due to substrate viscosity. A sample was taken at the end of the fermentation and determined for ethanol, sugars, acetic acid, formic acid and glycerol with HPLC (section 6.2.8). The ethanol yield was calculated based on the amount of sugars consumed by the yeast relative to the

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total fermentable sugars in the residue and theoretical yield of 0.51 $g_{ethanol}$/g of glucose or fructose consumed.

### 6.2.9. Analytical methods

Ethanol, glucose, xylose, rhamnose and galactose concentrations were determined through a high-performance liquid chromatography (HPLC) system fitted with an Aminex HPx-87 column, a cation-H Micro Guard Cartridge, RI-101 detector, pump and an AS3000 Auto Sampler (Thermo Scientific Products, Bio-Rad, South Africa). The column was operated at 65 °C with a 5 mM sulphuric acid mobile phase at the flowrate of 0.6 mL/min. Fructose concentration was analysed using HPLC with a Dionex 3000 System equipped with a Grace® Prevail Carbohydrate ES Column (250 x 4.6 mm) and a Varian® evaporative light scattering detector. The operating conditions were an injection volume of 10 uL, column temperature of 30 $^{o}$C, flow rate of 0.6 mL/min and a gradient mobile phase of acetonitrile.

## 6.3. Results and Discussion

### 6.3.1. Chemical composition of the JA tuber extraction residue

Table 6.1 is a summary of the chemical composition of the JA residues after protein-inulin extraction, in comparison to the raw tubers. The inulin, cellulose, and hemicellulose content of the residue was 38.98, 11.88 and 12.62%, respectively (Maumela *et al*., 2019). The amount of lignocellulosic fibres was approximately 24% and higher in the JA residues after extractions, compared to the raw tubers (ca. 12%), due to the removal of protein and inulin during the extraction. The inulin content in tuber residues was approximately 45% lower than in the raw tubers. The total fermentable sugars (inulin and cellulose) comprised ca. 50% of dry matter in JA tuber residues and is comparable to agricultural residues such as bagasse and cane leaf (Yuda *et al.,* 2014; Mokomele *et al*., 2018), thus making the residues a potential feedstock for ethanol production. Lignin and ash comprised of 17.41 and 8.45%, respectively, of the dry matter (DM). Meanwhile the rhamnose, galactose and mannose contents were 1.02, 2.01 and 0.88%, respectively, thereby confirming the presence of pectin in the tubers of JA.

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Table 6.1: Compositional analysis of the raw tuber and tuber residues[a] on dry matter basis. Data reported as mean values ±standard deviation of triplicates.

| Component | Raw tuber* (% w/w) | Tuber residue* (% w/w) |
|---|---|---|
| Inulin | 71.33±4.34 | 38.98±2.01 |
| Cellulose[b] | 6.23±.05 | 11.88±.08 |
| Hemicellulose[c] | 6.48±.02 | 12.62±.09 |
| Lignin | 8.82±0.6 | 17.41±1.09 |
| Ash | 5.99±.07 | 8.45±.08 |
| Pectin[d] | 1.04±.01 | 3.91±0.21 |
| Protein | 7.5±0.04 | 5.63±0.03 |

[a]Residue obtained after sequential protein and inulin extraction

[b]Calculated as sum of glucose and cellobiose

[c]Calculated as a sum of arabinose and cellobiose

[d]Calculated as a sum of rhamnose, galactose and mannose

### 6.3.2. Screening for enzymes for hydrolysis of the extraction residue

Screening experiments for enzymatic hydrolysis were used to determine the enzymes to be included and optimized in the cocktail for the saccharification of the extraction residues. The tuber extraction residue, like other agricultural waste feedstock, is composed of a diverse group of sugar polymers with a complex chemical structure (Table 6.1). These polymers require different enzymes for effective solubilisation and saccharification during ethanol fermentation (Kádár *et al*., 2004; Berlin *et al*., 2007). Moreover, appropriate enzyme cocktails should be designed and optimised to minimize the overall enzyme loading thereby, enabling cost-effective utilisation of biomass in biorefining (Hunag *et al*., 2012; Berlin *et al*., 2007). Exo- and endoinulinases, cellulases and hemicellulases were used in the hydrolysis experiments. The screening did not however, include hydrolysis for exo- and endoinulinase separately since the combined use of both enzymes has synergistic benefits, with the latter randomly cleaving inulin to make more reducing ends available for cleavage by the former (Hu *et al*., 2013). Pectinase was excluded from the screening since it is not directly involved in the hydrolysis of either inulin or lignocellulosic fibres into fermentable sugars. Separate inulinases assays were not included because endo does not make reducing sugars. The enzymes were selected based on the chemical composition of the residue (Table 6.1). The addition of cellulose to exo- and endoinulinases during hydrolysis, resulted in a significant ($p < 0.05$) increase in the

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concentration of total fermentable sugars (Fig. 6.1). The sugar concentration increased from 4.5 to 7 g/L when cellulase was added compared to an increase to 6 g/L when hemicellulases was added (Figure 6.1). Exo- and endoinulinases as well as cellulases were subsequently selected for inclusion in the enzyme cocktail for the saccharification of the extraction residue during ethanol production. Although the residue is composed of approximately 12% hemicellulose (Table 6.1), the exclusion of hemicellulases from the cocktail will be compensated by the background hemicellulases activity in the commercial cellulases (Cellic® CTec3) preparation (Berlin *et al.*, 2007).

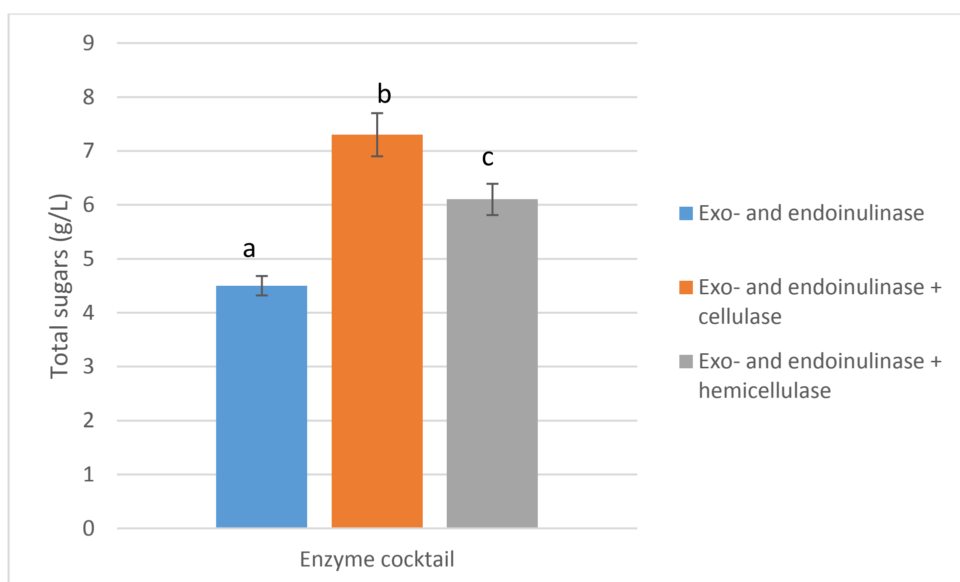


Figure 6.1: Screening enzymes for inulin and fibres hydrolysis to obtain sugars for ethanol fermentation. The data represents mean values of triplicates with standard deviation. The different letters represent differences that are statistically significant

### 6.3.3. Optimization of the enzyme proportions in the cocktail and synergistic interactions of the enzymes during enzymatic hydrolysis

Cellulases (Cellic® CTec3) supplementation to exo- and endoinulinases during hydrolysis resulted in significant ($p<0.05$) increase in the total sugars recovery (Fig. 6.1). Therefore, the mixture design approach was subsequently used to determine the optimal proportions of the enzymes in the cocktail, to maximise the enzyme saccharification efficiency. The presence of pectin, a significant contributor to viscosity (Srichuwong *et al.*, 2009), in the waste residues (Table 6.1) has the potential to reduce the saccharification and consequently the fermentation efficiency. Thus, the inclusion of pectinases into the cocktail was considered.

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An augmented simplex lattice mixture design (Table 6.2) was subsequently used to determine the optimum proportions of exo- and endoinulinases, cellulase and pectinase for the enzymatic hydrolysis of the tuber residues. The enzyme dosage used in the hydrolysis experiment was limited to 20 $mg_{protein}/g_{sugars}$ based on the minimum inulinases dosage, from literature, for the fermentation of JA tubers (Ge *et al.,* 2005; Zhang *et al.,* 2010; Mokomele *et al*., 2013). The mixture design was composed of 15 experimental points including center points (Table 6.2). The experimental data was used for regression analysis to evaluate the significant enzymes and interactions thereof, during hydrolysis and impact on the total sugar recoveries. The results of the regression analysis are summarised in the Pareto chart (Figure 6.2). Saccharification with different combinations of the cocktail resulted in total fermentable sugars recoveries of between 30 and 390 $mg/g_{biomass}$ (Table 6.2). The corresponding xylose and glucan recovery yield for this data point was 62.5 and 66.7%, respectively. The cocktail thereby resulted in extensive LCF conversion without prior pre-treatment to the tuber residues. This could be attributed to either the synergistic benefits of using accessory enzymes (pectinases) in the cocktail, the protein and inulin extraction process prior to fermentation or both. Hu and co-workers (2013) reported the synergistic benefits for LCFs hydrolysis when xylan degrading accessory enzymes were added to cellulases in the hydrolysis of corn stover. The pressing and hot water extraction, to extract proteins and inulin (Pfariso and co-workers 2019), could have conditioned and improved the porosity of the tuber residues thereby improving the LCFs accessibility and hydrolysis by the enzyme. The lowest sugar recovery (30 $mg/g_{biomass}$) was obtained at run 4 when pectinases was used alone, while the highest recovery (390 $mg/g_{biomass}$) was at run 11 with a combination of all the four enzymes (Table 6.2). In addition, glucose and xylose contributed to ca. 90% of the total sugar recovery at run 4 and this could be attributed to the fact that the fungal-derived pectinase (Pectinex Ultra-SP) has been reported to have some background cellulase and hemicellulases activity (Lim *et al*., 2013). Pectinases addition also resulted in the accumulation of rhamnose and galactose monomers thereby, further confirming the presence of pectin in the tuber residue (Poonsrisawat *et al*., 2011).

The results from the regression analysis showed that all enzymes except pectinases had a significant ($p<0.05$) impact on the sugar recovery. However, the data showed significant ($p<0.05$) interaction of pectinases with exo- and endoinulinases as well as the cellulases

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(Figure 6.2). Thus, the above observations and data showing simultaneous accumulation of pectin monomers as well as significant increase in the sugar recovery when pectinase was added to the cocktail (Table 2), demonstrates that pectinases is not directly involved in the hydrolysis of inulin and cellulose. Pectin has been reported to coat cellulose fibres, consequently causing an access barrier for enzyme (Berlin *et al*., 2007). Furthermore, the reducing ends of inulin oligomers have been reported to be capable of forming interactions with other components such as proteins and polysaccharides (Mensink *et al*. 2015). Thus, the role of pectinases could have been the disruption of pectin-cellulose/inulin interaction and liberation thereof, and subsequently improving saccharification efficiency. Pectin disintegration may also be important in enhancing inulin and cellulose permeability or accessibility by their respective hydrolytic enzymes, through increasing the tuber residue porosity (Hu *et al*., 2011).

Furthermore, the degree of synergy (DS) was determined between/amongst the different enzyme in the cocktail to evaluate the relationship and degree of cooperation. Although pectin did not significantly ($p>0.05$) affect the sugar recoveries as an individual enzyme, it demonstrated synergy when used with other enzymes (Table 6.2). For instance, a 50:50 combination of pectinase and exoinulinase resulted in an increase of sugar recoveries from 145 to 172 mg/$g_{biomass}$, compared to when exoinulinase was used alone; the resulting degree of synergy was 1.97, thereby demonstrating that the cooperation between pectinase and exoinulinase was synergistic rather than additive. The Pareto chart (Figure 6.2) summary also illustrates that pectinase showed synergistic interactions with exoinulinase and endoinulinase at binary level. Furthermore, the regression analysis data showed that the synergy between exoinulinase and pectinase was higher than that of endoinulinase and pectinase. Cellulase showed a higher level of synergy with pectinase compared with endo- and exoinulinase (Figure 6.2). The estimated degree of synergy was consistent with the regression analysis results and showed a DS between cellulose and pectinase to be 2.48 compared to 1.04 with exoinulinase (Table 6.2). The regression analysis predicted optimal proportions of the cocktail were; exoinulinases: endoinulinases: cellulases: pectinases = 0.625, 0.125, 0.125 and 0.125, (cocktail). Moreover, this proportion was similar to where the highest sugar recoveries (run 11 were obtained from the experimental data (table 6.2)

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Table 6.2: Mixture design summarising the cocktail components and proportions as well as sugar recoveries

| | | | | | | | Sugar recoveries (mg/$_{gbiomass}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Run | Exo | Endo | Cellulase | Pectinase | Glucose | Arabinose | Xylose | Fructose | Pectin | Total sugar recoveries[a] | DS |
| 1 | 1 | 0 | 0 | 0 | 42.62 | 0.00 | 0.00 | 102.15 | 0.00 | 145.00 | 1 |
| 2 | 0 | 1 | 0 | 0 | 29.37 | 0.00 | 0.00 | 68.02 | 0.00 | 98.17 | 1 |
| 3 | 0 | 0 | 1 | 0 | 109.88 | 4.50 | 60.10 | 20.70 | 0.00 | 195.03 | 1 |
| 4 | 0 | 0 | 0 | 1 | 24.70 | 0.00 | 3.70 | 1.70 | 18.11 | 30.00 | 1 |
| 5 | 0.5 | 0.5 | 0 | 0 | 34.21 | 0.00 | 0.00 | 69.84 | 0.00 | 104.82 | 0.86 |
| 6 | 0.5 | 0 | 0.5 | 0 | 111.20 | 4.90 | 19.68 | 40.71 | 0.00 | 176.58 | 1.04 |
| 7 | 0.5 | 0 | 0 | 0.5 | 48.20 | 0.00 | 0.00 | 123.40 | 17.8 | 172.50 | 1.97 |
| 8 | 0 | 0.5 | 0.5 | 0 | 118.20 | 1.21 | 60.25 | 25.21 | 0.00 | 204.89 | 1.39 |
| 9 | 0 | 0.5 | 0 | 0.5 | 37.21 | 0.00 | 0.00 | 78.89 | 17.59 | 116.25 | 2.29 |
| 10 | 0 | 0 | 0.5 | 0.5 | 129.86 | 3.03 | 65.02 | 19.21 | 18.21 | 217.50 | 2.48 |
| 11 | 0.625 | 0.125 | 0.125 | 0.125 | 133.45 | 2.21 | 71.98 | 184.95 | 8.24 | 390.00 | 2.97 |
| 12 | 0.125 | 0.625 | 0.125 | 0.125 | 130.20 | 1.98 | 52.40 | 147.57 | 8.45 | 330.00 | 2.90 |
| 13 | 0.125 | 0.125 | 0.625 | 0.125 | 145.20 | 1.01 | 53.10 | 151.20 | 7.21 | 350.00 | 2.24 |
| 14 | 0.125 | 0.125 | 0.125 | 0.625 | 126.08 | 2.46 | 49.21 | 114.76 | 18.19 | 292.50 | 2.78 |
| 15 | 0.25 | 0.25 | 0.25 | 0.25 | 131.20 | 3.01 | 50.89 | 136.91 | 13.21 | 322.50 | 2.75 |

[a]Sum of glucose, arabinose, xylose and fructose

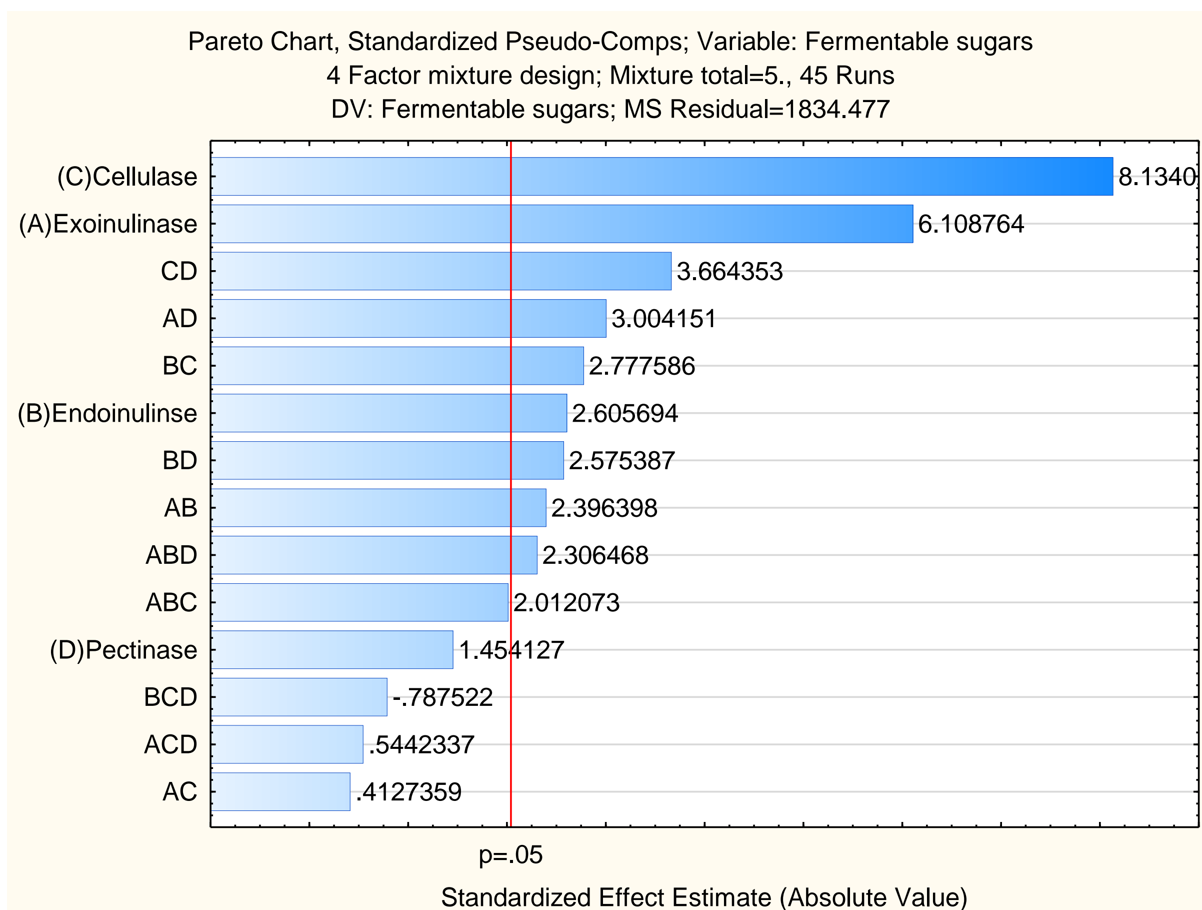


Figure 6.2: Pareto chart summarizing the effects of the different cocktail proportions on the sugar recovery

In contrast, the use of equal proportions of exo- and endoinulinase (50:50) resulted in a severe reduction in sugar recoveries. The recovery was reduced from 145 to 104 mg/$g_{biomass}$, in comparison to when exoinulinase was used alone, and consequently the DS was lower at 0.8 (Table 6.2). Inulin is a linear polymer (Naskar *et al.*, 2010), capable of forming helical structures when in solutions (Mensink *et al.*, 2015). Endoinulinase is important in the disruption of these supramolecular structure (Liu et al., 2016) due the enzyme's ability to randomly cleave inulin polymers into oligomers of low DP (Hu *et al.*, 2013) that are more soluble and accessible for exoinulinase hydrolysis. The synergetic interaction between endo- and exoinulinase is, however, dependent on the relative proportions of the different components of the cocktail. Hu and co-workers (2011) also demonstrated that increasing the proportion of xylanase relative to cellulase significantly improved cellulose and xylan hydrolysis and the degree of synergy increased. Increasing the exoinulinase content in the cocktail to 62.5% of the total protein dosage and reducing the proportion of endoinulinase to 12.5% as well as the inclusion of 12.5% cellulase and pectin, significantly increased the synergy degree to 2.97 (Table 6.2). In contrast, a reduction in the exoinulinase proportion

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below 62.5%, with a relative increase in at least endoinulinase, cellulase or pectinase, resulted in a reduction in both the sugar recoveries and DS (Table 6.2). For instance, using equal proportions (25%) of all the four enzymes (Run 15, Table 6.2) resulted in a decrease in the sugar recoveries and fructose content from 390 to 322 mg/$g_{biomass}$ and 184 to 136 mg/$g_{biomass}$, respectively. On the other hand, using a cocktail with 62.5% (Run 12, Table 6.2) of pectinase resulted in a decrease of sugar recoveries from 390 to 292 mg/$g_{biomass}$. The reduction in sugar recoveries and consequent reduction in the DS with an increase in the proportion of either, endoinulinase, cellulase or pectinase relative to the proportion of exoinulinase, could be attributed to unproductive binding, which shields exoinulinase accessibility to the reducing ends of inulin (Hu et al., 2011). Although inulin is a linear polymer (Naskar et al., 2010), it is also capable of forming helical structures when in solutions (Mensink *et al*., 2015) and endoinulinase is capable of the disruption of these supramolecular structure (Liu *et al*., 2016), due the enzyme's ability to randomly cleave inulin polymers into oligomers of low DP (Hu *et al*., 2013). Inulin cleavage into oligomers subsequently makes it more soluble and accessible to exoinulinase hydrolysis into fermentable monomers. Consequently, the cleavage of these oligomers by exoinulinase is important in reversing endoinulinase inhibition by the oligomers (Andersen *et al*., 2004). Therefore, this demonstrates that the synergetic interactions between endo- and exoinulinase are also reciprocal.

**6.3.4. Fed-batch simultaneous saccharification and fermentation**

Fed-batch SSF was used for the conversion of the extraction residues into ethanol. Three enzyme loadings (20, 40 and 60 $mg_{protein}/g_{sugars}$) based on the proportions, with the highest sugar recoveries, from the mixture design, exoinulinases: endoinulinases: cellulases: pectinases = 0.625, 0.125, 0.125 and 0.125, (cocktail 1) were used with biomass solids loading of 6, 11, 16 and 21%. The solids loading was selected based on the previous studies on the fermentation of JA tubers for ethanol production. Ethanol concentrations in the range of 15-32 g/L were demonstrated, corresponding to 40-80% ethanol yields (Fig. 6.3a and b, respectively).

The data demonstrated that increasing the enzyme dosage increased both the ethanol yield and concentration with solids loading in the range of 6-21% w/v. An increase of the dosage from 20-60 $mg_{protein}/g_{sugars}$ resulted in an ethanol concentration increase from 15 to 17 g/L (Fig. 6.3a), equivalent to an increase in the yield from 77-90% (Fig. 6.3b), at the solids loading

of 6% w/v. Moreover, an ethanol concentration increase, from 22 to 32 g/L (Fig. 6.3a), corresponding to a 40-62% (Fig. 6.3b) yield increase, was recorded at 21% w/v solids loading.

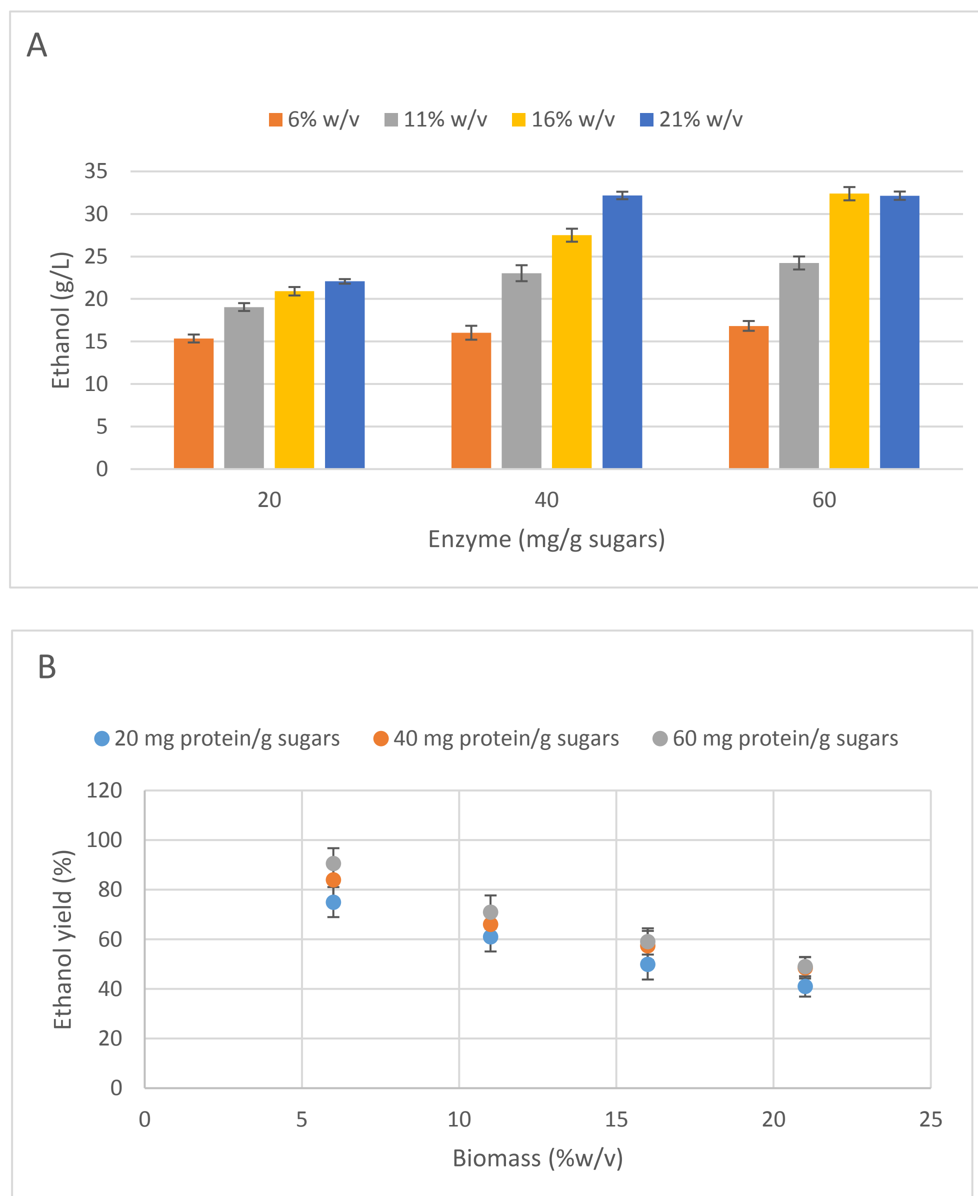


Figure 6.3: Ethanol concentrations (g/L) and sugar conversion yields (A and B, respectively) for fed-batch simultaneous saccharification and fermentation of JA tubers solid residues obtained after sequential extraction

In contrast, the ethanol yield decreased with an increase in the solids loading at all enzyme dosages (Fig. 6.3b). For instance, the ethanol yield decreased from ca. 90% to ca. 50%, at an enzyme dosage of 60 $mg_{protein}/g_{sugars}$, when the solids loading increased from 5 to 21% w/v. A final ethanol concentration of 32 g/L was obtained at a 21% w/v solids loading with an enzyme dosages of 40 and 60 $mg_{protein}/g_{sugars}$, illustrating that enzyme dosage was not a limitation to improving fermentation productivity at high solids loadings.

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The decrease in the ethanol yield with a solid loading increase could be attributed to the increase in the broth viscosity and poor mixing efficiency. Moreover, the accumulation of non-utilized tuber components, by the yeast, such as lignin and xylose contribute to severe stearic obstruction consequently limiting enzymatic hydrolysis (Hu *et al*., 2013; Saha *et al*., 2003). Lignins have been reported to unproductively bind to enzyme thereby, significantly restraining hydrolytic enzymes and reducing performance, thereof (Saha et al., 2005a). High solids loading has been reported to increase the external osmotic stress of the yeast resulting in loss of cell viability, growth and fermentation productivity (Olofsson *et al*., 2009; Liu *et al*., 2010).

### 6.3.5. Effects of solids loading on enzyme synergy and fermentation productivity in fed-batch SSF

Enzyme synergy and consequently saccharification efficiency are not only affected by variations in the proportion of the enzyme components of a cocktail. The accumulation of non-utilized and recalcitrant material in the residual solids (Saha *et al*., 2005b; Berlin *et al*., 2007) and variations in the relative biomass components as results of fermentation and feeding, both have an impact on enzyme synergy and saccharification efficiency. A {2, 4} simplex lattice design, with an enzyme loading of 60 $mg_{protein}/g_{sugars}$, was used to determine the impact of changing the enzyme proportion on the ethanol yields. Fed-batch SSF fermentation was performed with solids loading of 16 and 21% w/v. Regression analysis summary (Table 6.3) shows that changes in the solids loading from 5 to 16 and 21% % w/v resulted in differences in the effects of the cocktail components on fermentation productivity and the corresponding synergistic interaction. In contrast to low solids loading saccharification where cellulase had the most significant ($p<0.05$) impact on sugar recoveries (Table 6.3) when used alone, endoinulinases had the most significant impact at high solids loading. Moreover, the data illustrated that all the four enzymes had a significant effect on the final ethanol concentration (Table 6.3). Exoinulinase showed the highest synergy with both cellulase and pectinase. Endoinulinase had minimal synergy with exoinulinase and pectinase while there was no marked synergy with pectinase (Table 6.3). The regression analysis results showed marked synergy among cellulase, endo- and exoinulinase. The refined cubic regression model generated for the ethanol concentration is represented by equation 2,

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$$\text{Ethanol (g/L)} = 2.17*Exo + 26.28*Endo + 20.62*Cellu + 12.89*Pectinase + 42.06*Exo*Endo + 86.8*Exo*Cellu + 96.09*Exo*Pectinase + 60.32*Cellu*Pectinase + 472.12*Exo*Endo*Cellu - 873.48*Exo*Endo*Pectinase - 416.55*Exo*Cellu*Pectinase \quad (2)$$

The data demonstrated that a change in the cocktail enzyme proportion improved the ethanol concentration (14-38 g/L) and yield (reaching a maximum of 83% of theoretical max), at higher solids loading (16% w/v) without changing the total protein dosage of 60 $mg_{protein}/g_{sugars}$ (Table 4). Regression analysis was used to determine the optimum proportion of the cocktail components for maximum saccharification and fermentation of the tuber residues based on the final ethanol concentration. The optimum proportion of enzymes for high solids loading (21% w/v) SSF was estimated to be exoinulinase: endoinulinase: cellulase: pectinase = 0.4:0.22:0.21:0.1 (cocktail 2) compared to 0.625:0.125:0.125:0.125 in cocktail 1. The highest ethanol concentration and ethanol yield obtained at a solids loading of 16% w/v with cocktail 2 was 34.5 g/L and 83%, respectively compared to 32 g/L and 78%, respectively, with cocktail 1 (optimised at low solids loading). On the other hand, the highest concentration and yield for the 21% solids loading was 38 g/L and 70%, respectively, with cocktail 2 compared to 32 g/L and 59%, respectively, with cocktail 1 (Table 6.4). The ethanol concentration and yields for the tuber residues were comparable to those for the fermentation of pre-treated lignocellulosic residues such as bagasse (Mokomele *et al*., 2018), wheat straw (Saha *et al*., 2005a) and rice hull (Saha *et al*., 2005b).

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Table 6.3: Summary of the regression analysis for the saccharification and SSF mixture designs illustrating the significant factors for the hydrolysis of JA tubers at different solids loading

| | Solids loading (%w/v) | | | | | |
|---|---|---|---|---|---|---|
| | 3 | | 16 | | 21 | |
| **Factor** | **Coefficient** | **p-value** | **Coefficient** | **p-value** | **Coefficient** | **p-value** |
| **Exo (A)** | 150.92 | 0.001 | 17.7 | 0.000 | 2.177 | 0.018 |
| **Endo (B)** | 64.37 | 0.013 | 29.33 | 0.000 | 26.28 | 0.000 |
| **Cellu (C)** | 200.95 | 0.0003 | 22.58 | 0.000 | 20.62 | 0.000 |
| **Pectinase (D)** | 35.92 | 0.155 | 14.22 | 0.01 | 12.89 | 0.013 |
| **AB** | 290.13 | 0.022 | 13.61 | 0.002 | 42.06 | 0.000 |
| **AC** | 49.97 | 0.682 | 63.9 | 0.000 | 86.8 | 0.000 |
| **AD** | 363.71 | 0.005 | 74.3 | 0.000 | 96.09 | 0.000 |
| **BC** | 336.28 | 0.009 | 7.49 | 0.077 | 8.26 | 0.000 |
| **BD** | 311.8 | 0.015 | 5.86 | 0.163 | 7.24 | 0.063 |
| **CD** | 443.64 | 0.001 | 65.78 | 0.000 | 60.32 | 0.101 |
| **ABC** | 6068.02 | 0.052 | 411.1 | 0.000 | 472.12 | 0.000 |
| **ABD** | 6956.86 | 0.027 | -1065.56 | 0.000 | -873.48 | 0.000 |
| **ACD** | 1641.86 | 0.059 | -532.56 | 0.000 | -416.55 | 0.000 |
| **BCD** | -2375.02 | 0.436 | 565.27 | 0.000 | 151.63 | 0.160 |

Optimisation of the proportions of the cocktail components, considering the specific composition of the substrate, is important to ensure maximum synergy among the enzyme and substantial conversions of the polymers into fermentable sugars (Liu *et al*., 2010; Zhang *et al*., 2010). The optimal ratio of cocktail 2, which was suitable for efficient hydrolysis (ca. 80% conversion) at high solids loading of the tuber residues, had higher activities of cellulase

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and endoinulinases, compared to cocktail 1 which was sufficient for low solids loading hydrolysis. In contrast, exoinulinases and pectinases ratios were lower in cocktail 2. The high solids loading SSF of the tuber residues therefore, necessitated a change in the proportions of the cocktail enzymes to alleviate the poor substrate accessibility, because of the high viscosity, and non-productive enzyme and lignin binding. The accumulation of non-fermentable lignin material has reported to result in uncompetitive binding to the substrate thereby, interfering with the activity of hydrolytic enzymes (Saha *et al*., 2005b; Berlin *et al*., 2007; Gonzalez *et al*., 2015). Furthermore, the accumulation of xylan oligomers has been reported to interfere with cellulases hydrolysis by either competitive binding or being a physical barrier to the active sites. Therefore, an increase in the proportion of Cellic® CTec3 (with background hemicellulases activity), for high solids loading SSF, was necessary to disrupt the significant amount of non-utilised lignocellulosic components and their interference with hydrolytic enzymes (Zhang *et al*., 2010; Berlin *et al*., 2007; Gonzalez *et al*., 2015) The inefficient mixing, resulting from the high broth viscosity, could have negatively impacted the solubility of inulin and accessibility by exoinulinases. Therefore, the increase in the activity of endoinulinases, with a higher affinity and specificity for polymeric inulin (Liu *et al*., 2016), was important for inulin hydrolysis into soluble oligomers which were easily accessible and further hydrolysed into fermentable sugars by exoinulinases.

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Table 6.4: Mixture design illustrating the ethanol yields and residual sugars from the fed-batch simultaneous saccharification and fermentation of the tuber residue

| | **Solids loading (%w/v)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **16** | | | | **21** | | | |
| | **Etoh** | **Cellobiose** | **Xylose** | **Arabinose** | **Etoh** | **Cellobiose** | **Xylose** | **Arabinose** |
| **Run** | **(g/L)** | **(g/L)** | **(g/L)** | **(g/L)** | **(g/L)** | **(g/L)** | **(g/L)** | **(g/L)** |
| 1 | 15.47 | 2.28 | 0.73 | 0.39 | 17.50 | 2.01 | 0.85 | 0.33 |
| 2 | 26.08 | 2.10 | 0.59 | 0.47 | 29.13 | 1.98 | 0.76 | 0.38 |
| 3 | 20.42 | 0.24 | 5.35 | 0.76 | 22.38 | 0.43 | 8.38 | 2.25 |
| 4 | 12.69 | 0.11 | 7.03 | 1.78 | 14.02 | 0.19 | 7.73 | 0.87 |
| 5 | 24.33 | 1.93 | 0.72 | 0.19 | 26.52 | 2.15 | 0.85 | 0.49 |
| 6 | 32.68 | 0.71 | 7.49 | 0.71 | 35.71 | 0.46 | 8.67 | 1.49 |
| 7 | 31.14 | 0.22 | 6.95 | 1.84 | 34.13 | 0.47 | 7.88 | 2.10 |
| 8 | 25.10 | 0.00 | 8.18 | 1.76 | 27.43 | 0.00 | 8.96 | 1.58 |
| 9 | 20.98 | 0.00 | 6.27 | 1.70 | 22.84 | 0.00 | 7.36 | 1.69 |
| 10 | 31.42 | 0.16 | 9.15 | 1.04 | 34.44 | 0.17 | 9.38 | 1.70 |
| 11 | 21.58 | 0.00 | 6.91 | 1.72 | 23.54 | 0.00 | 7.73 | 1.95 |
| 12 | 27.62 | 0.00 | 7.48 | 1.27 | 30.13 | 0.00 | 7.73 | 2.25 |
| 13 | 34.47 | 0.12 | 9.81 | 1.72 | 37.81 | 0.22 | 10.15 | 2.00 |
| 14 | 20.61 | 0.13 | 8.05 | 1.80 | 22.64 | 0.14 | 8.55 | 2.13 |
| 15 | 20.53 | 0.14 | 7.88 | 2.17 | 22.46 | 0.18 | 9.10 | 2.09 |

Analysis of the residual sugars (Table 6.4), from the fermentation broth, was performed to determine the changes and potential underlying mechanism of synergism at high solids loading SSF. Residual cellobiose from fermentation runs was highest in the run where cellulases was excluded (Table 6.4). However, the addition of cellulases resulted in a decrease in the amount of detectable residual cellobiose. Although, increasing the cellulases proportion in the cocktail resulted in general trend showing a decrease in residual cellobiose, the lowest cellobiose concentration was observed when cellulases was used in combination with at least one of the other three enzymes in the cocktail. In addition, the xylose content followed a similar trend with the cellobiose content (Table 6.4). This illustrates potential reciprocal synergy between cellulases and other enzyme cocktail components (Jalak *et al.*,

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2012; Hu *et al.*, 2013). Moreover, the data demonstrates that 50% of residual xylose was recovered in fermentation broth when using a cocktail (cocktail 2) proportion optimised at a high solids loading (Run 13 Table 6.4). In contrast ca. 37% was recovered using cocktail proportion optimised at a low solids loading (cocktail 1).

## 6.4. Conclusions

The fermentation of JA residues after protein-inulin extraction demonstrated the potential of a biorefinery based approach to ethanol production from JA tubers. High solids, SSF culture with the extraction residue resulted in an ethanol concentration and yield of 38 g/L and 83%, respectively. The use of an enzyme cocktail of exo- and endoinulinases, cellulases, and pectinases significantly improved the fermentation performance with xylose and glucan recoveries of at least 62 and 66%, respectively. The optimised cocktail, therefore, resulted in extensive LCFs conversion without prior pre-treatment. The synergistic interactions among the enzyme resulted in ethanol yield increases from 37% to 83%. Furthermore, the use of an optimized cocktail significantly improved the fermentation performance without changing the total protein loading. The ethanol concentration and yield were 32g/L and 59% with cocktail 1 compared to 38g/L and 83% with cocktail 2. The substantial increase in the accumulation of the recalcitrant residual substrate, during high solids loading, thereby, warranted a change in the ratio of the optimum cocktail to enable efficient conversion of the polymers into fermentable sugars.

## 6.5. Competing interests

The authors declare that there have no competing interests.

## 6.6. Acknowledgements

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# CHAPTER SEVEN: CONCLUSIONS AND RECCOMENDATION

## 7. Conclusions and recommendations

The increasing food and energy demand coupled with changing consumption behaviour towards healthy foods, necessitate prospecting for innovative and sustainable approaches to utilise currently available water and land resources for food and energy production while simultaneously addressing the effects of global warming. The current study demonstrated and optimised the technical feasibility of integrating processes for a multiproduct JA tuber-based biorefinery for the co-production of protein and inulin with potential nutraceutical, food, or feed applications, as well as ethanol, a biofuel product (Figure 7.1). This approach will ultimately result in synergistic benefits among food, feed, fuel production and mitigating global warming. This section will give insight into key findings and conclusions as well as recommendation for future work.

### 7.1. Conclusions

The experimental data demonstrated the technical feasibility for the development of JA tuber-based biorefinery for the co-production of nutraceuticals, food or feed products and ethanol (Figure 7.1) with potential incorporation of onsite endoinulinases production. This illustrated the potential synergistic benefits for food, feed and fuel production that can be derived through a biorefinery approach for the use of JA tubers. The experimental data showed that a three-step protein and inulin sequential extraction process coupled with the fermentation of the extraction residues into ethanol, is a potential strategy towards maximising the economic value derived from Jerusalem artichoke.

The sequential extraction approach with protein extraction in the first step, at room temperature and high solids loading (15% w/v) and inulin extraction in the second step, was enough for the selective protein and inulin extraction. The pressing of the tuber mash and protein extraction step, prior to inulin extraction, were important in reducing the viscosity of the tubers thereby enabling high solids (10% w/v) extraction of inulin from wet tubers and achievement of high yields (67%). Moreover, the protein extraction steps enabled inulin extraction at relatively lower temperature (74$^{o}$C) compared to conventional hot water extraction done at temperatures between 80-90$^{o}$C for wet tubers in particular. The above

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observations are also important in contributing towards the sequential extraction processes being water and energy efficient.

The integration of a centralised inulinases, for inulin hydrolysis, producing facility will improve the feasibility of the JA-based biorefinery. Recombinant endoinulinases production from *A. niger* was growth associated at higher growth, where the maintenance energy requirements are lower. Moreover, the use of a peptone, yeast extract and casa-amino acids nitrogen cocktail in the culture medium supported fungal growth in pellet morphology under oxygen rich conditions. In contrast, the high cell density induced broth viscosity resulted in the pellet morphology disruption and fungal growth in mycelial form. Pellet disruption was a result of complex interactions between factors such as low DO, inefficiency in mixing, oxygen and nutrients diffusion, high biomass concentration as well as high agitation. The low DO and pellet disruption, and subsequent fungal growth in mycelial form, did not significantly impact biomass growth and enzyme production by the recombinant *A. niger* system.

Furthermore the use of an optimized cocktail of lab produced enzymes (chapter 5) and commercial enzymes, resulted in substantial sugar conversion from the tuber residues derived from the sequential protein and inulin extraction process (chapter 4). The optimized cocktail resulted in a substantial conversion of LCF with no prior pre-treatment of the tuber residues. In addition, optimisation of the enzyme ratios in the cocktail enabled effective enzyme hydrolysis and fermentation productivity with high gravity fermentation without a change in the total protein content. The use of an optimized enzyme cocktail was crucial in minimizing the enzyme dosage for the hydrolysis of and fermentation of tuber residues, which was a result of the synergistic cooperation amongst the enzymes.

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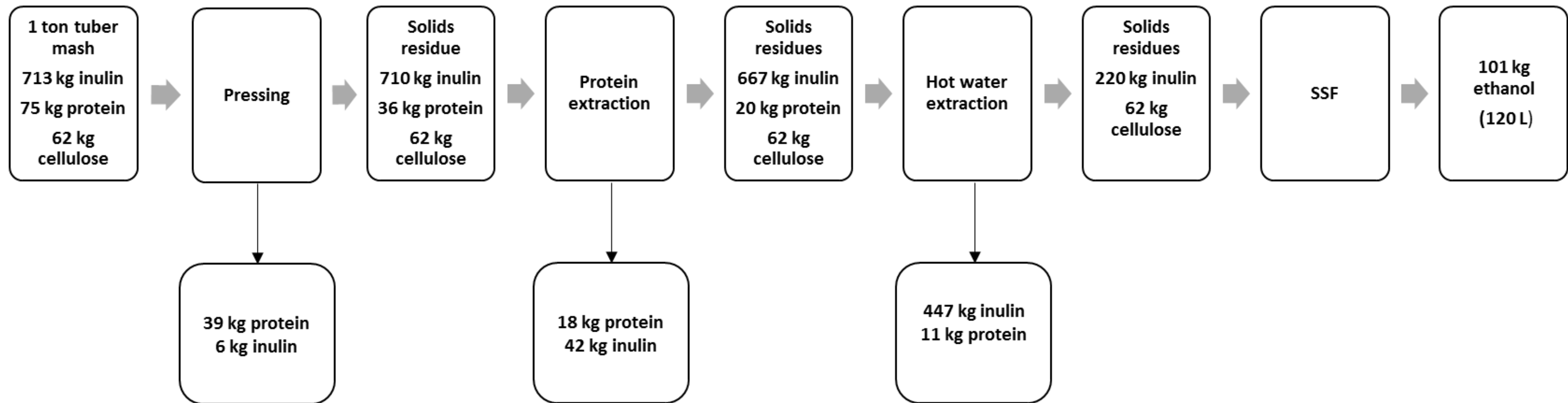


1

2

3 Figure 7.1: Process flow for the integrated process of sequential protein and inulin extraction as well as SSF of the extraction residue

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### 7.2. Recommendations

The current recommendations are aimed at improving the overall technical and economic performance of a JA based biorefinery.

The solvent protein extraction step need improvement to increase the yields because, only 50% of the protein from the solid residues was recovered. Possible improvements to protein extraction could include the use of cell wall degrading enzymes, such as pectinases, to improve the porosity of the biomass and thereby, enable solubilisation of the protein.

Although the biomass achieved in *A. niger* culture was high comparable to literature, it was not adequate to produce volumetric activities comparable to other recombinant systems such as *P. pastoris*. High cell density fermentation is associated with poor mass transfer and consequently reduced productivity. Thus, strategies that improve the specific productivity of the *A. niger* system will enable attainment of improved volumetric activity, considering the culture has limitations in the maximum attainable biomass concentration.

Ethanol production was performed in shake flasks with mixing limitations and thus, the effects of upscaling to bioreactors, on ethanol productivity should be explored. Although SSF has advantages, the use a temperature that is sub-optimal for hydrolytic enzyme impacts fermentation, thus the use of separate hydrolysis and fermentation should be explored, and the two methods compared.

Finally, technoeconomic studies should be performed to evaluate the overall technical and economic feasibility of the developed JA-tuber based biorefinery as well as make recommendations on areas of improvements.

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# CHAPTER EIGHT: APPENDIX

## 8.1. Exponential feeding equation derivation

### 8.1.1. Overview

An exponential feeding regime, during the feeding phase of fed-batch culture, was used to regulate a specific growth rate of *A niger* and maintain a constant biomass concentration and steady state conditions. The residual glucose concentration in the fermenter was assumed to be zero during the feeding phase. The fed batch culture was operated in the variable volume mode, where the growth limiting nutrient was added at a flow rate that increased the volume of the broth thereby resulting in constant changes in the biomass concentration and products.

### 8.1.2. Biomass balance

During the steady-state condition of the feeding phase, the change in biomass concentration and products is independent of time.

The biomass balance for the fed-batch is thus defined by:

Biomass accumulation = biomass in + biomass accumulation – death

However, in fed-batch biomass in = 0

$$\frac{dX}{dt} = 0 + \mu xV - \alpha xV \quad (1)$$

where *X* is the mass of biomass (g) = *x*V, x is biomass concentration (g/L), V is volume of broth (L), μ is the specific growth rate ($h^{-1}$) and α is the specific death rate ($h^{-1}$).

During the exponential growth phase when steady-state growth has been attained and μ » α, equation is simplified to:

$$\frac{d(xV)}{dt} = \mu xV \quad (2)$$

The change in biomass concentration and broth at the beginning of the feeding, for the variable volume mode fed-batch is derived from the biomass balance equation through application of differential calculus:

$$\frac{xdV}{dt} + \frac{Vdx}{dt} = \mu xV \quad (3)$$

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Since the change in volume with time ( $\frac{dV}{dt}$ ) is equivalent to the flowrate (F), equation 3 can be simplified to:

$$xF + \frac{Vdx}{dt} = \mu xV \tag{4}$$

At steady-state condition, when the total biomass and broth volume increase in equal proportion,

$$\frac{dx}{dt} = 0 \tag{5}$$

Thus from equation 4 and 5 μ is derived as

$$\mu = D \tag{6}$$

8.1.3. Glucose substrate balance

The amount of substrate (glucose) required to regulate a pre-determined μ is described by the substrate balance equation below that is based on the relationship mass of glucose fed and cell growth:

$$\frac{d(SV)}{dt} = S_f F - \frac{\mu x V}{Y_{x/s}} \tag{7}$$

Where S is the concentration of glucose in the reactor (g/L), V is the volume of the broth (L), $S_f$ is the concentrated glucose feed, F is the volumetric flow rate of the feed (L/h), *x* is the biomass concentration (g/L) and $\frac{\mu}{Y_{x/s}}$ = $q_s$ is the specific substrate consumption rate ($g_{Glucose}.g_{DW}^{-1}.h^{-1}$).

The constant change in the volume of the feed and glucose can be derived from the substrate balance equation through application of differential calculus:

$$\frac{SdV}{dt} + \frac{VdS}{dt} = S_f F - \frac{\mu x V}{Y_{x/s}} \tag{8}$$

Since feeding begin upon depletion of glucose and feeding is maintained to regulate a specified μ less than $\mu_{max}$, the amount of residual glucose (g/L) is expected and assumed to remain at zero during exponential feeding.

Therefore, $\frac{dS}{dt}$ = 0

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And since $\frac{dV}{dt}$ = F, equation 8 can be reduced to:

$$SF = S_f F - \frac{\mu x V}{Yx_{/s}} \quad (9)$$

From the biomass balance,

$$\frac{V}{F} = \frac{1}{D} \quad (10)$$

Thus combining equation 6 from the biomass balance, equation 8 and 9, the biomass yield coefficient ($Yx_{/s}$) is derived and shown below:

$$Yx_{/s} = \frac{x}{S_f - S} \quad (11)$$

Since $x$ is the biomass produced and $S_f - S$ is the glucose consumed to produced, $Yx_{/s}$ can be rewritten to show the change in biomass relative change in substrate ($\frac{dx}{dS}$):

$$Yx_{/s} = \frac{x_1 - x_0}{S_f - S} \quad (12)$$

The mass of glucose fed $S_f$ into the reactor at a given point in time to achieve $X$ total biomass (g) at a specified μ is derived as following:

$$S_f = \frac{x_1 - x_0}{Yx_{/s}} + S \quad (13)$$

Since $X_1 = X_0 e^{\mu t}$ (14)

and $X = xV$ (15) (from the biomass balance),

$$S_f = \frac{x_0 V_0 (e^{\mu t} - 1)}{Yx_{/s}} + S \quad (16)$$

Where $S_f$, $x_0$, $V_0$, μ and $Y_{x/s}$ is mass of glucose to be fed into the reactor, biomass concentration (g/L) at the start of the fed batch phase, volume of reactor (L) at the start of the fed batch

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phase, set growth rate, amount of glucose fed at $t_{i-1}$, and biomass yield coefficient, respectively.

### 8.2 Application of the method

This section describes how fed batch fermentation was carried out, by demonstrating how the equations derived from the previous section were used to estimate biomass growth, amount of glucose fed and volume of glucose fed during the fed batch phase of the fermentation. The fed batch fermentation at u = 0.07 was used for illustration. The amount and volume of glucose fed to achieve x biomass at a given time point is dependent on the specified growth rate, concentration of feed, and the $Y_{x/s}$ derived during the batch culture, biomass (g) and volume of broth at the end of the batch phase. Table A1 is a summary of the specified parameters and those derived from the batch culture.

Table A1: Specified and experimental parameters for batch and fed-batch cultures

| **Parameter** | **Value** | **Source** |
|---|---|---|
| Maximum growth rate ($\mu_{max}$) | 0.075 $h^{-1}$ | Batch |
| Biomass yield coefficient ($Y_{x/s}$) | 0.49 $g_{biomass}.g_{glucose}^{-1}$ | Batch |
| Biomass concentration at the beginning of the fed-batch phase | 9.23 g/L | Batch |
| Reactor volume at the beginning of the fed-batch phase ($V_0$) | 4 L | Batch |
| Glucose feed concentration ($S_f$) | 300 g/L | Specified |
| Pre-determined specific growth rate ($\mu_{set}$) | 0.07 $h^{-1}$ | Specified |

The amount of biomass accumulation at $t_i$ is estimated by equation 14 and is dependent on biomass at $t_{i-1}$, specified μ, and $Y_{x/s}$. For instance, to estimate biomass at $t_1$ (1 min)

equation (1) was applied and estimated biomass derived as follows;

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$$X_t = X_0 \, e^{\mu t} \qquad (1)$$

$\mu$ *(min$^{-1}$) = 0.07/60 = 0.00117 min$^{-1}$* (2)

$$X_1 = 36.92 \, e^{0.00117 \, x \, 1} = 36.96 \qquad (3)$$

Biomass at t = 4 is estimated as follows $X_4 =$

$$36.92 \, e^{0.00117x4} = 37.093 \qquad (4)$$

Since the programmable logic controller (P) pumps the feed every 10 seconds, the biomass for the first 10 seconds was estimated with the following,

$$X_{10S} = 36.92 \, e^{0.00117 \, x \, 0.1667} = 36.93 \qquad (5)$$

Figure A1 and A2 shows the predicted biomass profile for estimated at 1 min and 10 sec interval, respectively

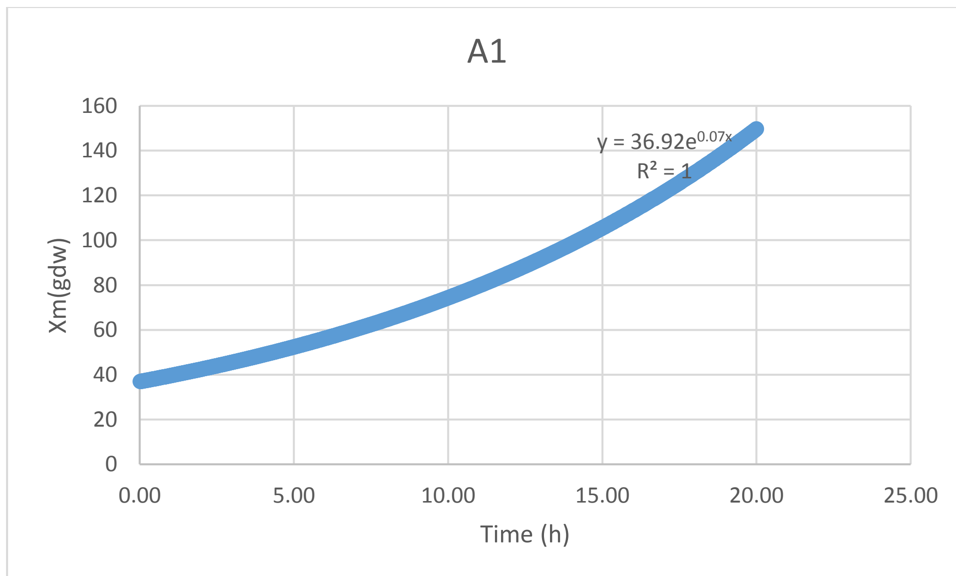


Figure A1: Estimated biomass profile at 1 min interval during the fed batch phase a $\mu_{set}$ = 0.07

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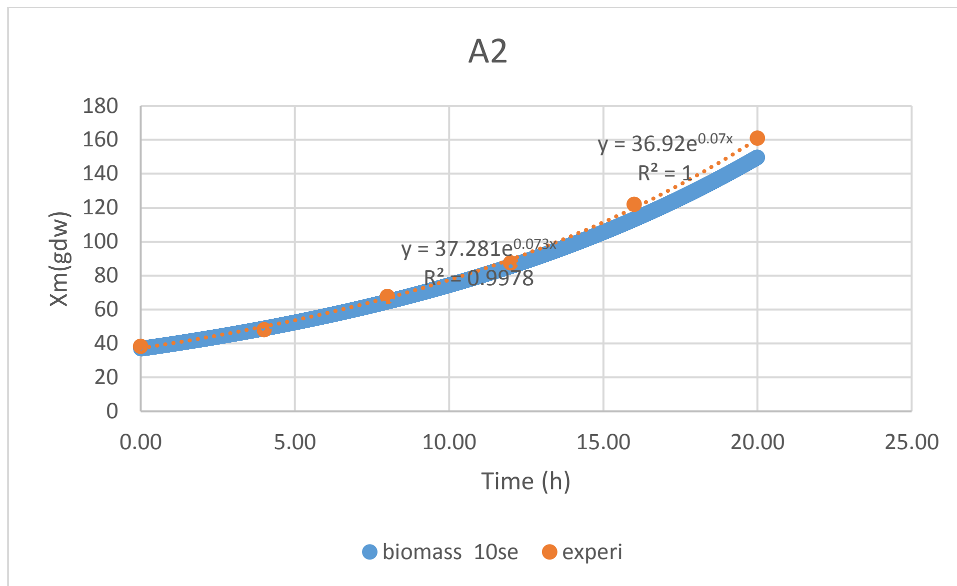


Figure A2: Estimated biomass profile every 10s during the fed batch phase a $\mu_{set}$ = 0.07. The figure also includes a summary of the experimental biomass profile

Moreover, to estimate the amount of glucose to achieve the desired biomass at $t_1$, equation 16 was applied. The glucose required is dependent on the biomass to be achieved, $Y_{x/s}$, growth rate and is estimated as shown below

$$S_f = \frac{36.96\ (e^{0.00117\ x\ 1} - 1)}{0.49} + 0 = 0.0882 \qquad (6)$$

Biomass at t = 4 likewise would be estimated as follows

$$S_f = \frac{37.093\ (e^{0.00117\ x\ 3} - 1)}{0.49} + 0.0882 = 0.3543 \qquad (7)$$

Therefore, change the biomass from 36.96 to 37.093, the amount of glucose required is determined as follow (equation 8);

$$\Delta S_f = S_{f4} - S_{f1} = 0.3543 - 0.0882 \qquad (8)$$

$$\Delta S_f = 0.2661\ g_{glucose} \qquad (9)$$

The mass of glucose to be pumped in the 1st 10 seconds is calculated as follows;

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$$S_f = \frac{36.93 \ (e^{0.00117 \ x \ 0..1667} - 1)}{0.49} + 0 = 0.08797$$

(10)

Figure A3 and A4 is an illustration of predicted mass of glucose required during the exponential feeding phase to maintain the desired growth rate, pumped at 1 min and 10 sec, respectively.

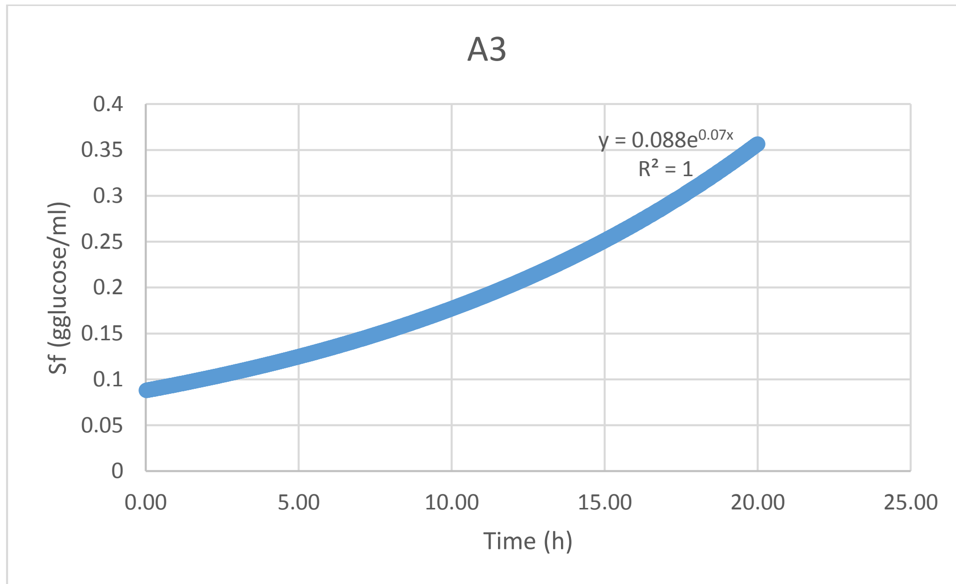


Figure A3: Predicted mass of glucose required during the exponential feeding phase to maintain the desired growth rate (a $\mu_{set}$ = 0.07), pumped at 1 min intervals

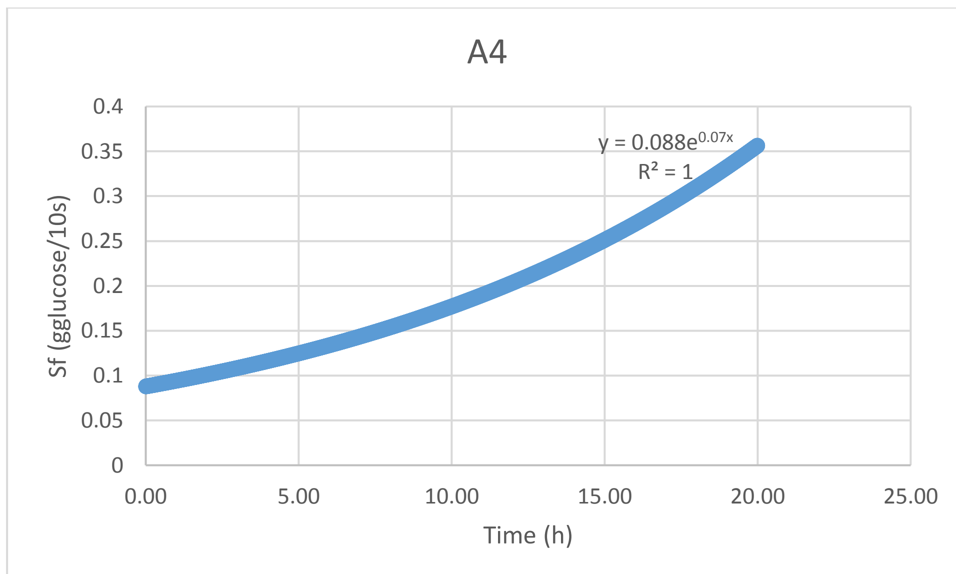


Figure A4: Predicted mass of glucose required during the exponential feeding phase to maintain the desired growth rate (a $\mu_{set}$ = 0.07), pumped every 10 sec

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Once the amount of glucose required to achieve x biomass (g) has been estimated, the volume of glucose to be fed into the reactor can be estimated using equation 11. The volume to be fed is calculated based on the feed concentration and amount of glucose (g) required

$$V_f = \frac{0.2661\ g_{glucose}}{300 \frac{g_{glucose}}{L}} X \frac{1000\ ml}{1L}$$

(11)

$V_f$ = 0.887 ml

(12)

Thus, 0.887 ml of the 300 g/L glucose feed is required between the 1$^{st}$ and 4$^{th}$ minute to ensure the estimated biomass is attained at the specified growth rate.

The volume of the glucose feed to be within the 1$^{st}$ 10 sec is estimated as;

$$V_f = \frac{0.08797\ g_{glucose}}{300 \frac{g_{glucose}}{L}} X \frac{1000\ ml}{1L}$$

(13)

$V_f$ = 0.2932 ml

(14)

Figure A5 and 6 shows the estimated feed flow rates required to meet the culture substrate need at 1 min and 10 sec intervals.

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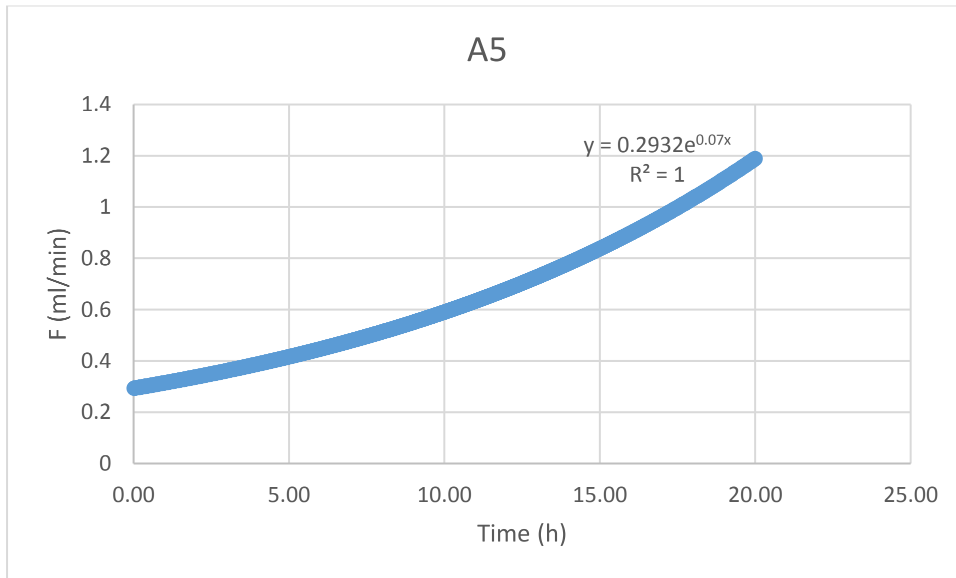


Figure A5: Estimated feed flow rates required to meet the culture substrate need at 1 min and 10 sec intervals to maintain a $\mu_{set}$ = 0.07

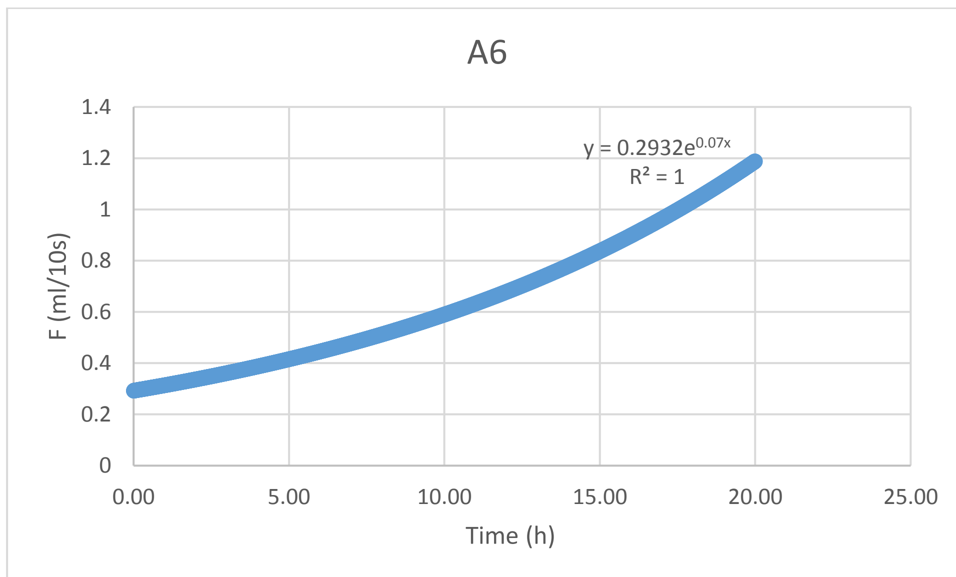


Figure A6: Estimated feed flow rates required to meet the culture substrate need at 10 sec intervals $\mu_{set}$ = 0.07

The Watson-Marlow® U505A pump was calibrated according to the specific size of a silicon tube to enable the pump to be set according to the desired flowrate to achieve the predetermined growth rate at 10 sec intervals. Figure 6 is an illustration of the calibration curve used to set the pump to pump set point required to achieve $\mu_{set}$ (0.07).

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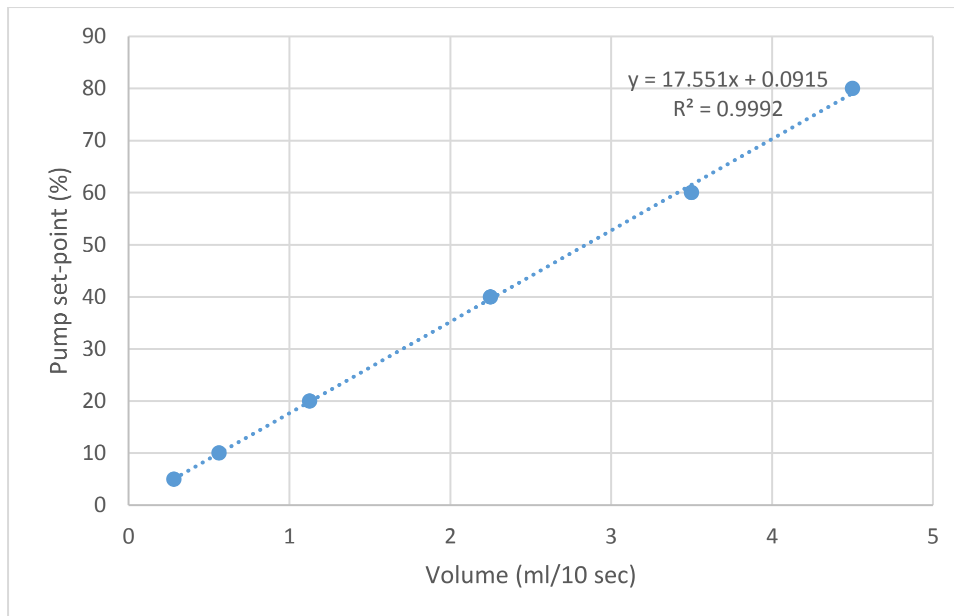


Figure A7: An illustration of the calibration curve used to set the pump to pump set point

The required feed volume of glucose (ml) derived from equation 18 was converted into the corresponding pump set-point (PST) using equation derived from the calibration curve (Figure A7).

$$PST(t_i) = 17.551V_{fti} + 0.0915 = 17.551x\ 0.2932 + 0.0915 \quad (15)$$

$$PST(t_i) = 5.23 \quad (16)$$

The programmable logic controller was programmed to the exponential feeding phase with an initial pump set-point of 5.23 derived from equation 19 and to increase the flow rate with a factor of 1.000194 every 10 sec.

The increment factor was calculated based on the set growth rate and accounting for the 10 sec pumping interval of the fermenter pump.

Stellenbosch University https://scholar.sun.ac.za

**CHAPTER TEN: REFERENCES**

Abozed, S. S., Abdelrashid, A., El-kalyoubi, M., Hamad, K. I. (2009) Production of inulin and high-fructose syrup from Jerusalem artichoke (*Helianthus tuberosus* L.). Annals of agricultural sciences. 54, 417-423.

Aguilera, E., Guzman, G., Alfonso, A. (2015) Greenhouse gas emissions from convectional and organic cropping systems in Spain. I. Herbaceous crops. Agronomy for sustainable development. 35, 713-724.

Alla, N. A., Domokos-Szabolcsy, E, El-Ramady H., Hodossi S, Fári, M., Ragab, M., Taha, H. (2014) Jerusalem artichoke (Helianthus tuberosus L.): A review of in vivo and in vitro propagation. International journal of horticultural science and technology. 20, 131-136.

Alriksson B., Rose, S. H., van Zyl, W, H., Sjode, A., Nilvebrant, N., Jonsson, L. J. (2009) Cellulase production from spent lignocellulose hydrolysates by recombinant *aspergillus niger*. Applied and environmental microbiology. 75, 2366-2374.

Karboune, A. S., Alli, I. (2014) Potato protein isolates: Recovery and characterization of their properties. Food chemistry. 142, 373-382.

Andersen, N., Johansen, K. S., Michelsen, M., Stenby, E.H., Krogh, K. B. R. M., Olsson, L. (2008) Hydrolysis of cellulose using mono-component enzymes shows synergy dur-ing hydrolysis of phosphoric acid swollen cellulose (PASC), but competition on Avicel. Enzyme and microbial technology. 42, 362–70.

AOAC International (2002) Official methods of analysis (17th edition), Arlington, Virginia, USA: Association of Official Analytical Chemists Inc. Protein (Crude) in Animal Feed and Pet Food. AOAC Official Method.

AOAC Method 11.: Dairy One Corporative Inc., Ithaca, NY, USA. (2011).

Apolinário, A. C., de Lima Damasceno, B. P. G., de Macêdo Beltrão, N. E., Pessoa, A., Converti, A., da Silva, J. A., Liebhard P., Praznik, W. *(*2014). Inulin-type fructans: A review on different aspects of biochemical and pharmaceutical technology. Carbohydrate polymers. 101, 368-378.

Stellenbosch University https://scholar.sun.ac.za

Apprich, S., Tirpanalan, O., Hell, J., Reisinger, M., Böhmdorfer, S., Siebenhandl-Ehn, S., Novalin, S., Kneifel, W. (2014) Wheat bran-based biorefinery 2: Valorization of products. Food science and technology. 56, 222-231.

Arrizon, J., Gschaedler, S., Pierre, A. M. (2010) Comparison of the water-soluble carbohydrate composition and fructan structures of Agave tequilana plants of different ages. Food chemistry. 122, 123–130.

Bajpai, P. K. and Bajpai, P. (1991) Cultivation and utilization of Jerusalem artichoke for ethanol single cell protein, and high-fructose syrup production. Enzyme and microbial technology. 13, 359-362.

Baldini, M., Danuso, F., Rocca, A., Bulfoni, E., Monti, A. and De Mastro, G. (2011) Jerusalem artichoke (*Helianthus tuberosus* L.) productivity in different Italian growing areas: a modelling approach. Italian journal of agronomy. 6, 126-132.

Barbosa, A. C. P., Lajolo, F. M. and Genovese, M. I. (2006) Influence of temperature, pH and ionic strength on the production of isoflavone-rich soy protein isolates. Food chemistry. 98, 757–766.

Barclay, T., Ginic-Markovic, M., Cooper, P. and Petrovsky, N. (2010) Inulin - a versatile polysaccharide with multiple pharmaceutical and food chemical uses. Journal of excipients and food chemicals. 1, 27-50.

Bartova V. and Bárta, J. A. N. (2009) Chemical composition and nutritional value of protein concentrates isolated from potato (*Solanum tuberosum* L.) fruit juice by precipitation with ethanol or ferric chloride. Journal of agricultural and food chemistry. 19, 9028-9034.

Bech-Larsen, T. and Grunert, K. G. (2003). The perceived healthiness of functional foods: A conjoint study of Danish, Finnish and American consumers' perception of functional foods. Appetite. 40, 9-14.

Bekers, M., Grube, M., Upite, D., Kaminska, E., Danilevich, A. and Viesturs, U. (2008) Inulin syrup from dried Jerusalem artichoke. Proceedings of the Latvia University of agriculture. 21, 116-121.

Stellenbosch University https://scholar.sun.ac.za

Berlin, A., Maximenko, V., Gilkes, N. and Saddler, J. (2007) Optimisation of enzyme complexes for lignocellulose hydrolysis. Biotechnology and bioengineering. 97, 287-297.

Bhargava, S., Nandakumar, M. P., Roy, A, Wenger, K. S. and Marten, M. R. (2003) Pulsed feeding during fed-batch fungal fermentation leads to reduced viscosity without detrimentally affecting protein expression. Biotechnology and bioengineering. 81, 341-347.

Bouaziz, M.A., Rassaoui, R., Besbes, S. (2014) Chemical Composition, Functional Properties, and Effect of Inulin from Tunisian Agave americana L. Leaves on Textural Qualities of Pectin Gel. Journal ofcChemistry. /dx.doi.org/10.1155/2014/758697.

Cai, R., Klaczynska, B., Baik, B. K. (2001) Preparation of bean curds from protein fractions of six legumes. Journal of agricultural and food chemistry. 49, 3068-3073.

Casas Lopez, J. L., Sanchez Perez, J. A., Fernandez Sevilla, J. M., Rodriguez Porcel, E. M. and Chisti, Y. (2005) Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*. Journal of biotechnology. 116, 61-77.

Can Karaca, A., Low, N. and Nickerson, M. (2011). Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. Food research international. 44, 2742-2750.Chi, Z., Zhang, T., Cao, T., Liu, X., Cui, W. and Zhao, C. (2011) Biotechnological potential of inulin for bioprocesses. Bioresource technology. 102, 4295–4303.

Chen, M., Lei, X., Chen, C., Zhang, S., Xie, J. and Wei, D. (2015). Cloning, Overexpression, and Characterization of a Highly Active Endoinulinase Gene from *Aspergillus fumigatus* Cl1 for Production of Inulo-Oligosaccharides. Applied biochemistry and biotechnology. 175, 1153-1167.

Cheng, L., Wu, J. and Chen, T. (2002) A pseudo-exponential feeding method for control of specific growth rate in fed-batch cultures. Biochemical engineering journal. 10, 227-232.

Cherubin, F. (2010) The biorefinery concept: using biomass instead of oil for producing energy and chemicals. Energy conversion and management. 51, 1412-1421.

Cho, Y. J., Sinha, J., Park, J. P., Yun, J. W. (2001) Production of inulooligosaccharides from inulin by a dual endoinulinase System. Enzyme and microbial technology. 29, 428-433.

Stellenbosch University https://scholar.sun.ac.za

Christopher, G. K., Phipps, A. G., Gray, R. J. (1998) Temperature-dependent solubility of selected proteins. Journal of crystal growth. 191, 820-826.

Cieślik, E., Gębusia, A, Florkiewicz, A., Mickowska, B. (2011) The content of protein and of amino acids in Jerusalem artichoke tubers (*Helianthus tuberosus* l.) of red variety Rote Zonenkugel. ACTA ccientiarum polonorum technologia alimentaria. 10, 433-441.

Coban, H. B., Demirci, A. (2015) Improved submerged *Apergillus ficuum* phytase production in bench-top bioreactors by optimisation of fermentation medium. Acta alimentaria. 4, 549-560.

Cooper, C. L., Dubin, P. L., Kayitmazer, A. B., Turksen S. (2005) Polyelectrolyte-protein complexes. Current opinion in colloid & interface science. 10, 52-78.

da Silva, T. P., Gouveia, L., Reis, A. (2014) Integrated microbial processes for biofuels and high value-added products: the way to improve the cost effectiveness of biofuel production. Applied microbiology and biotechnology. 98, 1043-1053.

De Mastro, G., Manolio, G., Marzi, V. (2004) Jerusalem artichoke (*Helianthus tuberosus* L.) and Chicory (*Cichorium intybus* L.): Potential crops for inulin production in the mediterranean area. Future for medicinal and aromatic plants. 629, 365-374.

de Moura, J.M., Campbell, L.N., Mahfuz, K. (2008) Enzyme-Assisted Aqueous Extraction of Oil and Protein from Soybeans and Cream De-emulsification. Journal of the American oil chemists' society. 85, 985–995.

Dobre, T., Parvulescu, O., Stoica, A.,Stroescu, M. (2010) Experimental investigation and modelling of inulin and Glycyrrhizin extraction. Revista de chimie. 61, 82-86.

Driouch, H., Roth, A., Dersch, P., Wittmann, C. (2010) Optimised bioprocess for production of fructofuranosidase by recombinant *Apergillus niger*. Applied microbiology and biotechnology. 87, 2011-2024.

Driouch, H., Hansch, R., Wucherpfennig, R., Krull, R., Wittmann, C. (2012) Improved Enzyme Production by Bio-Pellets of *Aspergillus niger*: Targeted Morphology Engineering Using Titanate Microparticles. Biotechnology and bioengineering. 109, 462-471.

Stellenbosch University https://scholar.sun.ac.za

Dumas, J. B. A. (1831). Procedes de l'Analyse Organique. Annales de chimie et de physique. 247, 198-213.

Duong-Ly, K. C., Gabelli, S. B. (2014) Salting out of proteins using ammonium sulfate precipitation. Methods in enzymology. 541, 85-94.

Edelman, J., Jefford, T. G. (1968). The mechanism of fructosan metabolism in higher plants as exemplified in *Helianthus tuberosus.* New phytologist. 67, 517-531.

Elisashvili, V. I., Kachlishvili, E. T., Wasser, S. P. (2009) Carbon and nitrogen source effects on basidiomycetes exopolysaccharide production. Applied biochemistry and microbiology. 45, 531-535.

Fabian, C., Ju, Y. (2011) A review on rice bran protein: its properties and extraction methods. Critical reviews in food Science and nutrition. 51, 816-827.

Femia, F. P., Luceri, C., Dolara, P., Giannini A., Biggeri, A., Salvadori, M., Clune, Y., Collins, K. J., Paglierani, M., Caderni, G. (2002) Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane-induced colon carcinogenesis in rats. Carcinogenesis. 23, 1953–1960.

Feyzi, S., Milani, E., Golimovahhed, Q. A. (2018) Grass Pea *(Lathyrus sativus* L.) Protein Isolate: The Effect of Extraction Optimization and Drying Methods on the Structure and Functional Properties. Food hydrocolloids. 74, 187-196.

Fan, Z. L., South, C., Lyford, K., Munsie, J., van Walsum, P.,Lynd, L. R. (2003) Conversion of paper sludge to ethanol in a semicontinuous solids-fed reactor. Bioprocess biosystems engineering. 26, 93-101.

Favaro, L., Cagnin, L., Basaglia, M., Pizzocchero, V., van Zyl, W. H., Casella, S. (2017) Production of bioethanol from multiple waste streams of rice milling. Bioresource technology. 244: 151-159.

Franck, A. (2002) Technological functionality of inulin and oligofructose. British Journal of Nutrition. 87, 87– 91.

Stellenbosch University https://scholar.sun.ac.za

Gafaar, A. M., El-Din, M. F. S., Boudy, E. A. , El-Gazar, H. H. (2010) Extraction conditions of inulin from Jerusalem artichoke tubers and its effects on blood glucose and lipid profile in diabetic rats. Journal of american science. 6, 36-43.

Ge, X. and Zhang, W. (2005) A Shortcut to the Production of High Ethanol Concentration from Jerusalem Artichoke Tubers. Food technology and biotechnology. 43, 241-246.

Gilani, G. S., Xiao, G.W., Cockell, K. A. (2012) Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. British journal of nutrition. 108, 315–332.

Glibowski, P.,Bukowska, A. (2011) The effect of pH, temperature and heating time on inulin chemical stability. Acta scientiarum polonorum technologia alimentaria. 10, 189-196.

Godfray, H. C. J., Beddington, J. R, Cute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M., Toulmin, C. (2010). Food security: the challenge of feeding 9 billion people. Science. 327, 812-818.

Gonzalez, G. A., Takasugia, Y., Jiaa, L., Moria, Y., Nodab, S., Tanakac, T., Hirofumi I., Kamiyaa, N (2015) Synergistic effect and application of xylanases as accessory enzymes to enhance the hydrolysis of pretreated bagasse. Enzyme and microbial technology. 72, 16-24

Gramblicka, M.,Polakovic, M. (2007) Adsorption equilibria of glucose, fructose, sucrose, and fructooligosaccharides on cation exchange resins. Journal of Chemical and Engineering Data. 52, 345-350

Gujska, E.,Khan, K. (1991) High temperature extrusion effects on protein solubility and distribution in navy and pinto beans. Journal of food science. 56, 1013-1016.

Gunnarsson, I. B., Svensson, S. E., Johansson, E.J., Karakashev, D.,Angelidaki, I. (2014) Potential of Jerusalem artichoke (Helianthus tuberosus L.) as a biorefinery crop. Industrial crops and products. 56, 231–240.

Gupta, A. K., Kaur N., Kaur N. (2003) Preparation of inulin from chicory roots. Journal of scientific and industrial research. 62, 916-920.

Stellenbosch University https://scholar.sun.ac.za

Haack, M. B., Olsson L., Hansen, K.,Lantz, A. E. (2006) Change in hyphal morphology of *Aspergillus oryzae* during fed-batch cultivation. Applied microbiology and biotechnology. 70, 482-487.

Haq, I., Nawaz, A., Mukhtae, H., Ur-Rehman, A. (2015). Optimisation of inoculum volume, fermentation medium and aeration rate for the production of glucose oxidase by UV mutant strain of *Aspergillus niger* AN-14. Pakistan journal of botany. 329, 329-332

Herrero, M.,Ibánez, E. (2015) Green processes and sustainability: An overview on the extraction of high added-value products from seaweeds and microalgae. Journal of supercritical fluids. 96, 211-216.

Hoyer, K., Galbe, M., Zacchi, G (2010) Effects of enzyme feeding strategy on ethanol yield in fed-batch simultaneous saccharification and fermentation of spruce at high dry matter. Biotechnology for biofuels. 3, 14-25.

Hu, J., Arantes, V., Pribowo, A., Saddler, J. N. (2014) The synergistic action of accessory enzymes enhances the hydrolytic potential of a “cellulase mixture” but is highly substrate specific. Biotechnology for biofuels. 6, 112-124.

Hunag, W., Shieh, G. S.,Wang, F. (2012) Optimization of fed-batch fermentation using mixture of sugars to produce ethanol. Journal of the taiwan institute of chemical engineers. 43, 1-8.

Itaya, N. M., Figueiredo-Ribeiro, R. C.,Buckeridge, M. S. (1999). Synthesis of fructans by fructosyltransferase from the tuberous roots of *Viguiera discolor* (Asteraceae). Brazilian Journal of medical and biological research. 32, 435-442.

Izsaki, Z.,Kadi, G. N. (2013). Biomass accumulation and nutrient uptake of Jerusalem artichoke (*Helianthus tuberosus* L.). American journal of plant sciences. 4, 1629-1640.

Jalak, J., Kurasin, M., Teugjas, H., Valjamae, P. (2012) Endo-exo Synergism in Cellulose Hydrolysis Revisited. Journal of biological chemistry. 34, 28802-28815.

James, E., van Zyl, W., van Zyl, P., Görgens, J. (2007) Increased Hepatitis B surface antigen production by recombinant *Aspergillus niger* through the optimization of agitation and dissolved oxygen concentration. Applied microbiology and biotechnology. 75, 279-288

Stellenbosch University https://scholar.sun.ac.za

James E, van Zyl W, van Zyl P, Görgens J (2012) *Recombinant Hepatitis B surface antigen production in Aspergillus niger.* Thesis, Stellenbosch University

Johansson, E., Prade, T., Angelidaki, I., Svensson, S., Newson, W. R., Gunnarsson, I. B., Hovmalm, H. P. (2015). Economically viable components from Jerusalem artichoke (*Helianthus tuberosus* L.) in a biorefinery concept. International Journal of molecular sciences. 16, 8997-9016.

Jurgonski, A., Joanna, M., Juśkiewicz, J., Zenon, Z.,Bogus, K. (2011). Composition of Chicory Root, Peel, Seed and Leaf Ethanol Extracts and Biological Properties of Their Non-Inulin Fractions. Food technology and biotechnology. 49, 40-47.

Kachlishvili, E., Penninckx, M. J., Tsiklauri, N., Elisashvil, V. (2006) Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. World journal of microbiology and biotechnology. 22, 391-397.

Kachrimanidou, V., Kopsahelis, N., Alexandri, M., Strati, A., Gardeli, C., Papanikolaou, S., Komaitis, M., Kookos, I. K., Koutinas, A. A. (2015). Integrated sunflower-based biorefinery for the production of antioxidants, protein isolate and poly(3-hydroxybutyrate). Industrial rops and products. 71,106–113.

Kádár, Z., Szengyel, Z.,d Réczey, Z. (2004) Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. Industrial crops and products. 20, 103-110.

Kato, A., Takahashi, A., Matsudomi, M., Kobayashi, K. (1983) Determination of foaming properties of proteins by conductivity measurements. Journal of food science. 48, 62-65.

Kaur, N.,Gupta, A. K. (2002). Applications of inulin and oligofructose in health and nutrition. Journal of biosciences. 27, 703-714.

Karaca, A. C., Low, N.,Nickerson, M. (2011) Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. Food research international. 44, 2742–2750.

Stellenbosch University https://scholar.sun.ac.za

Karahalil, E., Demirel, F., Evcan, E., Germec, M., Tari, C., Turhan, I. (2017) Microparticle-enhanced polygalacturonase production by wild type *Aspergillus sojae*. Biotechnology. 7, 361-368.

Karazhiyan, H., Razavi, S. M. A.,Phillips, G. O. (2011) Extraction optimization of a hydrocolloid extract from cress seed (*Lepidium sativum*) using response surface methodology. Food hydrocolloid. 25, 915-920.

Kim, Y., Faqih, M. N.,Wang, S. S. (2001). Factors affecting gel formation of inulin. Carbohydrate polymers. 46, 135–145.

Kim, B. S., Lee, S. C., Lee, S. Y., Chan, Y. K., Chan, H. N. (2004) High cell density fed-batch cultivation of Escherichia coli using exponential feeding combined with pH-stat. Bioprocess and biosystems engineering. 26, 147-150.

Kosaric, N., Cosentino, G. P., Wieczorek, A., Duvnjak, Z. (1984) The Jerusalem artichoke as an agricultural crop. *Biomass*. 5, 1-6.

Kocsis, L., Liebhard, P.,Praznik W. (2007). Effect of seasonal changes on content and profile of soluble carbohydrates in tubers of different varieties of Jerusalem artichoke (*Helianthus tuberosus* L.). Journal of agricultural and food chemistry. 55, 9401–9408.

Koutinas, A. A., Garcia, I. L., Kopsahelis, N., Papanikolaou, S., Webb, C., Villar, M. A., Lopez, J. A. (2013) Production of fermentation feedstock from Jerusalem artichoke tubers and its potential for polyhydroxybutyrate Synthesis. Waste and biomass valorisation. 4, 359-370

Krull, R., Wucherpfennig, T., Esfandabadi, M. E., Walisko, R., Melzer, G., Hempel, D. C., Kampen, I., Kwade A., Wittmann C (2013). Characterisation and control of fungal morphology for improved production performance in biotechnology. Journal of biotechnology. 163, 112-123.

Kuo, S., Merhige, P. M., Hagey, L. R. (2013) The effect of dietary prebiotics and probiotics on body weight, large intestine indices, and fecal bile acid profile in Wild type and IL102/2 Mice. PubliclLibrary of sciences ONE.8:e60270. doi:10.1371/journal.pone.0060270.

Lai, Y. P., Mondor, M., Moresoli, C., Drolet, H., Gros-Louis, M., Ippersiel, D., Lamarche, F., Arcand, Y. (2013) Production of soy protein isolates with low phytic acid content by

Stellenbosch University https://scholar.sun.ac.za

membrane technologies: Impact of the extraction and ultrafiltration/diafiltration conditions. Journal of food engineering. 114, 221–227.

Lam, A., Can Karaca, R. T., Tyler, M. T. (2016) Nickerson, Pea protein isolates: Structure, extraction, and functionality. Food reviews international. 34, 126-147.

Leelaram, S., Sivanesha, N. E., Surianarayanan, M., Deepab, P. R., Balaje, S. A. (2016) Effect of feeding strategies on inulinase production analyzed in a biocalorimeter. Process biochemistry. 51, 692-703.

L'Homme, C., Arbelot, M., Puigserver, A., Biagin, A. (2003) Kinetics of hydrolysis of fructooligosaccharides in mineral-buffered aqueous solutions: Influence of pH and temperature. Journal of agricultural and food chemistry. *51,* 224-228.

Li, H., Zhu, H., Qiao, J., Du, J.,Zhang, H. (2012) Optimization of the main liming process for inulin crude extract from Jerusalem artichoke tubers. Frontiers of chemical science and engineering. 6, 348-355.

Li, W., Zhang, J., Yu, C., Li, Q., Dong, F., Wang, G., Gu, G.,Guo, Z. (2015) Extraction, degree of polymerization determination and prebiotic effect evaluation of inulin from Jerusalem artichoke. Carbohydrate polymers. 121, 315–319.

Li, X., Kang, Y., Yu, C., Dai, J., Wang, Z., Li, Z., Yao, J., Li, P., Zheng, G.,Chen, X. (2016) Exponential feeding strategy of high-density cultivation of a salt-tolerant aroma-producing yeast *Zygosaccharomyces rouxii* in stirred fermenter. Biochemical engineering journal. 111, 18-23.

Li, Y., Liu, G., Wang, K., Chi, Z.,Madzak, C. (2012) Overexpression of the endo-inulinase gene from Arthrobacter sp. S37 in Yarrowia lipolytica and characterization of the recombinant endo-inulinase. Journal of molecular catalysis B: Enzymatic. 74, 109-115.

Liu, W., Wang, Y., Yu, Z.,Bao, J. (2012) Simultaneous saccharification and microbial lipid fermentation of corn stover by oleaginous yeast *Trichosporon cutaneum*. Bioresource technology. 118, 13-18.

Liu, K., Lin, X., Yue, J., Li, X., Fang, X., Zhu, M., Lin, J., Qua, Y., Xiao, L. (2010) High concentration ethanol production from corncob residues by fed-batch strategy. Bioresource technology. 101, 4952-4958.

Stellenbosch University https://scholar.sun.ac.za

Lim, Y., Jang, Y. and Kim, K. (2013) Production of a high concentration of ethanol from potato tuber by high gravity fermentation. Food science and biotechnology. 22, 441-448.

Lim, S., Lee, J., Shin., D., Sim, H. S. (1999). Comparison of protein extraction solutions for rice starch isolation and effects of residual protein content on starch pasting properties. Starch. 51, 120-125.

Lin, C. S.,Zayas, J. (1987) Protein, solubility, emulsifying stability and capacity of two defatted corn germ proteins. Journal of food science. 52, 1615-1619.

Lingyun, W., Jianhua, W., Xiaodong, Z., Da, T., Yalin, Y., Chenggang, C., Tianhua, F.,Fan, F. (2007) Studies on the extracting technical conditions of inulin from Jerusalem artichoke tubers. Journal of food engineering. 79, 1087-1093.

Liu, G., Chi, Z., Chi, Z. (2013). Molecular characterisation and expression of microbial inulinase genes. Critical review in microbiology. 39, 152-165.

Li, H., Zhu, H., Qiao, J., Du, J.,Zhang, H. (2012) Optimization of the main liming process for inulin crude extract from Jerusalem artichoke tubers. Frontiers of chemical science and engineering. 6, 348-355.

Liu, H., Zhang, Y.X., Hou, T., Chen X., Gao, C., Han, L., Xiao, W. (2018) Mechanical deconstruction of corn stover as an entry process to facilitate the microwave-assisted production of ethyl levulinate. Fuel processing technology. 174, 53-60.

Liu, K., Lin, X., Yue, J., Li, X., Fang, X., Zhu, M., Lin, J., Qua, Y., Xiao, L. (2010) High concentration ethanol production from corncob residues by fed-batch strategy. Bioresource technology. 101, 4952-4958.

Liu, Y., Zhou, S., Cheng, Y., Chia, Z., Chia Z., Liu, G. (2016) Synergistic effect between the recombinant exo-inulinase and endo-inulinase on inulin hydrolysis. Journal of molecular catalysis. 128, 27-38.

Li, H., Zhu, H., Qiao, J., Du, J., Zhang, H. (2012) Optimization of the main liming process for inulin crude extract from Jerusalem artichoke tubers. Frontiers of chemical science and engineering. 6, 348-355.

Stellenbosch University https://scholar.sun.ac.za

Liu, Z. X., Huub, S., Jing, S., Shuai, X., Guang, X. (2012) Growth and Yield Performance of Jerusalem Artichoke Clones in a Semiarid Region of China. Agronomy journal. 104, 1538-1546.

Løkra, Si., Strætkvern, K. (2009). Industrial Proteins from Potato Juice. A review in food. 3. 88-95.

Long, X., Shao, H., Liu, L.,Liu, Z. (2016) Jerusalem artichoke: A sustainable biomass feedstock for biorefinery. Renewable and sustainable energy reviews. 54, 1382-1388.

Long, X. H., Zhao, J., Liu, Z. P., Rengel, Z., Liu, L., Shao, H. B., Tao, Y. (2014) Applying geostatistics to determine the soil quality improvement by Jerusalem artichoke in coastal saline zone. Ecological engineering. 70, 319-326.

Low, E. W.,Chase, H. A. (1999) The effect of maintenance energy requirements on biomass production during wastewater treatment. Water research. 33, 847-853.

Merino, S. T.,Cherry J. (2006) Progress and challenges in enzyme development for Biomass utilization. Biofuels. 108, 95-120.

Maity, S. K. (2015) Opportunities, recent trends and challenges of integrated biorefinery: Part I. renewable and sustainable energy reviews.43: 1427-1445

Manamperi, W. A. R., Wiesenborn, D. P., Chang, S. K. C., Pryor, S. W. (2011) Effects of protein separation conditions on the functional and thermal properties of canola protein isolates. Journal of food science. 76, 266-273.

Martín-Cabrejas, M. A., Esteban, R. M., Waldron, K. W., Maina, G., Grant, G., Bardocz, S., Pusztai, A. (1995) ‘Hard-to-cook phenomenon in beans: changes in antinutrient factors and nitrogenous compounds during storage.’, Journal of the science of food and agriculture. 69, 429–435.

Matusek, A., Meresz, P., Le, T. K. D., Orsi, F. (2009) Effect of temperature and pH on the degradation of fructo-oligosaccharides. European food research and technology. 228, 355-365.

Maumela P., van Rensburg, E., Chimphango, A. F. A., Görgens, J. F. (2019) Sequential extraction of protein and inulin from the tubers of Jerusalem artichoke (Helianthus tuberosus L.). Journal of food science and technology. 57, 775-786.

Stellenbosch University https://scholar.sun.ac.za

Meuser, F., Bauer, I., Hellwege, E., Pilling, J. (2009) Inulin extraction patent. Patent No. US 2009/0099129 A1.

Mellado-Mojica, E., López, M. G. (2012). Fructan metabolism in A. tequilana Weber blue variety along its development cycle in the field. Journal of agricultural and food chemistry. 60, 11704-11713.

Milani, E., Koocheki, A.,Golimovahhed, Q. A. (2011) Extraction of inulin from Burdock root (*Arctium lappa*) using high intensity ultrasound. International journal of food science and technology. 46, 1699-1704.

Mitra, R. K., Sinha, S. S.,Pal, S. K. (2007) Hydration in protein folding: Thermal unfolding/refolding of human serum albumin. Langmuir. 23, 10224-10229.

Mizubuti, I. Y., Junior, O. B., Souza, L. D. W., da Silva, R. S. D. F., Ida, E. I. (2000). Response surface methodology for extraction optimization of pigeon pea protein. Food chemistry. 70, 259-265.

Mokomele, T., da Costa Sousa, L., Balan, V., van Rensburg, E., Dale, B. E., Görgens, J. F. (2018) Ethanol production potential from AFEX™ and steam exploded sugarcane residues for sugarcane biorefineries. Biotechnology for biofuels. 11, 127-148

Moshfegh, J. E., Goldman J. P. ,Ahuja, J. K. (1999). Presence of Inulin and Oligofructose in the Diets of Americans. The journal of nutrition. 129, 1407-1411.

Monti, A., Amaducci, M. T., Venturi, G. (2005). Growth response, leaf gas exchange and fructans accumulation of Jerusalem artichoke (*Helianthus tuberosus* L.) as affected by different water regimes. European journal of agronomy. 23, 136-145.

Montgomery, M. C. (2013). Design and analysis of experiments. 8$^{th}$ edition. John Wiley and sons.

Mutanda, T., Wilhelmi, B. S., Whiteley, C. G. (2008) Response surface methodology: Synthesis of inulooligosaccharides with an endoinulinase from *Aspergillus niger*. Enzyme and microbial technology. 43, 362-368.

Stellenbosch University https://scholar.sun.ac.za

Naskar,B., Dan, A., Ghosh, S., Moulik, S. P. (2010) Viscosity and solubility behavior of the polysaccharide inulin in water, water +dimethyl sulfoxide, and water + isopropanol media. Journal of chemical and engineering data. *55,* 2424–2427.

Nguyen, Q. D., Rezessy-Szabo, J. M., Czukor, B., Hoschke, A. (2011) Continuous production of oligofructose syrup from Jerusalem artichoke juice by immobilized endo-inulinase. Process biochemistry. 46, 298-303.

Nsabimana, C.,Jiang, B. (2010) The chemical composition of some garden Dahlia tubers. British food journal. 113, 1081-1093.

Nunes, M. C., Raymundo, A., Sousa, L. (2006) Gelled vegetable desserts containing pea protein, k-carrageenan and starch. European food research and technology. 222, 622-628

Pace, C. N., Treviño, S., Prabhakaran, E., Scholtz, J. M. (2004). Protein structure, stability and solubility in water and other solvents. Philosophical transactions of the royal society B. 359, 1225-1235.

O'Connell, K.,Stults, J. T. (1997) Identification of mouse liver proteins on two-dimensional electrophoresis gels by matrix-assisted laser desorption/ionization mass spectrometry of in situ enzymatic digests. Electrophoresis. 18, 349-59.

Olofsson, K., Bertilsson, M.,Lidén, G. (2008) A short review on SSF- an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnology for biofuels. 1, 7-21.

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

Palthur, M. P., Palthur, S. S., Chitta, S. K. (2010). Nutraceuticals: A conceptual definition. International journal of pharmacy and pharmaceutical Sciences. 3, 19-27.

Papagianni, M., Mattey, M. (2006) Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level. Application of neural network and cluster analysis for characterization of mycelial morphology. Microbial cell factories. 5, 3-15.

Stellenbosch University https://scholar.sun.ac.za

Pandino, G., Lombardo, S., Mauromicale, G. (2011) Chemical and morphological characteristics of new clones and commercial varieties of Globe Artichoke (Cynara cardunculus var. scolymus). Plant foods for human nutrition. 66, 291–297.

Park, J. P., Yun, J. W. (2001) Utilization of chicory roots for microbial endoinulinase production. Letters in applied microbiology. 33, 183-187.

Pelegrine, D. H. G. and Gasparetto, C. A. (2005) Whey proteins solubility as function of temperature and pH. Swiss Society of Food Science and Technology. 38, 77-80.

Plüddemann, A. ,van Zyl, W. H. (2003) Evaluation of *Aspergillus niger* as host for virus-like particle production, using the hepatitis B surface antigen as a model. Current genetics. 43, 439-446.

Poonsrisawat, A., Paemanee, A., Wanlapatit, S., Piyachomkwan, K., Eurwilaichitr, L., Champredag, V. (2011) Simultaneous saccharification and viscosity reduction of cassava pulp using a multi-component starch- and cell-wall degrading enzyme for bioethanol production. Biotechnology. 7, 290-390.

Porcel, E. M. R., Lopez, J. L. C., Perez, J. A. S., Chisti, Y. (2007). Enhanced production of lovastatin in a bubble column by *Aspergillus terreus* using a two-stage feeding strategy. Journal of chemical technology and biotechnology. 82, 58-64.

Praznik, W.,Beck, R. H. F. (1987). Inulin composition during growth of tubers of *Helianthus tuberosus*. Agricultural and biological chemistry. 51, 1593-1599.

Puligundla, P., Smogrovicova, D., Obulam, V. S. R., Ko, S. (2011) Very high gravity (VHG) ethanolic brewing and fermentation: a research update. Journal of industrial microbiology and biotechnology. 38, 1133-1144.

Punt, P. J., van den Hondel, C. A. J. (1992) Transformation of filamentous fungi based on hygromycin b and phleomycin resistance markers. Methods in enzymology. 216, 447-457.

Qui, Y., Lei, P., Zhang, Y., Sha, Y., Zhan, Y., Xu, Z., Li, S., Xu, H., Ouyang, P. (2018) Recent advances in bio‑based multi‑products of agricultural Jerusalem artichoke resources. biotechnology for Bbiofuels. 11, 151-165.

Stellenbosch University https://scholar.sun.ac.za

Rashid, A., White, E. D., Howes, T., Lister, J. D., Marziano, I. (2014) Effect of solvent composition and temperature on the solubility of ibuprofen in aqueous ethanol. Journal of chemical and engineering data. 59, 2699−2703.

Remize, F., Schorr-Galindo, S., Guiraud, J. P., Dequin, S., Blondin, B. (1998) Construction of a flocculating yeast for fructose production from inulin. Biotechnology letters. 20, 313-318.

Rasul, G. (2016) Managing the food, water, and energy nexus for achieving the Sustainable Development Goals in South Asia. Environmental development. 18, 14-2

Ravenscroft, N., Cescutti, P., Hearshaw, M. A., Ramsout, R., Rizzo, R., Timme, E. M. (2009) Structural analysis of fructans from Agave americana grown in South Africa for spirit production. Journal of agricultural and food chemistry. 59, 3995–4003.

Robbins, K. R., Ballew, J. E. (1982) Effect of Alkaline Treatment of Soy Protein on Sulfur Amino Acid Bioavailability. Journal of food science. 47, 2070-2071.

Roberfroid, M. B. (2005). Introducing inulin-type fructans. British journal of nutrition. 93, S13-S25.

Roberfroid, M. B. (2007). Inulin-type fructans: Functional food ingredients. The journal of nutrition. 137, 2493–2502.

Roller, M., Rechkemmer, G., Watzl, B. (2004). Prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* modulates intestinal immune functions in rats. The journal of nutrition. 134, 153–156.

Rose, S. H.,van Zyl, W. H. (2002) Constitutive expression of the *Trichoderma reesei* beta-1,4-xylanase gene (xyn2) and the beta-1,4-endoglucanase gene (egl) in *Aspergillus niger* in molasses and defined glucose media. Applied microbiology and biotechnology. 58, 461-468.

Rose, S. H.,van Zyl, W. H. (2008) Exploitation of Aspergillus niger for the Heterologous production of cellulases and hemicellulases. The open biotechnology journal. 2, 167-175.

Rubel, I. A., Perez, E. E., Genovese, D.B., Manrique G. D. (2014). In vitro prebiotic activity of inulin-rich carbohydrates extracted from Jerusalem artichoke (*Helianthus tuberosus L.*) tubers at different storage times by *Lactobacillus paracasei*. Food research international. 62, 59–65.

Stellenbosch University https://scholar.sun.ac.za

Saha, B. C., Iten, L. B., Cotta, M. A., Wu, Y. V. (2005) Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process biochemistry. 40, 3693-3700.

Saha, B. C., Iten, L. B., Cotta, M. A.,Wu, Y. V. (2005) Dilute Acid Pretreatment, Enzymatic Saccharification, and Fermentation of Rice Hulls to Ethanol. Biotechnology progresa. 21, 816-822.

Sambrook, J., Fritsch, E. F.,Miniatis, T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sari, Y. W., Bruins, M. E., Sanders, J. P. M. (2013). Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. Industrial crops and products. 43, 78-83.

Sereewatthanawut, I., Prapintip, S., Watchiraruji, K, Goto, M., Sasaki, M.,Shotipruk, A. (2008). Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. Bioresource technology. 99, 555–561.

Silva-Santisteban, B., Converti, A..Filho, F. M. (2009) Effects of carbon and nitrogen sources and oxygenation on the production of inulinase by Kluyveromyces marxianus. Applied biochemistry and biotechnology. 152, 249-261

Singh, P., Kumar, R., Sabapathy, S. N.,Bawa, A. S. (2008). Functional and edible uses of soy protein products. Comprehensive reviews in food science and food safety. 7, 14-28.

Sing, R. S., Chauhan, K. (2017) Inulinase production from a new inulinase producer, Penicillium oxalicum BGPUP-4. Biocatalysis and agricultural biotechnology. 9, 1-10.

Singh, R. S.,Chauhan, K. (2018) Production, purification, characterization and applications of fungal inulinases. Current biotechnology. 7, 242- 260.

Singh, R. S., Dhaliwal, R., Puri, S. (2007) Production of high fructose syrup from Asparagus inulin using immobilized exoinulinase from *Kluyveromyces marxianus* YS-1. Journal of Industrial microbiology and biotechnology. 34, 649-655.

Singh, R. S., Singh, R. P. (2010) Production of fructooligosaccharides from inulin by endoinulinases and their prebiotic potential. Food technology and biotechnology. 48, 435–50.

Stellenbosch University https://scholar.sun.ac.za

Singh R. S.,Singh R. P. (2014) Response surface optimization of endoinulinase production from a cost effective substrate by *Bacillus safensis* AS-08 for hydrolysis of inulin. Biocatalysis and agricultural biotechnology. 3, 365–372.

Singh, R. S., Singh, R. P.,Yadav, M. (2013) Molecular and biochemical characterization of a new endoinulinase producing bacterial strain of *Bacillus safensis* AS-08. Biologia. 68, 1028-1033.

Sirisansaneeyakul, S., Worawuthiyanan, N., Vanichsriratana, W., Srinophakun, P., Chisti, Y. (2007) Production of fructose from inulin using mixed inulinases from *Aspergillus niger* and *Candida guilliermondii*. World Journal of microbiology and biotechnology. 23, 543-552.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J. ,Templeton, D. (2005) Determination of Ash in Biomass. National Renewable Energy Laboratory. *Technical Report.* NREL/TP-510-42622.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. (2008) Determination of Structural Carbohydrates and Lignin in Biomass. National Renewable Energy Laboratory. *Technical Report.* NREL/TP-510-42618.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Wolfe, J. (2008) Determination of total solids in biomass and total dissolved solids in liquid process samples. National Renewable Energy Laboratory. *Technical Report.* NREL/TP-510-42621.

Starovoitov, V. I., Starovoitov, O. K., Aldoshin, N., Manokhina, A. A. (2018) Technology and mechanization of cultivation of Jerusalem artichoke healthier. Research in agricultural engineering. 64, 151-156.

Stone A. K., Karalash, A., Tyler, R. T., Warkentin, T. D., Nickerson, M. T. (2015) Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. Food research international. 76, 31-38.

Srichuwong, S., Fujiwara, M., Wang, X., Seyama, T., Shirom, R., Arakame, M., Mukojima, N.,Tokuyasu., K. (2009) Simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol. Biomass and bioenergy. 33, 890-898.

Stellenbosch University https://scholar.sun.ac.za

Sun, X., Zhang, R..Zhang, Y. (2004) Production of lignocellulolytic enzymes by *Trametes gallica* and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels. Journal of basic microbiology. 44, 220-231.

Taherian, A. R., Mondor, M., Labranche, J., Drolet, H., Ippersiel, D.,Lamarche, F. (2011) Comparative study of functional properties of commercial and membrane processed yellow pea protein isolates. Food research international. 44, 2505-2514.

Tan, S. H., Mailer, R.J., Blanchard, C. L.,Agboola, S. O. (2011) Canola proteins for human consumption: extraction, profile, and functional properties. Journal of food science. 76, 16-28.

Tannler, S., Decasper, S.,Sauer, U. (2008) Maintenance metabolism and carbon fluxes in Bacillus Species. Micobial cell factories. 7, 19-32.

Tewari, S., Ramalakshmi, K., Methre, L., Rao, L. J. M. (2015). Microwave-assisted extraction of inulin from chicory roots using response surface methodology. Journal of nutrition and food sciences. 5, 342. doi:10.4172/2155-9600.1000342.

Tenenbaum, D. J. (2008) Food vs. Fuel: Diversion of Crops Could Cause More Hunger. environmental health perspectives. 116: A254–A257.

Thompson, P. B. (2012) The Agricultural Ethics of Biofuels: The Food *vs.* Fuel Debate. Agriculture. *2*, 339-358

Urala, N., Lahteenmaki, L. (2007) Consumers' changing attitudes towards functional foods. food quality and preference. 18, 1-12.

Valentine, J., Clifton-Brown, J., Hastings, A., Robson, P., Allison, G., Smith, P. (2012) Food vs. fuel: the use of land for lignocellulosic 'next generation' energy crops that minimize competition with primary food production. Global change biology bioenergy. 4, 1-19.

Vos, T., Hakkaart, X. D. V., de Hulster, E. A. F., van Maris, A. J. A., Pronk, J. T., Daran-Lapujade, P. (2016). Maintenance-energy requirements and robustness of Saccharomyces cerevisiae at aerobic near-zero specific growth rates. Microbial cell factories. 15, 111-131.

Stellenbosch University https://scholar.sun.ac.za

Wada, T., Suqatani, J., Terada, E., Ohquchim, M., Miwa, M. (2005) Physicochemical characterization and biological effects of inulin enzymatically synthesized from sucrose. Journal of agricultural and food chemistry. 53, 1246-1253.

Wagner, W., Wiemken, A. (1987). Enzymology of Fructan Synthesis in Grasses. Plant physiology. 85, 706-710.

Wang, F., Gao, C., Yang, Chun and Xu, P. (2007) Optimization of an ethanol production medium in very high gravity fermentation. Biotechnology letters. 29, 233-236.

Wargenau, A., Kwade, A. (2010) Determination of adhesion between single *Aspergillus niger* spores in aqueous solutions using an atomic force microscope. Langmuir. 26, 11071-11076.

Wellisch, M., Jungmeier, G., Karbowski, A., Patel, M. K., Rogulska, M. (2010) Biorefinery systems—potential contributors to sustainable innovation. Biofuel bioproducts and bioresources. 4, 275-286.

Wenling, W., Huiying, W. W. L.,Shiyuan, W. (1999) Continuous preparation of fructose syrups from Jerusalem artichoke tuber using immobilized intracellular inulinase from *Kluy6eromyces* sp. Y-85. Process biochemistry. 34, 643-646.

Woods, J., Williams, A., Hughes, J. K., Black, M.,Murphy, R. (2010) Energy and the food system. Philosophical ransactions of the royal society B 365, 2991-3006.

Xiao, Y. M., Li, H. Z., Hong, B. S., Gang, X., Feng, Z., Fu, T. N., Brestic, M. (2011) Jerusalem artichoke (Helianthus tuberosus), a medicinal salt-resistant plant has high adaptability and multiple-use values. Journal of Medicinal lants esearch. 5, 1272-1279.

Xu, Y., Zheng, Z., Xu, Q., Yong. Q.,Quyang, J. (2016) Efficient Conversion of Inulin to Inulooligosaccharides through Endoinulinase from Aspergillus niger. Journal of A\agricultural and food chemistry. 64, 2612-2618.

Yang, L., He, Q. S., Corscadden, K., Udenigwe, C. C. (2015) The prospects of Jerusalem artichoke in functional food ingredients and bioenergy production. Biotechnology reports. 5, 77-88.

Stellenbosch University https://scholar.sun.ac.za

Yanovsky, E.,Kingsbury, R. M. (1933) Solubility of inulin. Journal of the American chemical society. 55, 3658-3662.

Yi, H., Zhang, L., Hua, C., Sun, K.,Zhang, L. (2009) Extraction and Enzymatic Hydrolysis of Inulin from Jerusalem artichoke and their Effects on Textural and Sensorial Characteristics of Yogurt. Food and bioprocess technology. 3: 315-319.

Yingling, B., Zongcheng, Y., Honglin, W., Li, C. (2011) Optimization of bioethanol production during simultaneous saccharification and fermentation in very high-gravity cassava mash. Antonie van Leeuwenhoek. 99, 329-339.

Yuda, B., García-Aparicio, A. P., Görgens, J. F. (2014) Impact of cultivar selection and process optimization on ethanol yield from different varieties of sugarcane. Biotechnology for biofuels. 7, 60-77.

Yuan, W. J., Chang, B. L., Ren, J. G., Liu, J. P., Bai, F. W., Li., Y. Y. (2011) Consolidated bioprocessing strategy for ethanol production from Jerusalem artichoke tubers by Kluyveromyces marxianus under high gravity conditions. Journal of applied microbiology. 112, 38-44.

Zhang, J., Siika-aho, M., Tenkanen, M., Viikari, L. (2011) The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. biotechnology for biofuels 2011, 4, 60-68.

Zhang, J., Zhang, J. (2016) The filamentous fungal pellet and forces driving its formation. Critical reviews in biotechnology. 36, 1066-1077.

Zhang, L., Chen, Q., Jin, Y., Xue, H., Guan, J., Wang, Z., Zhao, H (2010) Energy-saving direct ethanol production from viscosity reduction mash of sweet potato at very high gravity (VHG). fuel processing technology. 19, 1845-1850.

Zhang, M., Wang, F., Su, R., Qi, W., He, Z. (2010) Ethanol production from high dry matter corncob using fed-batch simultaneous saccharification and fermentation after combined pretreatment. Bioresource technology. 101, 4959-4964.

Stellenbosch University https://scholar.sun.ac.za

Zhang, T., Chi, Z., Zhao, C. H., Chi, Z.M.,Gong, F. (2010) Bioethanol production from hydrolysates of inulin and the tuber meal of Jerusalem artichoke by Saccharomyces sp. W0. Bioresource technology. 101, 8166-8170.

Zhang, X.., Vesselinov, V. V. (2016) Energy-water nexus: Balancing the trade offs between two-level decision makers. Applied energy. 183, 77-87.

Zarroug, Y., Abdelkarim, A., Dorra, S. T., Hamdaoui, G., Felah, M. E. L. , Hassouna, M. (2016) Biochemical characterization of Tunisian Cichorium Intybus L. roots and optimization of ultrasonic inulin extraction. Mediterranean journal of chemistry. 6, 674-685.

**Websites**

AGMRC, (2017) https://www.agmrc.org/renewable-energy/ethanol/ethanol-profile

EIA, (2018a) https://www.iea.org/geco/emissions/

EIA )2018b), https://www.iea.org/geco/renewables/

FAO, (2011) http://www.fao.org/3/i2454e/i2454e00.pdf

IEA (2014) www.iea-bioenergy.task42-biorefineries.com

Frost and Sullivan (2011). www.frost.com/prod/servlet/cio/236145272 Last accessed 2015/09/02

Trading economic 2019 https://tradingeconomics.com/egypt/exports/united-kingdom/manioc-arrowroot-salep-jerusalem-artichokes-sweet-potatoes-roots

CABI, (2018). https://www.cabi.org/isc/datasheet/26716#todistributionTable

United Nations Sustainable Development, 1992https://sustainabledevelopment.un.org/content/documents/Agenda21.pdf

OECD/FAO.: “Biofuels”, in OECD-FAO Agricultural Outlook 2016-2025, OECD Publishing, Paris. DOI (2016): http://dx.doi.org/10.1787/agr_outlook-2016-13-en