



**Development of a flat sheet woven fabric  
membrane fermenter for xylanase  
production by *Thermomyces lanuginosus***

**Submitted in fulfilment of the requirements for the degree of  
Master of Engineering in the Department of Chemical Engineering in  
the Faculty of Engineering and the Built Environment at Durban  
University of Technology**

**Venessa Thorulsley**

**August 2015**

## DECLARATION

---

This study presents original work by the author, Venessa Thorulsley, student number (20620155). It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences and in the Department of Chemical Engineering, Faculty of Engineering and the Built Environment, Durban University of Technology, South Africa, under the supervision of **Dr Sudesh Rathilal, Mr Suresh Ramsuroop** and **Prof Visvanathan Lingamurti Pillay**.

Venessa Thorulsley



---

**Student's signature**

August2015

---

**Date**

---

**Supervisor's signature**

---

**Date**

## DEDICATION

---

*To my parents, Mr and Mrs K. Thorulsley for the motivation to always reach for the stars*

*and*

*to my fiancé Vickesh Bholai for sharing in this journey even when the road was potholed.*

## ACKNOWLEDGEMENTS

---

I wish to thank the following persons for their contribution to the completion of this dissertation:

Creator God of the heavens and earth, for the mental faculties, physical strength, endurance and will to embark on this study. “For the Lord gives wisdom; From His mouth come knowledge and understanding.” (Proverbs 2:6)

My supervisors, Dr. Sudesh Rathilal, Mr. Suresh Ramsuroop and Prof. Lingum Pillay for their guidance during the course of the research project.

The staff of the Department of Biotechnology and Food Technology especially Dr Aswani Kumar and Dr Adarsh Puri for their expertise and the staff of the Department of Chemical Engineering especially Jafaar Bux for his assistance.

All post – graduate students in the Department of Biotechnology especially Ashira, Evashnee, Khadija, Sanjana, Vashni, Kerisha, Meng, Melvin, Stephanie, Natasha, Nadine, Nivrithi, and Trishen, for welcoming me into their department, offering immeasurable assistance and advice, much needed coffee runs, and their friendship.

Viresh Deochand and Neeresh Bholai for their electrical and mechanical proficiency and company together with Sanisha Bholai who made my days on campus a little more enjoyable.

Lastly Vickesh Bholai for weekend and night time sampling trips to the lab (even Christmas), selfless aid, listening ears when I needed to voice my frustration, a shoulder to cry on when things went sour and the encouragement I needed to see this project to its conclusion.

## ABSTRACT

---

Fermentation processes are vital for the production of numerous bioproducts. Fermentation being the mass culture of micro – organisms for the production of some desired product, is an extensive field, with immense prospects for study and improvement. Enzyme production is of significance as these proteins are biological catalysts, finding niches in numerous industries, xylanase for example is utilized in the pulp and paper, animal feed, biofuel and food production processes. During enzyme production, a critical step is biomass separation, whereby the valuable product, the enzyme, is removed from the broth or micro – biological culture before it is denatured. This is typically achieved via centrifugation.

The aim of this study was to develop and evaluate a submerged membrane fermenter system with the specific outcome of increasing the rate of production of xylanase, from the thermophilic fungal species *Thermomyces lanuginous* DSM 5826. Preliminary shake flask experiments were performed to determine the optimal production conditions, followed by partial characterization of the enzyme. A bioreactor was then fabricated to include a flat sheet membrane module, with outlets for permeate and broth withdrawal and inlets for feed and sterile air input. Experiments were conducted to determine the optimal dilution rate for maximum volumetric productivity. Results from the shake flask experiments indicated that the best conditions for xylanase production, yielding xylanase activity of  $5118.60 \pm 42.76 \text{ U.mL}^{-1}$  was using nutrient medium containing beechwood xylan (1.5 % w/v), yeast extract (1.5 % w/v), potassium dihydrogen phosphate (0.5 % w/v), adjusted to a pH of 6.5 and inoculated with 1.0 mL of spore solution, rotating in a shaking incubator set to 150 rpm at 50 °C. Apart from analysis of the effect of the carbon source on xylanase activity, coarse corn cobs were used in the shake flask experiments as a cost saving initiative. The pH optima was determined to be 6.5 while the temperature optima of the enzyme was 70 °C. SDS PAGE analysis revealed that the molecular weight of the enzyme was between 25 and 35 kDa and qualitative analysis via a zymogram revealed clear zones of hydrolysis on a xylan infused agarose gel.

During short run membrane fermenter experiments the percentage increase in enzyme activity between the batch operation ( $610.58 \pm 34.54 \text{ U.mL}^{-1}$ ) and semi – continuous operation ( $981.73 \pm 55.54 \text{ U.mL}^{-1}$ ) with beechwood xylan nutrient replenishment was 60.78 %. The maximum volumetric productivity achieved with beechwood supplementation after 192 hours in semi – continuous operation ( $5.32 \pm 0.30 \text{ U.mL}^{-1}.\text{hr}^{-1}$ ) was 2.1 times greater than that of batch operation ( $2.54 \pm 0.14 \text{ U.mL}^{-1}.\text{hr}^{-1}$ ) which equates to an increase of 110.28 % in productivity measured at its peak. The increase in total activity between batch (610 576.92 U) and beechwood xylan medium supplemented semi – continuous mode (1 184 937.50 U) resulted in a 94.07 % increase.

During long run experimental periods, the increase in production of xylanase between the batch ( $873.26 \pm 61.78 \text{ U.mL}^{-1}$ ) and the xylan medium membrane system ( $1522.41 \pm 107.65 \text{ U.mL}^{-1}$ ) was determined to be 74.34 % while an overall average increase in productivity between the batch and xylan fed membrane system was 43.25%. The total enzyme activity with in membrane mode with beechwood xylan nutrient medium feed was 160 % greater than the batch process offering a 2.6 – fold increase. Experiments where de – ionized water was alternated with beechwood xylan nutrient medium had no significant impact on the productivity or enzyme activity. The optimal dilution rate for maximum volumetric productivity as determined to be  $0.0033 \text{ hr}^{-1}$ . The results are indicative of the potential viability of such a design, yielding the desired outcome of a membrane integrated system to significantly increase the production of enzymes during fermentation.

## TABLE OF CONTENTS

---

|   |              |
|---|--------------|
| <b>DECLARATION .....</b>  | <b>i</b>     |
| <b>DEDICATION.....</b>  | <b>ii</b>    |
| <b>ACKNOWLEDGEMENTS.....</b>  | <b>iii</b>   |
| <b>ABSTRACT .....</b>   | <b>iv</b>    |
| <b>TABLE OF CONTENTS .....</b>  | <b>vi</b>    |
| <b>LIST OF FIGURES .....</b>  | <b>xii</b>   |
| <b>LIST OF TABLES .....</b>   | <b>xv</b>    |
| <b>NOMENCLATURE .....</b>   | <b>xvii</b>  |
| <b>ABBREVIATIONS .....</b>  | <b>xviii</b> |
| <b>1. INTRODUCTION.....</b>   | <b>1</b>     |
| 1.1. Background .....   | 1            |
| 1.2. Objectives .....   | 2            |
| 1.3. Approach .....   | 3            |
| 1.4. Thesis organization .....  | 3            |
| <b>2. LITERATURE REVIEW .....</b>                                       | <b>5</b>     |
| 2.1. Fermentation technology .....                                      | 5            |
| 2.1.1. Classification of micro – organisms.....                         | 5            |
| 2.1.1.1. Fungi .....  | 7            |
| 2.1.2. Microbial growth.....  | 7            |
| 2.1.2.1. Lag phase.....   | 9            |
| 2.1.2.2. Growth phase .....   | 9            |
| 2.1.2.3. Stationary and death phase .....                               | 10           |
| 2.1.3. Nutritional requirements for fermentation .....                  | 11           |
| 2.1.3.1. Carbon source .....  | 11           |
| 2.1.3.2. Nitrogen source.....   | 11           |
| 2.1.3.3. Minerals.....  | 12           |
| 2.1.3.4. Inhibitors, precursors and inducers.....                       | 12           |
| 2.1.3.5. Antifoaming agents, buffers, growth factors and chelators..... | 12           |
| 2.1.4. Environmental conditions .....                                   | 13           |

|  |    |
|--|----|
| 2.1.4.1. Temperature.....  | 13 |
| 2.1.4.2. pH .....  | 13 |
| 2.1.4.3. Water activity .....                                    | 14 |
| 2.1.4.4. Oxygen.....   | 14 |
| 2.1.4.5. Radiation .....   | 14 |
| 2.1.4.6. Hydrostatic pressure .....                              | 15 |
| 2.1.5. Applications of fermentation .....                        | 15 |
| 2.1.5.1. Microbial cells.....                                    | 15 |
| 2.1.5.2. Microbial metabolites .....                             | 16 |
| 2.1.5.3. Microbial enzymes .....                                 | 18 |
| 2.1.5.4. Recombinant products and transformation processes ..... | 20 |
| 2.1.6. Fermenter technology.....                                 | 20 |
| 2.1.6.1. The aerated stirred tank batch fermenter .....          | 22 |
| 2.1.6.2. Anaerobic fermenters .....                              | 22 |
| 2.1.6.3. Continuous fermentation .....                           | 23 |
| 2.1.6.3.1. Classification of continuous fermenters .....         | 24 |
| 2.1.6.4. Fed – batch cultivation .....                           | 24 |
| 2.1.6.5. Other submerged culture fermenters .....                | 24 |
| 2.1.6.6. Construction and design of fermenters .....             | 25 |
| 2.1.6.6.1. Materials of construction .....                       | 25 |
| 2.1.6.6.2. Agitation and aeration .....                          | 26 |
| 2.1.6.6.3. Temperature control .....                             | 26 |
| 2.1.6.6.4. Foam production and control.....                      | 26 |
| 2.1.6.6.5. pH measurement and control .....                      | 27 |
| 2.2. Membrane Technology.....                                    | 28 |
| 2.2.1. Downstream biotechnological processing .....              | 28 |
| 2.2.2. Membrane filtration as a separation technique .....       | 31 |
| 2.2.3. Membrane processes .....                                  | 31 |
| 2.2.3.1. Reverse osmosis .....                                   | 33 |
| 2.2.3.2. Microfiltration .....                                   | 34 |
| 2.2.3.3. Ultrafiltration.....                                    | 35 |
| 2.2.3.4. Diafiltration .....                                     | 35 |
| 2.2.3.5. Nanofiltration .....                                    | 36 |
| 2.2.3.6. Depth filtration.....                                   | 37 |
| 2.2.3.7. Dialysis.....   | 38 |
| 2.2.4. Membrane structures and materials .....                   | 39 |
| 2.2.5. Membrane properties.....                                  | 41 |



|  |           |
|--|-----------|
| 2.2.5.1. Molecular weight cut – off or pore size .....                   | 41        |
| 2.2.5.2. Pure water permeability (PWP) .....                             | 41        |
| 2.2.5.3. Surface/pore charge (Zeta potential or isoelectric point) ..... | 42        |
| 2.2.5.4. Roughness .....   | 43        |
| 2.2.6. Membrane flow geometry .....                                      | 44        |
| 2.2.6.1. Dead – end filtration .....                                     | 44        |
| 2.2.6.2. Cross – flow filtration .....                                   | 44        |
| 2.2.7. Membrane configuration and modules .....                          | 44        |
| 2.2.7.1. Plate – and – frame membrane module .....                       | 45        |
| 2.2.7.2. Spiral wound membrane module .....                              | 46        |
| 2.2.7.3. Stirred cell membrane module .....                              | 47        |
| 2.2.7.4. Tubular membrane module .....                                   | 47        |
| 2.2.7.5. Hollow fibre membrane module .....                              | 47        |
| 2.2.8. Membrane bioreactors .....  | 48        |
| 2.2.8.1. Immersed versus non – immersed membranes in bioreactors .....   | 49        |
| 2.2.8.2. Film bioreactors .....  | 50        |
| 2.2.8.3. Applications of membrane bioreactors .....                      | 51        |
| 2.2.8.3.1. Wastewater treatment .....                                    | 51        |
| 2.2.8.3.2. Chemical fermentation .....                                   | 52        |
| 2.2.8.3.3. Food fermentation .....                                       | 52        |
| 2.2.8.3.4. Enzyme production .....                                       | 53        |
| 2.3. Enzymes as catalysts .....  | 54        |
| 2.3.1. Xylanase .....  | 55        |
| 2.3.1.1. Classification and structure .....                              | 56        |
| 2.3.1.2. Substrate and mechanism .....                                   | 57        |
| 2.3.1.3. Applications of xylanase .....                                  | 58        |
| 2.3.1.3.1. Supplementation of animal feed .....                          | 58        |
| 2.3.1.3.2. Production of biofuel .....                                   | 59        |
| 2.3.1.3.3. Food production processes .....                               | 60        |
| 2.3.1.3.4. Pulp and paper processing .....                               | 60        |
| 2.3.4. <i>Thermomyces lanuginosus</i> as a producer of xylanase .....    | 61        |
| <b>3. METHODOLOGY .....</b>  | <b>62</b> |
| 3.1. Maintenance of culture .....  | 62        |
| 3.1.1. Equipment and material .....                                      | 62        |
| 3.1.2. Methodology .....   | 63        |
| 3.2. Production of xylanase by shake flask submerged fermentation .....  | 64        |

|  |    |
|--|----|
| 3.2.1. Equipment and material.....   | 64 |
| 3.2.2. Methodology.....  | 65 |
| 3.2.2.1. Evaluation of the effect of carbon source.....                      | 65 |
| 3.2.2.2. Evaluation of the effect of nitrogen source.....                    | 67 |
| 3.2.2.3. Initial pH optimization.....  | 67 |
| 3.2.2.4. Rotational speed optimization.....                                  | 67 |
| 3.2.2.5. Inoculum size optimization.....                                     | 67 |
| 3.2.2.6. Production temperature optimization.....                            | 68 |
| 3.2.2.7. Fed – batch operation.....  | 68 |
| 3.2.2.8. Semi – continuous operation.....                                    | 68 |
| 3.3. Partial characterization of xylanase enzyme.....                        | 69 |
| 3.3.1. Equipment and materials.....  | 69 |
| 3.3.2. Methodology.....  | 70 |
| 3.3.2.1. Temperature optima.....   | 70 |
| 3.3.2.2. pH optima.....  | 70 |
| 3.3.2.3. SDS PAGE.....   | 70 |
| 3.3.2.3.1. Gel preparation.....  | 71 |
| 3.3.2.3.2. Gel electrophoresis and staining.....                             | 71 |
| 3.3.2.4. Xymogram.....   | 73 |
| 3.4. Translation to membrane fermenter system.....                           | 74 |
| 3.4.1. Equipment and materials.....  | 74 |
| 3.4.2. Methodology.....  | 74 |
| 3.4.2.1. Description of apparatus.....                                       | 74 |
| 3.4.2.2. Fabrication of apparatus.....                                       | 75 |
| 3.4.2.3. Membrane fermenter optimization.....                                | 78 |
| 3.4.2.3.1. Troubleshooting and modification of procedure.....                | 78 |
| 3.4.2.3.2. Effect of nutrient manipulation on activity and productivity..... | 80 |
| 3.4.2.3.3. Optimization of nutrient medium dilution rate.....                | 81 |
| 3.4.4. Prior concepts for the membrane fermenter system.....                 | 82 |
| 3.4.4.1. Film fermenter.....   | 82 |
| 3.4.4.2. Tank fermenter.....   | 83 |
| 3.5. Analytical methods.....   | 86 |
| 3.5.1. Determination of xylanase activity.....                               | 86 |
| 3.5.1.1. Equipment and materials.....  | 86 |
| 3.5.1.2. Methodology.....  | 86 |
| 3.5.1.2.1. Substrate solution preparation.....                               | 87 |
| 3.5.1.2.2. Sodium citrate buffer preparation.....                            | 87 |

|  |           |
|--|-----------|
| 3.5.1.2.3. DNS reagent preparation.....  | 87        |
| 3.5.1.2.4. Reagent blank preparation .....   | 88        |
| 3.5.1.2.5. Enzyme blank preparation.....   | 88        |
| 3.5.1.2.6. Standard curve preparation.....   | 89        |
| 3.5.2. Total protein analysis .....  | 89        |
| 3.5.2.1. Equipment and materials .....   | 89        |
| 3.5.2.2. Methodology .....   | 89        |
| 3.5.2.2.1. Preparation of the standard curve .....   | 91        |
| 3.5.3. Dry weight analysis.....  | 91        |
| 3.5.3.1. Equipment and materials .....   | 91        |
| 3.5.3.2. Methodology .....   | 91        |
| <b>4. RESULTS AND DISCUSION .....</b>  | <b>92</b> |
| 4.1. Experiments conducted in shake flasks.....  | 92        |
| 4.1.1. Effect of various carbon sources on xylanase activity.....  | 92        |
| 4.1.2. Effect of various nitrogen sources on xylanase activity .....                                     | 94        |
| 4.1.3. Effect of varying the initial pH on xylanase activity .....                                       | 96        |
| 4.1.4. Effect of varying the rotational speed on xylanase activity .....                                 | 98        |
| 4.1.5. Effect of varying the inoculum size on xylanase activity.....                                     | 99        |
| 4.1.6. Effect of varying production temperatures on xylanase activity.....                               | 101       |
| 4.1.7. Effect of operating in fed – batch mode .....   | 102       |
| 4.1.8. Effect of operating in semi – continuous mode .....   | 105       |
| 4.2. Experiments conducted to partially characterize the enzyme.....                                     | 107       |
| 4.2.1. pH optima.....  | 107       |
| 4.2.2. Temperature optima .....  | 109       |
| 4.2.3. SDS PAGE and zymogram analysis .....  | 110       |
| 4.3. Experiments conducted in the membrane fermenter .....   | 112       |
| 4.3.1. Effect of mode of operation on dry – weight of the micro – organism .....                         | 113       |
| 4.3.2. Effect of mode of operation on xylanase activity .....  | 115       |
| 4.3.3. Effect of mode of operation on volumetric productivity .....                                      | 116       |
| 4.3.4. Effect of mode of operation on total enzyme activity .....  | 121       |
| 4.4. Experiments conducted in the membrane fermenter over an extended period.....                        | 124       |
| 4.4.1. Effect of mode of operation on dry – weight of the micro – organism over an extended period ..... | 125       |
| 4.4.2. Effect of mode of operation on xylanase activity over an extended period.....                     | 128       |
| 4.4.3 Effect of mode of operation on volumetric productivity over an extended period .....               | 130       |

|  |            |
|--|------------|
| 4.4.4 Effect of mode of operation on total activity over an extended period .....            | 135        |
| 4.4.5 Effect of mode of operation on total protein concentration over an extended period ... | 139        |
| 4.4.6 Effect of mode of operation on pH over an extended period.....                         | 141        |
| 4.5. Optimization of dilution rate .....   | 143        |
| 4.5.1. Effect of different dilution rates on enzyme activity .....                           | 143        |
| 4.5.2 Effect of different dilution rates on volumetric productivity.....                     | 145        |
| <b>5. CONCLUSION AND RECOMMENDATIONS .....</b>   | <b>152</b> |
| 5.1. Conclusion .....  | 152        |
| 5.2. Recommendations.....  | 153        |
| <b>6. REFERENCES.....</b>  | <b>154</b> |

## LIST OF FIGURES

---

|  | <u>Page</u> |
|--|-------------|
| Figure 2-1: Phylogenetic – symbiogenetic tree of living organisms (Doridí, 2013).....                                      | 6           |
| Figure 2-2: Typical growth curve of a bacterial culture (Lee, 2003).....   | 8           |
| Figure 2-3: Types of fermenters based on different parameters, adapted from (Kale and Bhusari, 2007) .....                 | 21          |
| Figure 2-4: A typical aerated stirred tank batch fermenter (Reddy et al., 2012) .....                                      | 23          |
| Figure 2-5: Sequence of purification steps following fermentation, adapted from (Okafor, 2007) .....                       | 30          |
| Figure 2-6: Modes of membrane separation (Rathore and Shirke, 2011) .....  | 32          |
| Figure 2-7: Range of nominal pore diameters for commercially available membranes (Perry et al., 1997) .....                | 33          |
| Figure 2-8: Reverse osmosis schematic .....  | 34          |
| Figure 2-9: Basic diafiltration configuration .....  | 36          |
| Figure 2-10: Nanofiltration schematic .....  | 37          |
| Figure 2-11: Acid recovery by diffusion – dialysis.....  | 39          |
| Figure 2-12: Plate and frame membrane module .....   | 46          |
| Figure 2-13: Submerged (a) and side stream (b) membrane bioreactor configurations .....                                    | 50          |
| Figure 2-14: Schematic representation of enzyme – substrate ‘lock and key’ mechanism....                                   | 55          |
| Figure 2-15: (A) Topology diagram and (B) a schematic 3D structure of the family 11 xylanases (Purmonen et al., 2007)..... | 57          |
| Figure 2-16: Xylan degradation by xylanase (Held, 2012) .....  | 58          |
| Figure 3-1: <i>Thermomyces lanuginosus</i> agar plates during growth .....   | 64          |
| Figure 3-2: Processing of corn cobs .....  | 66          |
| Figure 3-3: Membrane module fabrication .....  | 76          |
| Figure 3-4: Schematic of membrane fermenter system.....  | 77          |
| Figure 3-5: Film fermenter (a) Diagrammatic representation and (b) Fabricated concept ....                                 | 83          |
| Figure 3-6: Tank fermenter (a) Diagrammatic representation and (b) Fabricated concept ...                                  | 85          |
| Figure 3-7: Reduction of 3,5 – dinitrosalicylic acid (Goel, 2007) .....  | 88          |

|   |     |
|---|-----|
| Figure 3-8: Coomassie dye changes from a red cationic form (a) (Congdon et al., 1993) to a blue anionic form (c) (Kilkowski and Gross, 1999) in the presence of proteins. The intermediate neutral form (b) is green (Katrahalli et al., 2010) .....  | 90  |
| Figure 4-1: Effect of various carbon sources on xylanase activity.....  | 93  |
| Figure 4-2: Effect of various nitrogen sources on xylanase activity .....   | 95  |
| Figure 4-3: Effect of varying the initial pH on xylanase activity .....   | 97  |
| Figure 4-4: Effect of varying the rotational speed on xylanase activity .....   | 98  |
| Figure 4-5: Effect of varying the inoculum size on xylanase activity .....  | 100 |
| Figure 4-6: Effect of varying production temperatures on xylanase activity .....  | 101 |
| Figure 4-7: Effect of fed – batch operation on xylanase activity .....  | 103 |
| Figure 4-8: Effect of semi – continuous operation on xylanase activity.....   | 106 |
| Figure 4-9: Cumulative effect of semi – continuous operation on xylanase activity .....   | 107 |
| Figure 4-10: pH optima of xylanase from <i>T. lanuginosus</i> DSM 5826 .....  | 108 |
| Figure 4-11: Temperature optima of xylanase from <i>T. lanuginosus</i> DSM 5826.....  | 110 |
| Figure 4-12: SDS PAGE and zymogram of xylanase from <i>Thermomyces lanuginosus</i> DSM 5826 (a) Spectra Multicolour Broad Range Protein Ladder, (b) Analysis of xylanase sample, Lane 1 – Protein marker, Lane 2 – Xylanase sample, Lane 3 – Zymogram for xylanase revealed with ethanol, Lane 4 – Protein staining ..... | 111 |
| Figure 4-13: Correlation between Daltons and microns (Koch membrane systems inc).....   | 112 |
| Figure 4-14: Effect of mode of operation on dry – weight of the micro – organism .....  | 113 |
| Figure 4-15: Effect of mode of operation on xylanase activity.....  | 115 |
| Figure 4-16: Effect of mode of operation on volumetric productivity .....   | 119 |
| Figure 4-17: Effect of mode of operation on total xylanase activity .....   | 123 |
| Figure 4-18: Effect of mode of operation on dry – weight of the micro – organism over an extended period.....   | 125 |
| Figure 4-19: Fungal growth in membrane vessel, (a) Growth after 216 hours, (b) Growth after 384 hours .....   | 127 |
| Figure 4-20: Effect of mode of operation on xylanase activity over an extended period.....  | 128 |
| Figure 4-21: Effect of mode of operation on volumetric productivity over an extended period .....   | 133 |

|   |     |
|---|-----|
| Figure 4-22: Effect of mode of operation on total xylanase activity over an extended period .....     | 138 |
| Figure 4-23: Effect of mode of operation on total protein concentration over an extended period ..... | 139 |
| Figure 4-24: Effect of mode of operation on pH over an extended period.....                           | 141 |
| Figure 4-25: Effect of varying dilution rates on xylanase activity.....                               | 143 |
| Figure 4-26: Effect of varying dilution rates on volumetric productivity .....                        | 148 |
| Figure 4-27: Optimization of dilution rates based on volumetric productivity.....                     | 150 |

## LIST OF TABLES

---

|  | <u>Page</u> |
|--|-------------|
| Table 2-1: Commercial metabolites and their significance .....   | 17          |
| Table 2-2: Enzymes, inducers and applications .....  | 19          |
| Table 2-3: Commonly used polymers for membrane separation processes (Wang and Zhou, 2013) .....  | 40          |
| Table 3-1: Preparation of 12 % separating gel.....   | 72          |
| Table 3-2: Preparation of stacking gel .....   | 72          |
| Table 3-3: Preparation of 10 x running buffer (1 L) .....  | 73          |
| Table 4-1: Volumetric productivity in batch operation .....  | 117         |
| Table 4-2: Volumetric productivity in semi – continuous operation with DI water supplementation.....   | 117         |
| Table 4-3: Volumetric productivity in semi – continuous operation with beechwood xylan nutrient medium supplementation .....                                       | 118         |
| Table 4-4: Total activity in batch operation.....  | 121         |
| Table 4-5: Total activity of permeate during semi – continuous operation with DI water supplementation.....  | 121         |
| Table 4-6: Total activity of retentate during semi – continuous operation with DI water supplementation.....   | 122         |
| Table 4-7: Total activity of permeate during semi – continuous operation with beechwood xylan nutrient medium supplementation.....                                 | 122         |
| Table 4-8: Total activity of retentate during semi – continuous operation with beechwood xylan nutrient medium supplementation.....                                | 123         |
| Table 4-9: Volumetric productivity in batch operation over an extended period .....  | 130         |
| Table 4-10: Volumetric productivity in semi – continuous operation with DI water and beechwood xylan nutrient medium supplementation over an extended period ..... | 131         |
| Table 4-11: Volumetric productivity in semi – continuous operation beechwood xylan nutrient medium supplementation over an extended period .....                   | 132         |
| Table 4-12: Total activity in batch operation over an extended period.....   | 135         |



|   |     |
|---|-----|
| Table 4-13: Total activity of permeate during semi – continuous operation with alternate DI water and beechwood xylan nutrient medium supplementation over an extended period | 135 |
| Table 4-14: Total activity of retentate during semi – continuous operation with alternate DI water and beechwood xylan nutrient medium supplementation .....                  | 136 |
| Table 4-15: Total activity of permeate during semi – continuous operation with beechwood xylan nutrient medium supplementation .....  | 136 |
| Table 4-16: Total activity of retentate during semi – continuous operation with beechwood xylan nutrient medium supplementation .....   | 137 |
| Table 4-17: Volumetric productivity in semi – continuous operation at a dilution rate of $0.0017 \text{ hr}^{-1}$ .....   | 145 |
| Table 4-18: Volumetric productivity in semi – continuous operation at a dilution rate of $0.0033 \text{ hr}^{-1}$ .....   | 146 |
| Table 4-19: Volumetric productivity in semi – continuous operation at a dilution rate of $0.0050 \text{ hr}^{-1}$ .....   | 147 |

## NOMENCLATURE

| Symbol         | Description                         | Unit                   |
|----------------|-------------------------------------|------------------------|
| A              | Cross sectional area                | m <sup>2</sup>         |
| J              | Linear fluid velocity               | (L/m <sup>2</sup> .hr) |
| k              | Electrical conductivity             | mS/m                   |
| M              | Molarity                            | mol.L <sup>-1</sup>    |
| n              | Number of doubling                  |                        |
| N <sub>0</sub> | Initial number of cells             |                        |
| OD             | Outer diameter                      | mm                     |
| R              | Pore radius                         | M                      |
| R <sub>m</sub> | Permeability of clean filter medium |                        |
| T              | Time                                | hr                     |
| t <sub>d</sub> | Doubling time                       | hr                     |
| V              | Volume                              | L                      |
| W              | Weight                              | g                      |
| β              | Beta linkage on molecule            |                        |
| ΔP             | Pressure gradient                   | N/m <sup>2</sup>       |
| Δx             | Membrane thickness                  | M                      |
| ΔΦ             | Streamlining potential              | V                      |
| ε              | Permittivity of solution            | s/m                    |
| ε              | Porosity                            |                        |
| ζ              | Zeta potential                      | V                      |
| η              | Viscosity                           | Pa.s                   |
| τ              | Tortuosity factor                   |                        |

## ABBREVIATIONS

---

|          |  |
|----------|--|
| AFM      | Atomic force microscopy                                    |
| AME      | Apparent metabolisable energy                              |
| AMPS     | Ammonium persulfate  |
| BSA      | Bovine serum albumin                                       |
| BW       | Beechwood  |
| CFF      | Cross flow filtration                                      |
| DI       | De – ionized   |
| DNA      | Deoxyribonucleic acid                                      |
| DNS      | Dinitrosalicylic acid                                      |
| EDTA     | Ethylenediaminetetraacetic acid                            |
| GS       | Gas separation   |
| MF       | Microfiltration  |
| MWCO     | Molecular weight cut – off                                 |
| NF       | Nanofiltration   |
| OD       | Outer diameter   |
| PDA      | Potato dextrose agar                                       |
| PV       | Pervaporation  |
| PVC      | Polyvinyl chloride   |
| RNA      | Ribonucleic acid   |
| RO       | Reverse osmosis  |
| SCP      | Single cell protein  |
| SDS PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SEM      | Scanning electron microscopy                               |
| TCF      | Thin film composite  |
| TEM      | Transmission electron microscopy                           |
| TEMED    | Tetramethylethylenediamine                                 |
| UF       | Ultrafiltration  |

XOS

Xylo oligosaccharides

# 1. INTRODUCTION

---

## 1.1. Background

Fermentation, an age old biochemical process which is responsible for the production of valuable commodities in almost all sectors, is developing significantly, as its applications have surpassed simple bread and wine making, into the field of biotechnology where microbial cells, enzymes and other metabolites have become the focus in the last century.

Fermentation may be conducted in various modes of operation viz., batch, fed – batch, semi – continuous, and continuous modes, each having their advantages and disadvantages. The major disadvantage of batch fermentation is low productivity due to the long turn – around times associated with cleaning and preparation of fermenters and the initial lag phase during the growth of the required micro – organism (Dunford, 2012). Continuous fermentation on the other hand offers higher conversion rates, improved product consistency, reduced product losses and especially higher volumetric efficiencies than batch fermentation due to the higher cell concentrations in the vessel (Verbelen et al., 2006).

Since fermentation is effected by the mass culture of micro – organisms in the appropriate apparatus and at optimal conditions, it is then necessary to separate the valuable products of fermentation from the biomass. The methods of separation include decanting, centrifugation, extraction and filtration. Membrane filtration processes provide a means of separation and concentration at the molecular and fine particle level and can be used to separate the range of species found in fermentation broths including microbial cells, viruses, colloids, proteins, polysaccharides, enzymes, antibiotics, simple sugars, organic acids and inorganic ions (Asenjo, 1990). The membrane may either be submerged or configured externally, also known as a side – stream system. Submerged systems offer constant reaction conditions, rule out additional shear and have less cell loss because of reduced dead volume (Carstensen et al., 2012) as well as easier sterilization and energy and space reduction when compared to non – immersed membrane systems. The development of a membrane fermenter which can be operated in semi – continuous mode to achieve high volumetric productivity is the focus of this study.

A commercially viable bio – product as well as a known producer micro – organism of the bio – product were selected for the study. The product of interest is xylanase. Xylanase is an enzyme which catalyses the hydrolysis of xylan which is a major component of plant tissue. Xylan is a polymer chain which can be hydrolysed by xylanase to its simple monomer sugar xylose. Xylose which is a primary carbon source is liberated and holds value due to the fact that it may be substituted for glucose. Xylanase is also used to supplement animal feed to aid digestion, break down hemicelluloses fractions in the bio – fuel as well as pulp and paper industries and as clarifiers, emulsifiers and oxidants in the food and beverage industries.

One of the hurdles in the commercialization of enzymatic processes is the bulk production of enzymes at a cost effective rate (Dhiman et al., 2008). A process that is able to exploit the ability of a micro – organism to metabolize waste materials and produce xylanase at high volumetric rates is required. As such, xylanase has been selected as the bio – product to be produced in this study.

The micro – organism to be used is *Thermomyces lanuginosus*, a fungal strain known to produce xylanase. Bacterial strains have been found to produce xylanase of lower activity levels than fungal strains, and these are restricted to internal fractions. *Thermomyces* is a thermophilic fungus, which is of interest as it is known to produce thermostable xylanases, which can be utilized in a wider array of applications.

## **1.2. Objectives**

The objectives of this research project are:

- To develop a membrane fermenter system using a flat sheet woven fabric membrane that is suitable for xylanase production by *Thermomyces lanuginosus* DSM 5826
- To determine the optimum operating conditions of the fermenter system
- To improve the volumetric productivity of the system by semi – continuous membrane fermentation

### **1.3. Approach**

1. A literature survey of existing information is to be conducted. The fields of microbiology, fermentation and membrane technology are to be reviewed with emphasis on xylanase production, woven fabric membranes and integrated membrane fermentation systems.
2. Review of laboratory production techniques and analysis required for xylanase production.
3. Conditions for optimal production will be investigated. The parameters to be investigated are the growth medium, production temperature, initial pH, rotational speed and time for production.
4. Enzyme characterization will be done to validate that xylanase has indeed been produced and to determine its molecular weight as well as the optimal pH and temperature that it is active in.
5. A membrane fermenter system whereby xylanase will be successfully produced will be developed. The specific outcomes desired from this system are to maintain the biomass of the micro – organism by operating the system in semi – continuous mode and to increase volumetric productivity by manipulating the nutrient feed to the system.

### **1.4. Thesis organization**

#### Chapter 2: Literature Review

The literature review will include information on fermentation and membrane technology as the two technologies being integrated into a membrane fermenter system. A review of current work on membrane bioreactors will be included as well as production studies on xylanase and *Thermomyces lanuginosus* systems.

### Chapter 3: Materials and Methods

The materials, equipment and methodology to grow and maintain the fungal culture as well as produce xylanase in shake flasks will be detailed here. Analytical techniques and methods of characterization employed will be discussed. The development procedure of translating the production system from shake flasks to a successful membrane fermentation system will be the culmination of this chapter.

### Chapter 4: Results and Discussion

The data that is collected will be refined and displayed as significant results which can then be interpreted. The results will be discussed in terms of the objectives of the research.

### Chapter 5: Conclusion and Recommendations

Conclusions regarding the effectiveness and usefulness of the research will be drawn. Recommendations to future work will be suggested.



## 2. LITERATURE REVIEW

---

This chapter details the technologies of fermentation and membrane separation, which when combined have yielded the concept of membrane fermenters or membrane bioreactors as they are sometimes referred to. The requirements, applications and technology involved in the fermentation process are described as well as the need for separation subsequent to the process. Membrane technology including the various membranes processes, properties of membranes and configurations are discussed, culminating in current membrane bioreactor applications. A brief review of xylanase and its applications is presented.

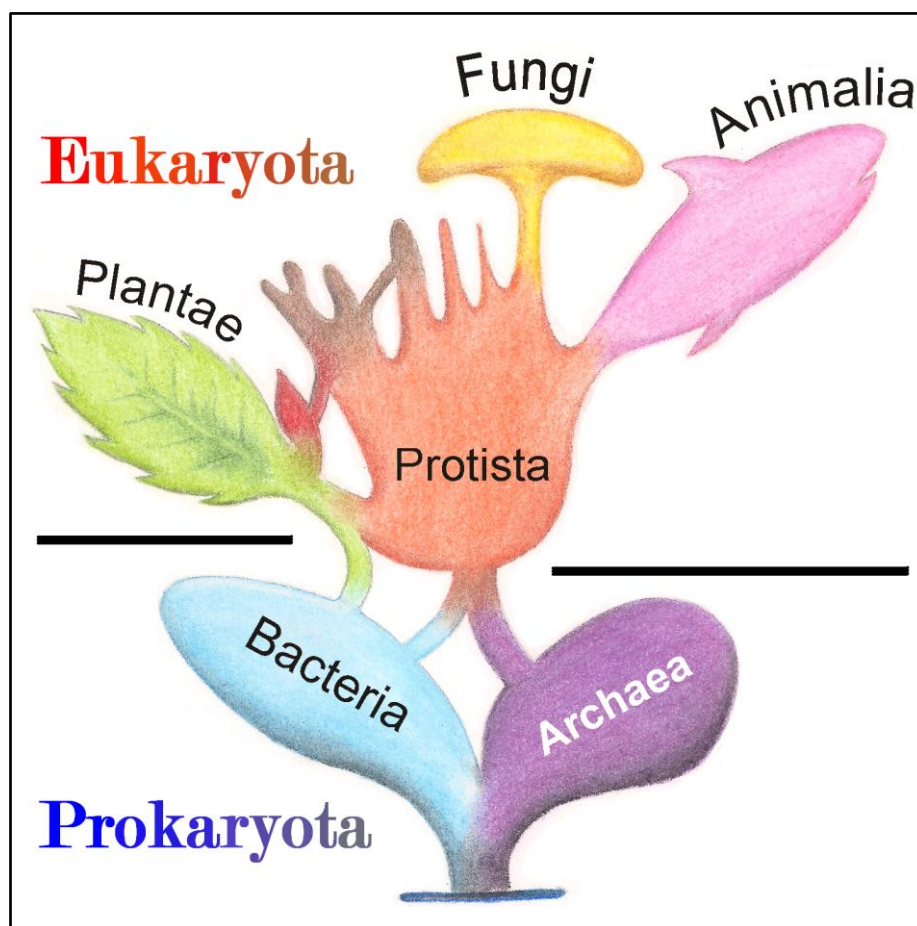
### 2.1. Fermentation technology

Fermentation is a biological process which has been studied and utilized for millennia. The term “fermentation” is derived from the Latin, “*fevere*” which means “to boil”, which describes the appearance of the process of alcohol production by yeasts acting on extracts of fruit or malted grain. The term is defined differently by microbiologists and biochemists, however. Microbiologists describe fermentation as any process for the production of a product by the mass culture of micro – organisms. Biochemists describe fermentation as an energy – generating process in which organic compounds act as both electron donors and acceptors, that is, an anaerobic process where energy is produced without the participation of oxygen or other inorganic electron acceptors (Stanbury et al., 1995). Fermentation is effected through the action of micro – organisms (with the appropriate metabolic apparatus) on specific substrates.

#### 2.1.1. Classification of micro – organisms

Micro – organisms can be found almost everywhere in the taxonomic organization of life on the planet. In the nineteenth century all life was classed as part of either the plant or animal kingdom. The assessment of the microbes to either kingdom was made on the basis of the most easily determinable differences between animals and plants which was the ability of active movement and the ability to photosynthesize. Algae and fungi were classed as plants

while protozoa and bacteria as animals. This distribution was inconsistent as fungi are not photosynthetic, and some fungi and algae have motile parts. A third kingdom, Protists, was proposed by E. Haeckel in 1886, to include protozoa, algae, fungi and bacteria (Whittaker, 1969). With the development of the electron microscope in 1950, the two kinds of cells that exist in the living world were distinguished. These are prokaryotic and eukaryotic cells. Prokaryotes lack a nucleus while eukaryotes possess a well defined nucleus (Parihar, 2008). A third domain called archaea was introduced by Carl Woese (Kapoor, 2010). It is recognized now that there are three domains, eukarya (protists, fungi, plant and animal kingdoms), bacteria and archaea. According to biologists bacteria and archaea are prokaryotes while protists, fungi, plants and animals are eukaryotes. The picture below depicts the six kingdoms. Of the kingdoms outlined in Figure 2-1 below, fungi have a great degree of application in fermentation processes, up to the industrial scale.



**Figure 2-1: Phylogenetic – symbiogenetic tree of living organisms (Doridí, 2013)**

#### **2.1.1.1. Fungi**

The fungi are a monophyletic group also called *Eumycota*, that is phylogenetically distinct from the morphologically similar slime moulds, *myxomycetes* and water moulds, *oomycetes* (Moore and Frazer, 2002). The fungi are heterotrophic organisms possessing a chitinous cell wall, with most species growing as multicellular filaments called hyphae forming a mycelium, although some species grow as single cells. Reproduction of the fungi occurs sexually and asexually via spores. Yeasts, moulds and mushrooms are examples of fungi. Occurring worldwide, most fungi are visible to the naked eye. They perform an essential role in ecosystems in decomposing organic matter and are indispensable in nutrient cycling and exchange. Fungi are significant pathogens of humans and animals. Losses due to diseases of crops and food spoilage have a large impact on the human food supply and local economies. Fungi grow in a wide range of habitats, including deserts, hyper – saline environments, the deep sea, on rocks, and in extreme temperatures. They have been used as a direct source of food such as mushrooms and truffles, and in fermentation of various food products such as wine, beer, and soy sauce (Khachatourians and Arora, 2001). More recently, fungi have been used as sources for antibiotics used in medicine and various enzymes which are important for industrial use or as active ingredients of detergents.

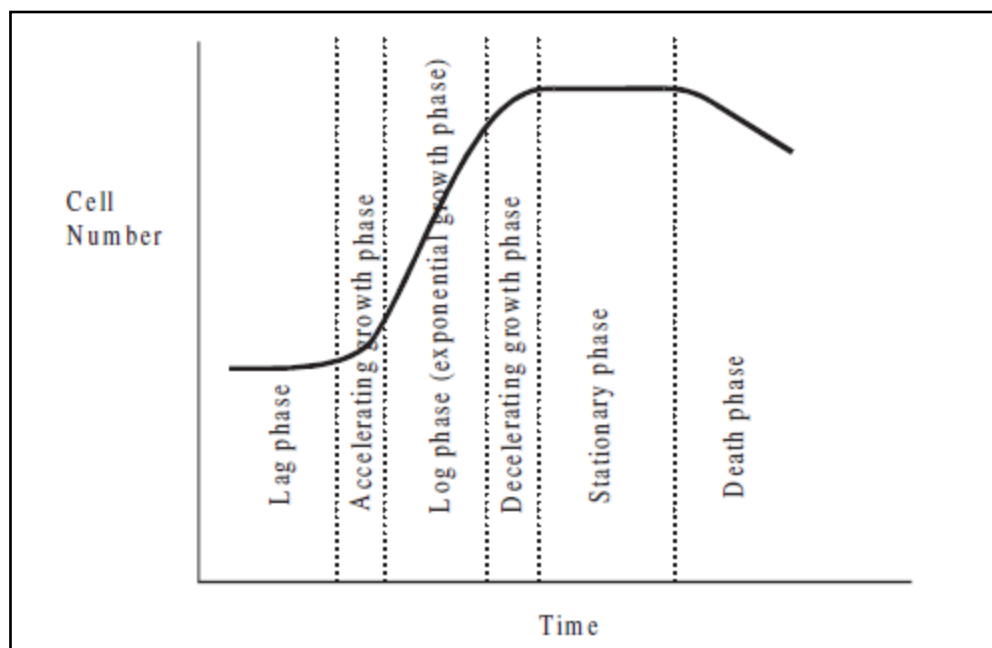
#### **2.1.2. Microbial growth**

Microbial growth kinetics is a key component to optimizing the fermentation process. Understanding this process and the parameters involved helps to identify the most important factors contributing to the maximum output of fermentation product. The factors influencing microbial proliferation and the manner in which they affect this phenomenon can be determined from the bacterial growth curve and observation of the phases of growth.

Most unicellular micro – organisms reproduce asexually when the nutrients required for cell growth are present at sufficiently high concentrations. The size and amount of the individual cells increase with time, resulting in biomass growth. Biomass is defined as the materials essential for the structure and reproduction of a living organism. Eventually, the DNA

content doubles in quantity and cell division occurs. DNA or Deoxyribonucleic acid is nucleic acid containing the genetic instructions used in the development and functioning of all known living organisms, with the exception of RNA viruses. The cell divides completely into two progenies of equal genome and approximately identical size, thereby increasing the population number (Lee, 2003).

Growth kinetics involves the rate of cell growth and the various chemical and physical conditions that affect it. During the course of growth, the heterogeneous mixture of young and old cells is continuously changing and adapting itself in the media environment, which is also continuously changing in physical and chemical conditions. There are distinct growth phases in the growth curve of a microbial culture. A typical growth curve includes the lag phase, growth phase and stationary and death phase. The growth phase is further divided into the accelerating, exponential and decelerating growth phase (Panikov, 1995). A typical growth curve is given in Figure 2-2 below.



**Figure 2-2: Typical growth curve of a bacterial culture (Lee, 2003)**

### 2.1.2.1. Lag phase

When an inoculum is introduced to a fresh culture medium, the culture may not immediately grow at the maximum rate, thus giving rise to a lag phase. This is essentially a period of adaptation of cells to a new environment. The cells increase in size and weight rather than increase in numbers. Lag phase is considered a non – productive period of a fermentation process (Ginovart et al., 2011). Thus, it is often desirable to minimize or control the duration of the lag phase. Based on its mechanism, growth lag can be differentiated into apparent lag and true lag. Immediately after inoculation, a portion of the seed culture may grow at the maximum rate while the rest fail to grow, thus resulting in an apparent lag phase. For example, if half the cells in the inoculum are not viable, and the other half of the cells grow at the maximum rate, it may appear that the culture is growing at half of the maximum rate (Monod, 1949). True lag occurs when the culture is not able to grow at its maximum rate initially due to factors such as a change in nutrient, change in culture conditions, presence of an inhibitor, spore germination, and inoculum effect, that is, the physiological stage of growth of the inoculum (Pirt, 1975).

### 2.1.2.2. Growth phase

There are three stages at the growth phase, namely the accelerating growth phase, the exponential or logarithmic growth and decelerating growth phase (Waites et al., 2009). At the late lag period, the cells have adjusted to the new environment and begin to grow and multiply, this is the accelerating growth phase. The cells then enter the exponential or logarithmic growth phase. Here the cells divide regularly by binary fission, and grow in geometric progression. The rate of exponential growth of a bacterial culture is expressed as generation or doubling time  $t_d$  of the population (Todar, 2006). The number of cells in an exponentially growing microbial culture could be expressed mathematically as follows:

$$2^0 N_0 \rightarrow 2^1 N_0 \rightarrow 2^2 N_0 \rightarrow 2^3 N_0 \rightarrow 2^n N_0 \quad \text{Equation 1}$$

Where

$N_0$  = Initial number of cells

$n$  = Number of doubling (generations)

The doubling time of bacterial cells measured at their optimal temperature varies widely. Some organisms take slightly longer than others to double their population. In general, the higher the optimal growth temperature, as in the thermophiles the shorter is the doubling time. The number of doubling,  $n$ , at a time interval  $t$ , is determined by the relation  $t/t_d$ . Thus, the number of cells,  $N_t$ , in an exponentially growing culture after incubated for some time,  $t$ , can be estimated as:

$$\begin{aligned}
 N_t &= N_0 \cdot 2^n \\
 &= N_0 \cdot 2^{t/t_d} \\
 N_t/N_0 &= 2^{t/t_d} \\
 \ln\left(N_t/N_0\right) &= (\ln 2) t/t_d
 \end{aligned}
 \tag{Equation 2}$$

During the exponential growth phase, the growth rate of the cells is proportional to the biomass of the cells. Since biomass generally can be measured more accurately than the number of cells, microbial growth equations can be expressed in terms of mass (Richmond, 2008). The decelerating growth phase follows the exponential growth phase. In order to withstand starvation, there are feed – back mechanisms that regulate the enzymes involved in important metabolic steps.

### **2.1.2.3. Stationary and death phase**

There is a phase when the growing culture does not experience a net increase in cell numbers. This is called the stationary phase. During this phase, the growth rate is equal to the death rate. This may occur when nutrients are exhausted, inhibitory end products accumulate, or when physical conditions are changed. The metabolites formed during this phase are of great biotechnological interest. When the micro – organism is unable to reproduce due to the conditions in the medium, the resulting phase is called the death phase. The number of viable cells begins to decline at an exponential rate (Sumbali and Mehrotra, 2009).

### **2.1.3. Nutritional requirements for fermentation**

A culture is any growth or cultivation of micro – organisms. Microbes which are cultivated deliberately in a laboratory are cultivated *in vitro*, that is, in glass such as flasks, tubes, vessels etc. A pure culture is one that contains only a single species of microbe. Culture media are nutrient preparations which serve as food for the microbes. Different micro – organisms require different nutrient materials, thus culture media vary in form and composition, depending on the species being cultivated (Sharma, 2007). Besides a source of energy, organisms require a source of materials for biosynthesis of cellular matter and products in cell operation, maintenance and reproduction. These materials must supply all the elements necessary to accomplish this. The four predominant types of polymeric cell compounds are lipids, polysaccharides, polydeoxyribonucleic acid and polyribonucleic acid (DNA and RNA), and proteins (Kampen, 1996). The nutrients should not only be physically present in the growth media, but should also be capable of entering the cell. Micro – organisms secrete extracellular enzymes to hydrolyse the macromolecules outside the organism, and the resultant lower molecular weight products are then assimilated.

#### **2.1.3.1. Carbon source**

Carbon serves as a major energy source for organisms. Product formation depends on the rate at which the carbon source is metabolised and the main product of fermentation depends on the type of carbon source used. Carbon sources for fermentation may be simple or complex carbohydrates, organic acids, oils, fats and hydrocarbons. The cost of substrate considerably affects the cost of the final product and it is therefore preferable to use industrial by – products and biomass derived carbon sources, as they are more cost effective.

#### **2.1.3.2. Nitrogen source**

Following carbon, nitrogen is the next most plentiful substance used in fermentation media. A few microbes can utilize nitrogen as an energy source but it is required as it is found in the organic compounds of the cell as well as in a reduced form in amino acids. The commonly used nitrogen sources in fermentation media are ammonia, ammonium salts, urea, amino

acids, and proteins. Nitrogen is used for the anabolic synthesis of DNA, RNA, purines and amino acids (Larroche et al.).

#### **2.1.3.3. Minerals**

Minerals supply the necessary elements to cells during their cultivation. Phosphorous occurs principally in the form of sugar – phosphates such as nucleotides. Sulphur is present to the greatest extent in the amino acids cysteine and methionine. Many of the other elements such as potassium, magnesium and sodium are found complexed with enzymes. Other trace elements required may include iron, zinc, manganese, molybdenum, cobalt, copper and calcium. The functions of each vary from serving in co – enzyme functions to reaction catalysis, vitamin synthesis and cell wall transport (Kampen, 1996).

#### **2.1.3.4. Inhibitors, precursors and inducers**

Inhibitors are added to the fermentation medium to effectively increase the yield of the desired product and reduce the yield of the undesirable products. Many a time, precursors are added to alter the structure and hence the permeability of the microbial cell wall for the release of the products. Similarly, with the majority of enzymes produced for industrial interest, the addition of an inducer to the fermentation medium is required. Inducers are often the substrate for the enzymes. Thus, when these are present in the environment, specific enzymes are synthesized (Kale and Bhusari, 2007).

#### **2.1.3.5. Antifoaming agents, buffers, growth factors and chelators**

Antifoaming agents prevent excessive foaming and frothing that occurs due to excessive aeration during the fermentation process. Examples include esters, fatty acids, silicones and propylene glycol. Buffers aid in maintaining pH. These include calcium carbonate and phosphate buffers amongst others. Some micro – organisms cannot synthesize the required cell components themselves and need to be supplemented with thiamine, biotin, and calcium pantothenate. Chelators are the chemicals used to avoid the precipitation of metal ions. Chelators such as EDTA, citric acid and polyphosphates are used in low concentrations (Singh and Kapoor, 2010).



#### **2.1.4. Environmental conditions**

The growth of micro – organisms is impacted by a range of chemical and physiochemical parameters. It is essential to determine these parameters in order to optimize the fermentation process by bringing together the best possible combination of factors in specific measurable units. Of interest are temperature, pH, water activity, oxygen, radiation and pressure.

##### **2.1.4.1. Temperature**

The rate of chemical reactions increases with an increase in temperature (Chang, 2006). However, cellular macromolecules, especially enzymes which are protein in nature are prone to denaturation by heat, thus limiting the reaction temperatures which can be tolerated (Wharton, 2002). Organisms that are able to thrive at relatively high temperatures are known as thermophiles, while those that are capable of growth in very low temperatures are referred to as psychrophiles. They have a minimal temperature at which growth may occur, below which, the lipids in the cell membrane are not sufficiently fluid. Organisms which prefer the less extreme range of temperature, from ten to forty degrees Celsius are referred to as mesophiles (Gerday, 2007). Identifying the organism type in this manner allows for setting of temperature conditions of the fermentation media at an optimal level.

##### **2.1.4.2. pH**

Organisms generally have a narrow range of pH within which they grow the best. For fungi the optimal pH tends to be lower than that for bacteria (Varnam and Evans, 2000). The optimal pH of the growth medium reflects the best compromise with respect to the impact of the surface charge of the cells and the impact it has on behaviours such as flocculation and adhesion. The cells must also be able to maintain a desirable intracellular pH and with this, the charge status of the macromolecules especially the enzymes, and the impact that it has on their ability to perform must be considered (Bamforth, 2008).

#### **2.1.4.3. Water activity**

Most micro – organisms comprise of between seventy to eighty percent water. When the organism is exposed to environments that contain too little water, that is, dehydrating or hypertonic locales, or an excess of water, that is, hypotonic regions, maintaining the appropriate levels is a challenge. The concept of water activity is the quantifiable amount of water available to an organism (Bamforth, 2008). Water activity is defined as the ratio of the vapour pressure of water in the solution to the vapour pressure of pure water (Stokes and Robinson, 1949). Micro – organisms differ greatly in the extent to which they tolerate changes in water activity. Most bacteria will not grow at values below 0.9 while fungi can grow at relatively low moisture levels (Pitt and Hocking, 2009). The reduction of water generally increases growth inhibition. Sporulation seems to occur at a lower water activity than that needed for growth, therefore water activity may also affect the shape of organisms and their mobility (Troller, 1980).

#### **2.1.4.4. Oxygen**

Oxygen requirements differ substantially for microbes. Oxygen is essential for some, while others are able to function in the absence of oxygen. Certain micro – organisms require relatively small proportions of oxygen to perform certain cellular activities while a group of microbes do not use molecular oxygen in their metabolism but are tolerant of it. The last group of microbes is killed by oxygen.

#### **2.1.4.5. Radiation**

Sunlight contains potentially damaging ultraviolet radiation and can also induce the generation of toxic free radicals and reactive oxygen species from compounds known as photo – sensitizers (Wilson, 2009). Ultraviolet light exposure can lead to DNA damage. Gamma rays cause the production of an especially reactive oxygen derived radical, hydroxyl. One of the numerous impacts of the presence of the hydroxyl radical in the bacterial cell is the breakage of DNA. Radiation is a very powerful technique for removing unwanted microbes (Bamforth, 2008).

#### **2.1.4.6. Hydrostatic pressure**

Microbes generally do not encounter forces exceeding atmospheric pressure. Increases in pressure tend to inhibit, if not destroy an organism. Pressure is relevant in fermentation as modern fermenters hold such large volumes that pressure may exceed 1.5 atm in some instances. A study on citric acid production showed a decrease in production above pressures of 1.7 atm (Clark and Lentz, 1961). Although they may not destroy the organisms, an increase in pressure impacts the behaviour of micro – organisms including their tendency to aggregate and certain elements of their metabolism, especially protein synthesis which parallels microbial growth (Pope and Berger, 1973). The latter is due partly to the accumulation of carbon dioxide that occurs when pressure is increased (Bamforth, 2008).

#### **2.1.5. Applications of fermentation**

The products of microbial fermentations can be classified into the following groups (Stanbury et al., 1995):

- Microbial cells or biomass
- Microbial metabolites
- Microbial enzymes
- Recombinant products
- Transformation products

##### **2.1.5.1. Microbial cells**

Microbial cells generally have high protein content. Compared to plants and animals, micro – organisms synthesize proteins more rapidly. They utilize cheap nitrogen and carbon sources to produce biomass which is high in protein content. Micro – organisms themselves are grown as a source of single cell protein (SCP) and are used for human and animal feed as well as in the baking industry. Various bacteria, fungi and algae are potential sources of large amounts of single cell proteins. The algae *Scenedesmus* and *Spirulina* are cultured as food supplements. There are bacteria that utilize methane, given off after sewage treatment, as a carbon source, while another group uses hydrogen and their biomass subsequently used as SCP. Yeast based SCP are very good sources of vitamins. Yeasts such as

*Sacchromyces*, *Candida* and *Torulopsis* are grown on waste material thus removing unwanted material as well as recycling the waste into useful food material. Baker's yeast called *Sacchromyces cerevisiae*, is produced in large quantities for the baking industry. Molasses is used as the growth medium (Litchfield, 1983).

#### **2.1.5.2. Microbial metabolites**

Metabolites are the products of metabolic pathways. The term 'trophophase' describes the log or exponential phase of a culture during which the sole products of metabolism are essential to growth (Demain, 1974). These include products such as amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates etc., or the by – products of energy – yielding metabolism such as ethanol, acetone and butanol (Carrier et al., 2012). The metabolites produced during the trophophase are referred to as primary metabolites (Madigan et al., 1997). The term 'idiophase' describes the phase of the culture during which products other than primary metabolites are produced, having no obvious role in cell metabolism (Williams et al., 1989). These metabolites are referred to as secondary metabolites. Secondary metabolites tend to be synthesized from the intermediates and end – products of primary metabolism. Examples of commercially important metabolites are listed in Table 2-1 below.

**Table 2-1: Commercial metabolites and their significance**

| Metabolite      | Producing micro – organism        | Commercial significance                                    | Reference  |
|-----------------|-----------------------------------|--|--|
| Ethanol         | <i>Sacchromyces cerevisiae</i>    | Active ingredient in alcoholic beverages                   | (Alfenore et al., 2002, Yadav et al., 1996)                  |
| Citric acid     | <i>Aspergillus niger</i>          | Various uses in food industry                              | (Taskin et al., 2012, Kumar et al., 2010)                    |
| Glutamic acid   | <i>Corynebacterium glutamicum</i> | Flavour enhancer   | (Abdenacer et al., 2012, Pasha et al., 2011)                 |
| Lysine          | <i>Corynebacterium glutamicum</i> | Feed additive  | (Brautaset and Ellingsen, 2011, Leuchtenberger et al., 2005) |
| Polysaccharides | <i>Xanthomonas spp</i>            | Applications in food industry and in enhanced oil recovery | (Savvides et al., 2012, Nasr et al., 2007)                   |
| Penicillin      | <i>Penicillium</i>                | Antibiotic   | (Fleming, 1932, Neushul, 1993)                               |
| Cephalosporin   | <i>Acremonium</i>                 | Antibiotic   | (Adinarayana et al., 2003, Araujo et al., 1996)              |
| Streptomycin    | <i>Streptomyces</i>               | Antibiotic   | (Waksman et al., 1946, Peng et al., 2008)                    |
| Pepstatin       | <i>Actinomyces</i>                | Treatment of ulcers  | (Takeuchi, 1973, Ito et al., 1972)                           |
| Cyclosporin A   | <i>Tolypocladium inflatum</i>     | Immunosuppressant  | (Survase et al., 2011, Thali, 1995)                          |
| Gibberellin     | <i>Gibberella fujikuroi</i>       | Plant growth regulator                                     | (Tudzynski, 1999, Hedden and Phillips, 2000)                 |
| Lovastatin      | <i>Pleurotus ostreatus</i>        | Cholesterol synthesis inhibitor                            | (El-Shami and Hamed, 2007, Rai et al., 2005)                 |

### **2.1.5.3. Microbial enzymes**

Microbial enzymes are commercially utilized in the chemical as well as food and beverage industry and have clinical and analytical applications. Most enzymes are synthesized in the logarithmic phase of culture and may be considered as primary metabolites. Although enzymes may be produced from plants and animals, production from microbial sources by fermentation is the most economic and convenient method (Stanbury et al., 1995). Enzymes produced by fermentation are mostly extracellular although there are a few important endocellular enzymes.

Many of the useful microbial enzymes are produced by the micro – organisms only when they grow in a medium containing the enzyme's substrate. To obtain high yield, the inducers are added slowly to the medium by maintaining their low concentration. Another method is to add a derivative form of the inducer. The incubation period as well as temperature for enzyme production varies according to the type of organism. The enzymes are either produced by semi solid culture technique or submerged culture technique.

Enzymes have wide applications in industry. Extremoenzymes are gaining much attention. These are the enzymes produced by prokaryotes that inhabit extreme environments. Since they grow at an exceedingly high temperature, the products including enzymes are heat – stable. As many industrial processes operate at high temperatures, extremoenzymes are gaining attention as biocatalysts for industrial applications (Kale and Bhusari, 2007). Table 2-2 below lists commercial enzymes, their inducers, and their applications.

**Table 2-2: Enzymes, inducers and applications**

| Enzyme            | Culture  | Inducer                | Application   | Reference   |
|-------------------|--|------------------------|---|---|
| Cellulase         | <i>T. viride</i>   | Cellulose<br>Celobiose | Commercial food processing.<br>Textile industry and laundry detergents.<br>Pulp and paper industry for various purposes.  | (Mandels and Weber, 1969, Bhat, 2000, Olempska-Beer et al., 2006, Kang et al., 2004)                              |
| Dextranase        | <i>P. funiculosum</i>  | Dextran<br>Isomaltose  | Sugar industry to break down dextran.   | (Sugiura et al., 1973, Khalikova et al., 2005)  |
| Invertase         | <i>A. Pullulans</i>  | Sucrose                | Confectionary industry  | (Berry and Paterson, 1990, Kumar et al., 2012)  |
| Amylase           | <i>A. niger</i><br><i>A. oryzae</i><br><i>Bacillus. sp</i>                           | Starch                 | Used as a sizing agent in the paper and textile industry.<br>Brewing industry.<br>Sweetener in soft drinks.   | (Ramachandran et al., 2007, Krishna and Chandrasekaran, 1996, Aiyer, 2005)  |
| B – galactosidase | <i>K. fragilis</i><br><i>A.oryzae</i>  | Lactose                | Marker to monitor gene expression in molecular biology  | (Park et al., 1979, Mahoney and Whitaker, 1978, Naylor, 1999)   |
| Proteases         | <i>B. subtilis</i><br><i>B. licheniformis</i><br><i>A. niger</i><br><i>A. oryzae</i> | Protein                | Detergent in laundry industry.<br>Baking industry.<br>Used in textile industry in freeing of silk fibres.<br>Used as a digestive in the food industry to soften meat. | (Kumar and Takagi, 1999, Sandhya et al., 2005, Coral et al., 2003, Cherry and Fidantsef, 2003, Kirk et al., 2002) |
| Pectinases        | <i>Aspergillus sp</i>  | Beet sugar molasses    | Used as a clarifying agent.   | (Nair and Panda, 1997, Pinelo et al., 2010, Patil and Dayanand, 2006)   |

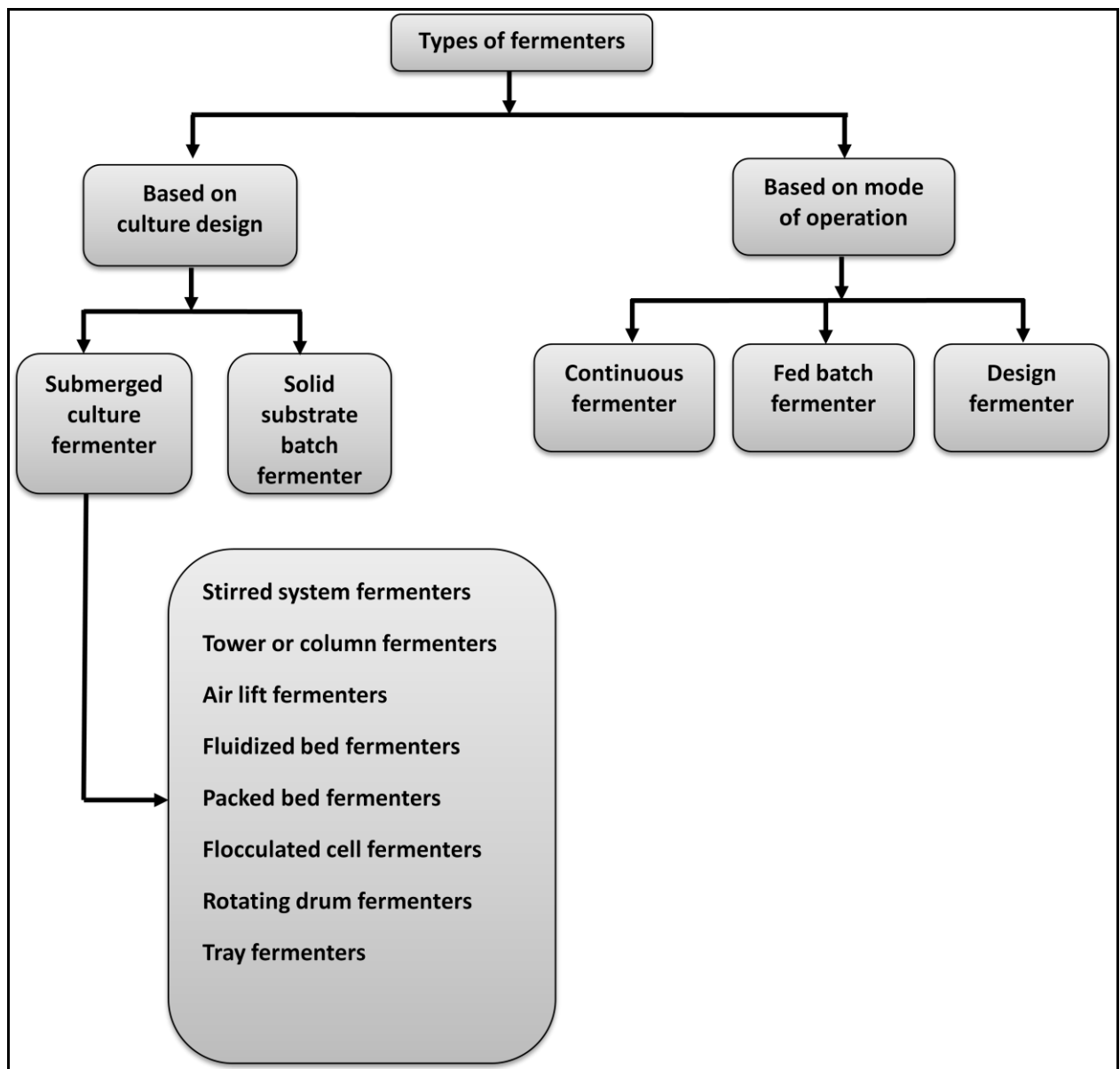
#### **2.1.5.4. Recombinant products and transformation processes**

The terms Recombinant DNA Technology, DNA cloning or gene cloning refer to the use of molecular techniques to select a sequence of DNA from an organism and transfer it into another organism to code for or alter specific traits (Chedrese, 2009). The products produced in the genetically manipulated organisms include insulin (Johnson, 1983, Ladisch and Kohlmann, 1992), interferon (Riesenberg et al., 1990, Babu et al., 2000), human serum albumin (Kobayashi, 2006, Dodsworth et al., 1996), epidermal growth factor (Araki et al., 1989, George-Nascimento et al., 1988), bovine somatostatin (Furu et al., 1999) and bovine chymosin (Cullen et al., 1987, Mohanty et al., 1999). Transformation processes, also termed biotransformations or bioconversions, is the use of microbial cells to catalyse the conversion of a compound into another compound which is similar in structure, but more financially valuable. Micro – organisms can behave as chiral catalysts, with microbial processes being more specific than chemical ones. A broad spectrum of chemical reactions can be catalysed by enzymes including hydrolysis, esterification, isomerization, addition and elimination, alkylation and dealkylation, halogenation and dehalogenation, as well as oxidation and reduction (Adam et al., 1999). Important industrial applications of transformation reactions are the production of certain steroid hormones, the conversion of glucose into mannitol and the saccharification of starch (Stanbury et al., 1995).

#### **2.1.6. Fermenter technology**

A fermenter is a vessel for the growth of micro – organisms, providing the necessary conditions for the maximum production of the desired products, while not permitting contamination. The ideal fermenter should provide the organism with its optimal pH, temperature, oxygen and other environmental conditions. Vessels in which reactions take place are called reactors in the chemical industry. Fermenters are therefore known as bioreactors. Fermenters may be liquid state, also known as submerged or they may be solid state, known as surface fermenters. Submerged fermenters are common in industry as they save space and are more amenable to engineering design and control. The types of fermenters used in industry are grouped by their shape or configuration, where aerated or anaerobic, or whether they are operated in batch or continuous mode, as shown in Figure 2-3 below.





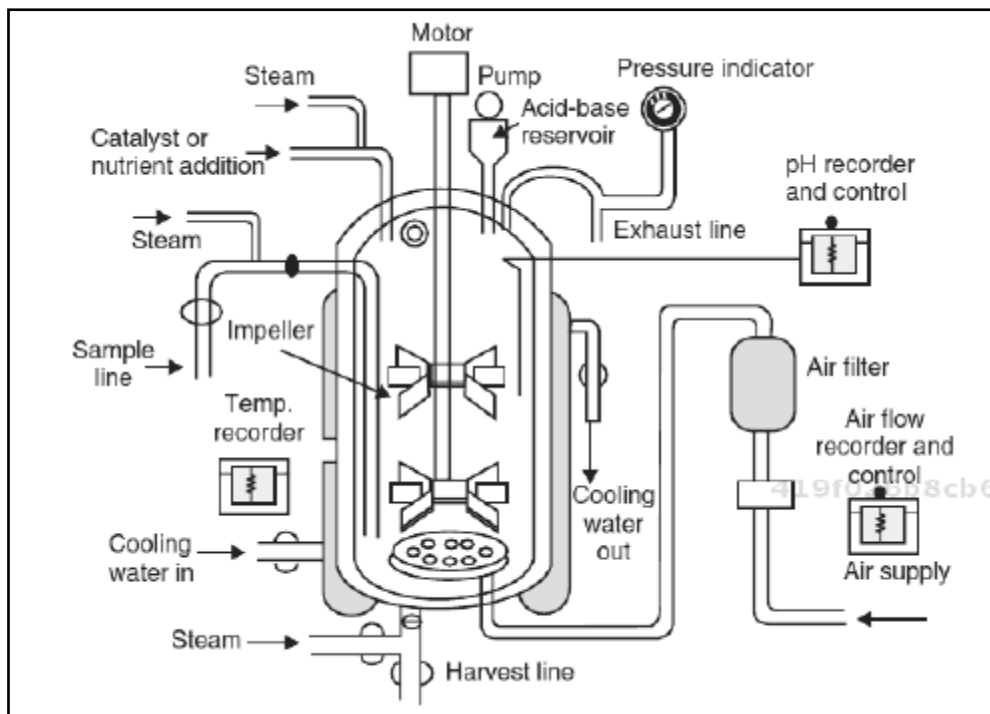
**Figure 2-3: Types of fermenters based on different parameters, adapted from (Kale and Bhusari, 2007)**

#### **2.1.6.1. The aerated stirred tank batch fermenter**

This is the most common type of fermenter vessel. Batch fermenters are used when the process is carried out in a batch basis. The fermenter is filled with the medium and the fermentation is allowed to proceed. At the end of the fermentation, the fermenter is cleaned, refilled, and reinoculated to start a new process. A typical fermenter of this type is an upright closed cylindrical tank, with baffles attached to the side of the wall, a water jacket for cooling or heating, a sparger for aeration, a mechanical agitator, inlets for introducing organisms and nutrients and outlets for sampling and exhaust gases as shown in Figure 2-4 below. It also has control systems for maintaining temperature and pH. It is important that the type of fermentation required be clearly understood before designing a fermenter.

#### **2.1.6.2. Anaerobic fermenters**

These are not commonly used in industry. This type of fermenter is essentially the same as the typical aerobic fermenter described above except that the micro – organisms do not require oxygen. However, differences occur in the construction and operation. These include the absence of air sparging since oxygen is not required, agitation only for even distribution of organisms and nutrients, introduction of hot media at the bottom of the fermenter to prevent absorption of gases, filling the fermenter to avoid air – space, and an initial introduction of nitrogen to remove oxygen. It is important that it is possible for both aerobic and anaerobic fermentations to be carried out in the same vessel as some fermentations require an earlier aerobic stage in which to produce cells in large number and a later anaerobic stage for the production of the desired product (Okafor, 2007).



**Figure 2-4: A typical aerated stirred tank batch fermenter (Reddy et al., 2012)**

### **2.1.6.3. Continuous fermentation**

When nutrients are continuously added and products are continuously removed, this mode of fermentation is called continuous fermentation. In batch fermentations, the products are collected, after which the fermenter is cleaned and charged for another round of fermentation. Continuous fermentation has replaced many industrial batch processes and offers many advantages. These include:

- i. Elimination of dead time used for emptying, cleaning, sterilizing, cooling and recharging in batch fermentations, thereby allowing more intensive use of the equipment and greater return on initial capital.
- ii. Minimizing labour requirements as the unproductive areas of batch fermentation are no longer required.
- iii. Ease of automation, thereby eliminating human error and ensuring uniformity of product.

A disadvantage is that the process may be easily contaminated.

#### **2.1.6.3.1. Classification of continuous fermenters**

- a) Single – state continuous fermentations: The entire operation is carried out in one vessel. Nutrient addition is simultaneous with broth removal.
- b) Multiple – stage continuous fermentation: There are numerous fermentation tanks with medium entering the first and broth entering the second and so on. The first tank is generally used for the growth phase and the tanks following, for production.
- c) Recycled – single or multiple – stage continuous fermentation: The micro – organisms are removed from the outflow by centrifugation and the supernatant returned to the system. This is useful when the nutrients are difficult to degrade or not easily miscible with water.
- d) Semi – continuous fermentations: Nutrient addition and outflow withdrawal are carried out intermittently instead of continuously. A volume of the fermentation broth is removed when the fermentation is nearing completion. Fresh medium of equal volume is then introduced. This method has a lower productivity than continuous fermentation and is termed cyclic semi – continuous fermentation. Cell reuse is another type of semi – continuous fermentation in which the cells are centrifuged and returned to the system.

#### **2.1.6.4. Fed – batch cultivation**

This is a modification of batch fermentations. Nutrient is added intermittently to a batch culture. This method is used to achieve higher yields and more efficient media utilization. Modifications to this method include the continuous addition of one or more media components and the withdrawal of a volume of the broth followed by immediate dilution with fresh medium.

#### **2.1.6.5. Other submerged culture fermenters**

Stirred tank fermenters as mentioned above are the most widely used in industry. In these types of fermenters, mechanical stirrers are used to mix the reactant mixture. Other types of fermenters use other means of agitation (Kale and Bhusari, 2007).

- Tower or column fermenters are tall vessels which use air alone to mix the reactant mixture. The air is introduced from the bottom. This type of fermenter is often used

in the production of citric acid, alcohol, SCP and vinegar since it is able to retain high biomass.

- Air lift fermenters contain a draft tube at the centre of the vessel. The draft tube is a hollow perforated tube that improves circulation and oxygen transfer. The air is introduced from the bottom of the fermenter that lifts the draft tube.
- In the fluidized bed fermenters, microbial cells are immobilized on small particles, these particles move along with the fluid and as a result, enabling a high rate of oxygen and nutrient transfer to the cells.
- In flocculated bed reactors the cells are immobilized on larger particles and thus do not move with the liquid. Flocculated bed reactors are used in sewage treatment.
- In the rotating drum fermenter, the rotating drums contain an inlet and outlet for circulation of humidified air. It also contains baffles to mix the reaction mixture.
- Tray fermenters consist of chambers containing deep stacked trays. The humidified air is forced into the chamber.

#### **2.1.6.6. Construction and design of fermenters**

##### **2.1.6.6.1. Materials of construction**

The materials of construction must be such that they will not adversely affect, nor be affected by the desired microbial activity, either by interaction with the fermentation medium or by harbouring unwanted organisms. They must be resistant to corrosion by the nutrient medium and products and to the effects of sterilization temperatures. The actual construction of the equipment from suitable materials such as stainless steel or pyrex glass, must also take account of these factors and of the stresses imposed by pressurization and the weight of the vessel contents (Blakebrough, 1973).

#### **2.1.6.6.2. Agitation and aeration**

Aerobic organisms require oxygen for growth as mentioned previously. The supply of oxygen to fermentations of this nature is critical. Efficient aeration depends on bringing the liquid into intimate contact with the largest obtainable surface of air. Oxygen is more efficacious than air but is too expensive for most industrial purposes. In order to bring the liquid into contact with air, sterilized air is sparged in order to disperse the air stream into fine bubbles. Concurrently, the liquid is mechanically agitated. This causes a further division of the air bubbles into finer bubbles and mixes them efficiently with all parts of the liquid (Chain et al., 1952). It is necessary to agitate the medium where filamentous fungi and actinomyces are grown or when the media is viscous. This is achieved with the aid of impellers. Agitators help to distribute the incoming air as fine bubbles, mix organisms uniformly, create turbulence and ensure uniform temperature. Baffles help eliminate the formation of a vortex and interfere with the upward throw of liquid against the side of the fermenter (Okafor, 2007).

#### **2.1.6.6.3. Temperature control**

The growth temperature of micro – organisms differs widely. Optimum fermentation temperature may be below or above ambient temperature. Due to metabolic activities of the micro – organism during fermentation, heat is generated. This leads to a rise in temperature in the fermenter. To maintain optimum temperature in the fermenter cold water may be sparged on the fermenter, circulated through the jacketed walls of a fermenter or through coils arranged along the inside walls of the fermenter (Reddy et al., 2012).

#### **2.1.6.6.4. Foam production and control.**

Foam is an agglomeration of gas bubbles that are separated from each other by a thin liquid layer. In fermentations, the aeration and agitation of the medium generate foam. Foam formation in submerged fermentation adversely affects productivity and downstream processing (Ghildyal et al., 1988). Foam generally is undesired because it has many

microbiological, economic and chemical engineering consequences. A few of these undesirable consequences are as follows:

- A substantial head space is wasted due to the need to accommodate foam.
- Excessive foaming inhibits maximum aeration and agitation and thus reduces the oxygen transfer rate.
- Contamination may be introduced when foam escapes and coalesces, falling back into the medium after wetting the filters and non – sterile portions of the fermenter.
- A loss of nutrient may occur if the organic nutrients or inorganic ions with complex organic compounds are removed from the medium by foam flotation. This will lead to reduced yields.
- The fermentation products itself may also be removed by foam flotation.
- Flotation of the micro – organism could also occur resulting in poor yield.

The use of antifoam is the most common method of preventing foam formation. Examples of antifoams are polypropylene glycol, silicones and vegetable oils. Antifoams used in industrial fermentation should be non – toxic, autoclavable, cheap, active in small concentrations, have no effect on taste and odour and should not be metabolized by the micro – organisms or impair oxygen transfer (Okafor, 2007).

#### **2.1.6.6.5. pH measurement and control**

The nature of the activities of micro – organisms is such that the pH of the environment of a metabolizing culture will not remain constant for long. These changes of pH are associated with the uptake of certain anions and cations, the degradation of proteins and other nitrogenous compounds yielding ammonia or other alkaline products and the metabolism of carbohydrates and hydrocarbons yielding organic acids (Munro, 1970). Control of pH is sometimes achieved by natural buffers present in the medium as well as phosphates and calcium carbonate. This buffering effect is temporary however, and the pH must be adjusted as desired by addition of an acid or base after sampling (Reddy et al., 2012). This is laborious and sterilized pH probes are now available which can be inserted in the fermenter. With these electrodes it is possible to monitor the pH and automatically induce either an acid or

alkali into the medium. Usually acidity is a problem and anhydrous ammonia is introduced into the fermenter (Okafor, 2007).

## **2.2. Membrane Technology**

### **2.2.1. Downstream biotechnological processing**

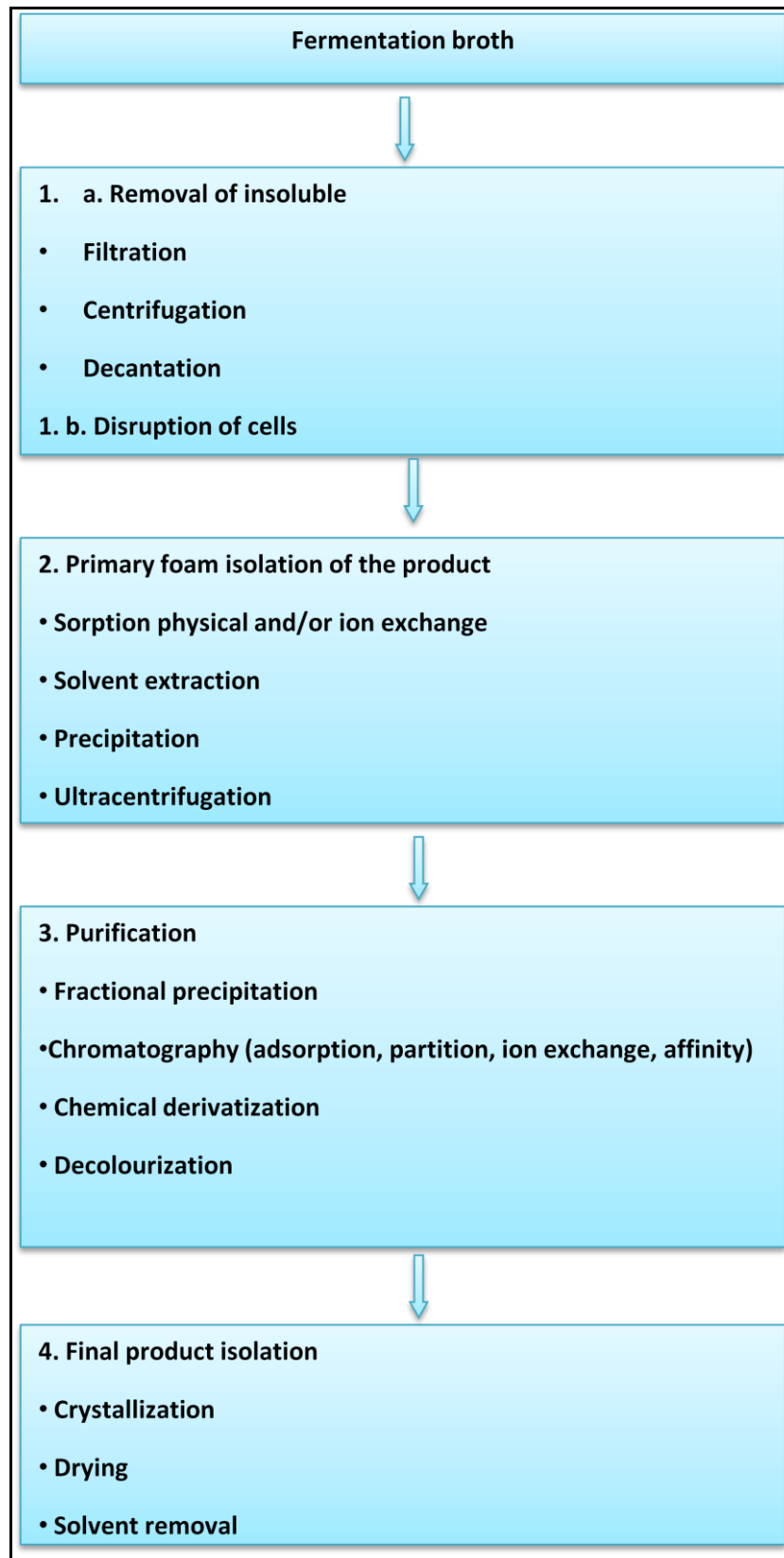
Downstream processing refers to the various separation and purification steps involved in achieving a final product. In biological processes, this is often the most expensive step, especially when the regulatory requirements are stringent (Tripathi, 2006). Bioproducts are often produced as aqueous solutions which are dilute and therefore must be concentrated. Improved downstream processing provides the potential for saving. The separation and purification of materials produced in a bioreactor is a critical part of the operation. High value bioproducts are usually fragile molecules such as proteins or peptides that require highly specialized and mild processing conditions and may need to be separated from a complex mixture of molecules including cell debris. These factors make separation difficult. At present most industrial separations are scaled up laboratory procedures and research is needed to improve their performance. The separation of biomolecules is largely focused on with respect to bio – specific interactions and properties such as molecular weight, charge distribution, hydrophobicity, immunogenic structure and structure (Jenkins, 1992). In many cases a large sequence of classical unit operations of low resolution is necessary to achieve the required purity. Numerous processing steps lead to a decrease in overall yield and this is a major problem in downstream processing.

The factors to consider when deciding on the method of extraction of the product include:

- The value of the final product
- The degree of purity necessary to make the final product acceptable
- The chemical and physical properties of the product
- The location of the product in the mixture, that is, whether it is cell – bound or free within the broth
- The location and properties of the impurities
- The cost – effectiveness of available procedures



The steps involved in the purification of fermentation follow the sequence represented by Figure 2-5 below. Of consequence to this study is the first stage of separation which is the removal of insoluble material.



**Figure 2-5: Sequence of purification steps following fermentation, adapted from (Okafor, 2007)**

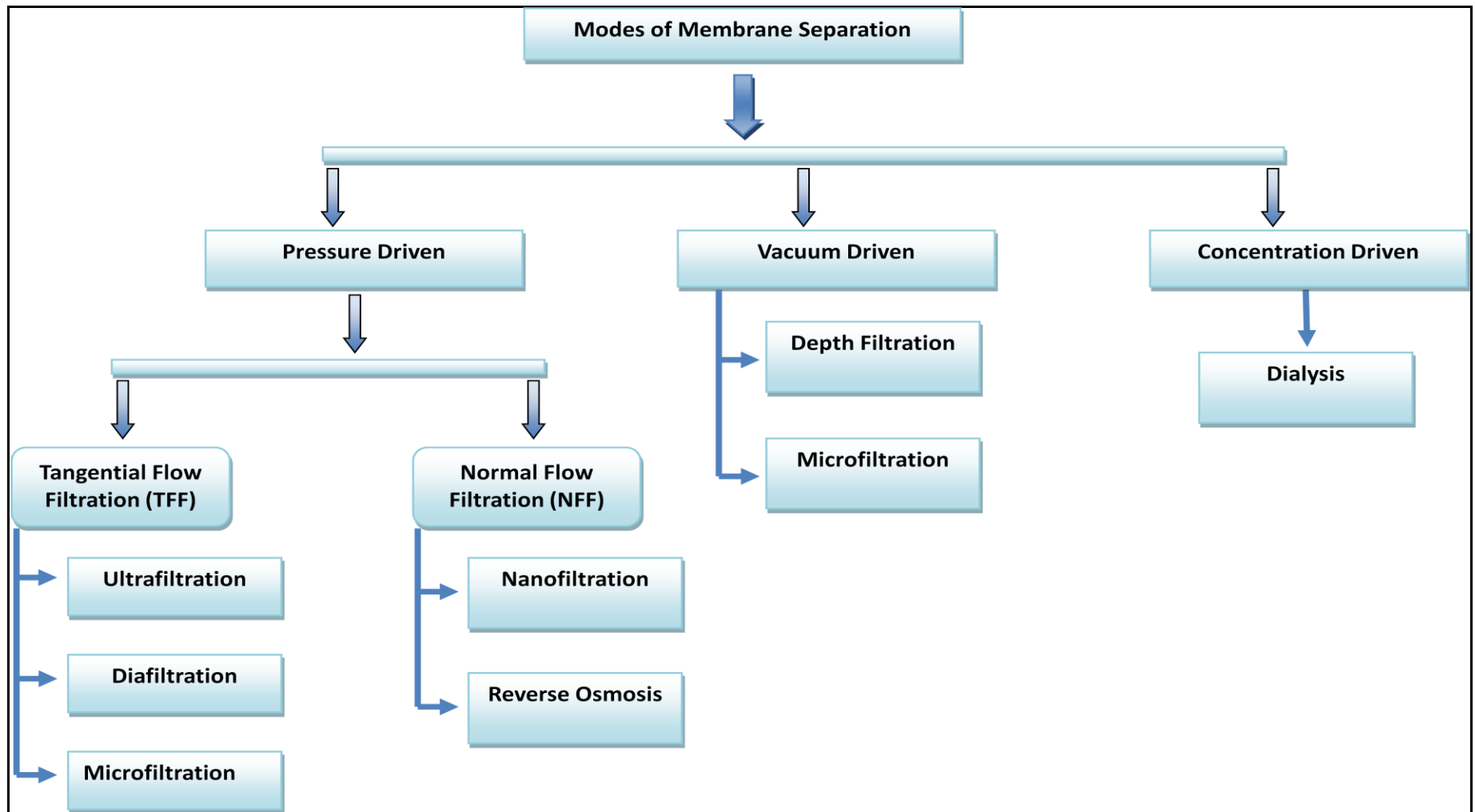
### **2.2.2. Membrane filtration as a separation technique**

A membrane is a barrier that separates two phases and restricts the transport of various chemical species in a specific manner. A membrane can be homogeneous or heterogeneous, symmetric or asymmetric in structure, solid or liquid, neutral or carry a positive or negative charge, or may be bipolar. The electrical resistance may vary from several megaohms to a fraction of an ohm. Mass transfer through a membrane may be caused by convection or by diffusion of individual molecules, induced by an electric field, or a concentration, pressure, or temperature gradient (Goldberg, 1997). Membrane processes provide a means of separation and concentration at the molecular and fine particle level. The family of liquid – phase pressure and concentration driven processes can be used to separate the range of species found in fermentation broths including microbial cells, viruses, colloids, proteins, polysaccharides, enzymes, antibiotics, simple sugars, organic acids and inorganic ions. Membrane processes offer the following advantages for the recovery of bioproducts:

- Processing can be at modest, even low, temperatures.
- Chemical and mechanical stresses can be minimized.
- No phase change is involved, and therefore the energy demand is modest.
- In many cases selectivity is good.
- Concentration and purification may be achieved in one step.
- Equipment is easily scaled up, is flexible in that it can be batch – driven or continuous, and provides a closed system for effective containment (Asenjo, 1990)

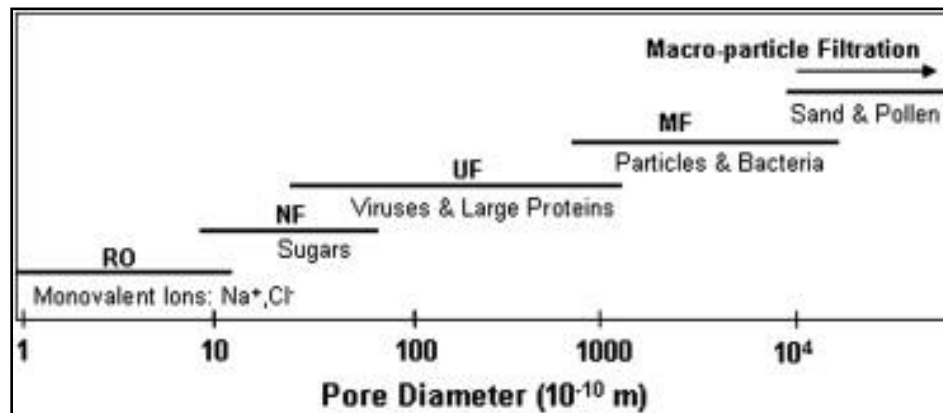
### **2.2.3. Membrane processes**

Membrane separations can be classified on the basis of the driving force and the flow direction with respect to the membrane surface. A typical membrane process step involves the passage of feed through the membrane such that the molecules are separated in the retentate and permeate on the basis of their size, charge, or other differential interaction. Selection of a membrane with appropriate characteristics example, pore size, molecular weight cut – off and surface chemistry, is one of the most important part of optimization of a membrane process step (Sirkar, 1997). The different kinds of membrane separations that are used in bio – processing are illustrated in Figure 2-6 below.



**Figure 2-6: Modes of membrane separation (Rathore and Shirke, 2011)**

Figure 2-7 below illustrates the range of nominal pore diameters for commercially available membranes.

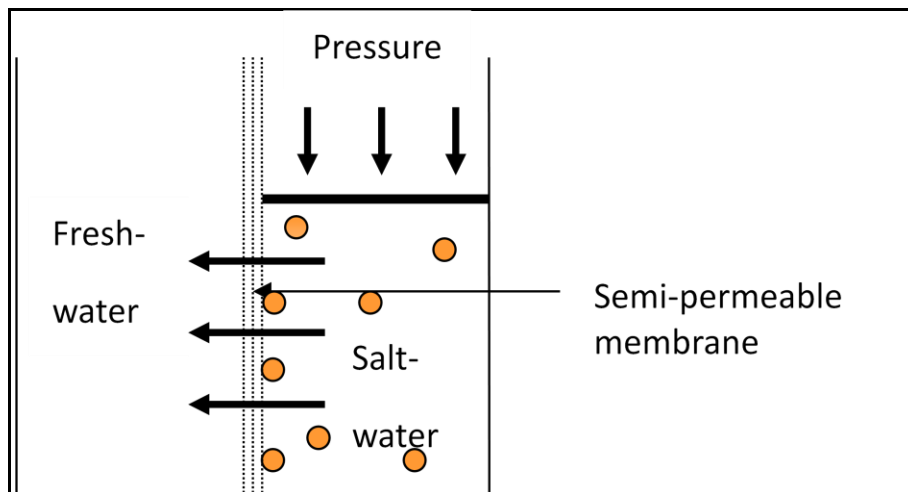


**Figure 2-7: Range of nominal pore diameters for commercially available membranes (Perry et al., 1997)**

### 2.2.3.1. Reverse osmosis

A reverse osmosis membrane acts as a physical barrier for hydraulic flow, allowing selective permeation of the solvent, mostly water and partial or total retention of the other dissolved substances, mostly salt. Osmosis is the natural phenomenon whereby water passes through a semi – permeable membrane from a solution of low salt concentration to a solution of higher salt concentration until equilibrium in the chemical potential of the water is established. In the state of equilibrium, the differential pressure over both sides of the membrane is identical to the osmotic differential pressure. In order to reverse the osmotic flow of water, external pressure, greater than the osmotic differential pressure of both solutions, is applied (Greenlee et al., 2009). This is illustrated in Figure 2-8 below. This additional pressure enables the dissolved salt to be separated from the water. The three categories of reverse osmosis are high – pressure reverse osmosis which is used in seawater desalination, low – pressure reverse osmosis which is used in brackish water desalination (Fritzmann et al., 2007) and nanofiltration which is used in softening of drinking water. Typical salt retention values for reverse osmosis

are 98% and 99.9% (Bartels et al., 2005). A reverse osmosis membrane will completely retain all molecules which have a molecular weight of more than 150 Dalton.



**Figure 2-8: Reverse osmosis schematic**

### **2.2.3.2. Microfiltration**

Microfiltration is defined as the filtration of a suspension with colloidal or other fine particles having a linear dimension of roughly 0.1  $\mu\text{m}$  to 10  $\mu\text{m}$  (Baker, 2012). Operating pressures for microfiltration typically lie between 0.02 MPa to 0.5 MPa. Typical applications of microfiltration include the separation of bacteria, yeasts, emulsified oils and fats as well as the separation of particles and fine dust from production processes (Ramakrishna et al., 2011). Microfiltration can be operated in the cross – flow as well as dead – end mode. The most important use of microfiltration is the filtration of aqueous solutions, namely in the treatment of drinking and beverage water. In beverage production, industrial applications include the filtration of beer and wine as well as in the processing of milk and whey. In biotechnology, microfiltration is used for the retention of biomass from fermentation broths. The most important microfiltration in the metal – working industry is oil – water separation.

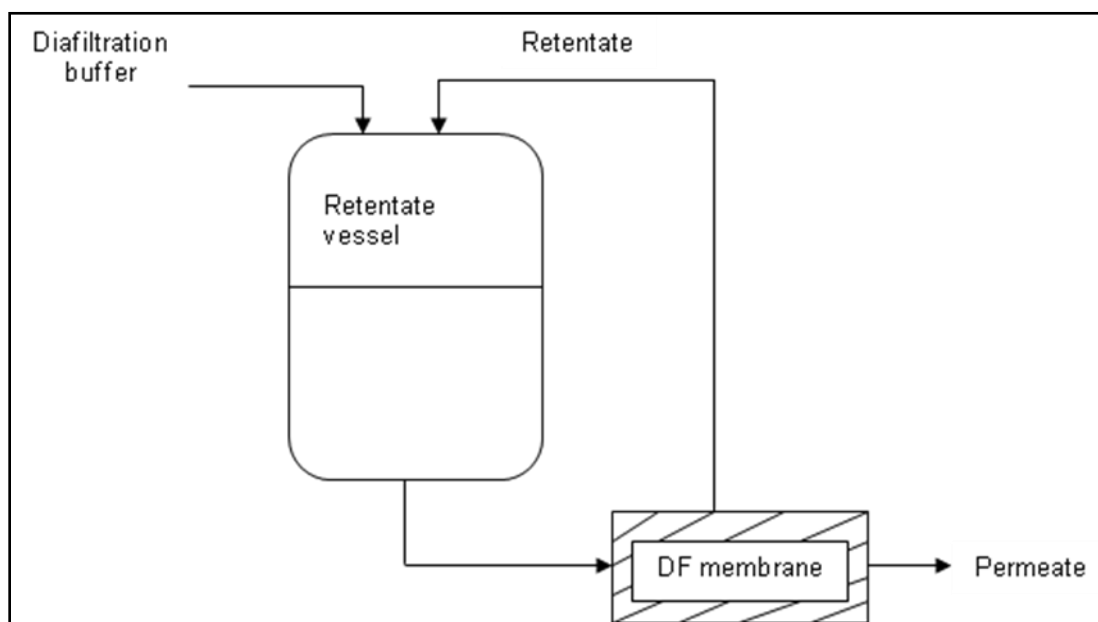
### **2.2.3.3. Ultrafiltration**

Ultrafiltration is another pressure – driven separation process, governed by a screening principle and dependant on particle size. Ultrafiltration membranes have a pore size between 1  $\mu\text{m}$  and 100  $\mu\text{m}$ , thus allowing retention of compounds with a molecular weight of 300 to 500000 Dalton. Typically, the process is suitable for retaining biomolecules, bacteria, viruses, polymers, colloidal particles and sugar molecules. Ultrafiltration membranes are defined by their nominal molecular weight cut – off (MWCO). The MWCO generally represents the smallest molecular weight for which the membrane has a retention value of more than 95% (Baker, 2012). Separation efficiency is also influenced by the interaction between the membrane and the raw solution. The operating pressure for ultrafiltration is usually between 0.1 and 1 MPa. Ultrafiltration applications include the filtration of raw solutions, concentration of substances and fractionating of substances. Applications of ultrafiltration can be found in nearly all industrial sectors, examples of which include the sterile filtration of drinking and beverage water (Sale et al., 1988), treatment of surface water (Li and Chu, 2003), separation of oil/water emulsions (Chakrabarty et al., 2008) and removal of heavy metals in wastewater treatment (Barakat and Schmidt, 2010).

### **2.2.3.4. Diafiltration**

Diafiltration is used to change the chemical properties of the retained solution under constant volume. Unwanted particles pass through a membrane while the make – up of the feed stream is changed to a more desirable state through the addition of a replacement solution. In biotechnology, diafiltration is commonly used to prepare the product stream for a chromatography step, to exchange drug product into a final formulation buffer, or other situations where higher or lower conductivity or pH levels are required. The diafiltration buffer is fed to the retentate vessel at the same rate that permeate leaves the system, thus the retentate volume remains relatively constant. Diafiltration continues until the desired conditions in the retentate vessel are achieved (Houp, 2009). Conventional techniques used for salt removal or buffer exchange such as membrane dialysis and column – based gel filtration

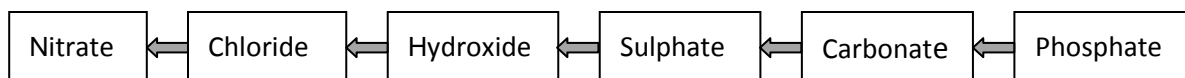
can be effective but have limitations. With diafiltration, salt or solvent removal as well as buffer exchange can be performed quickly and conveniently. Another significant advantage of diafiltration is that the sample is concentrated on the same system, minimizing the risk of sample loss or contamination (Schwartz, 2003).



**Figure 2-9: Basic diafiltration configuration**

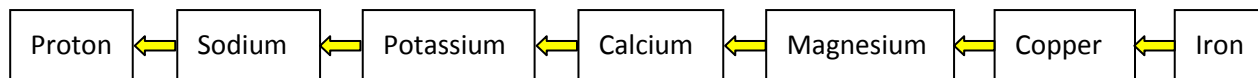
#### 2.2.3.5. Nanofiltration

Nanofiltration is a membrane separation process which has a cut – off between that of reverse osmosis and ultrafiltration, being specified as between 180 and 2000 Dalton. Operating pressures for nanofiltration are in the range of 0.3 to 4 MPa. The selectivity of a nanofiltration membrane is determined by two different parameters. Firstly retention depends on the size or molecular weight. Retention and permeability are also a function of electric charge and the valence of the salts and compounds in the solution. Diluted solutions of mono – valent ions can pass through a nanofiltration membrane unhindered whilst most of the multi – valent ions such as sulphates and carbonates are retained (Yacubowicz and Yacubowicz, 2005). In the case of anions, retention by a nanofiltration membrane increases in the order below:

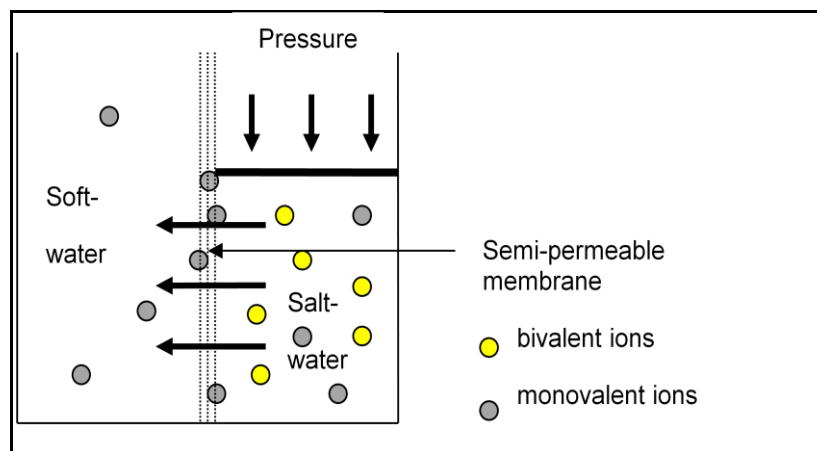




Cation retention by a nanofiltration membrane increases in the order below:



Membranes with good chemical resistance are now available for the treatment of acids, bases and oxidative media, making the process suitable for a number of separation operations including the removal of fluorides, heavy metals, pesticides and organic impurities (Goncharuk et al., 2011).



**Figure 2-10: Nanofiltration schematic**

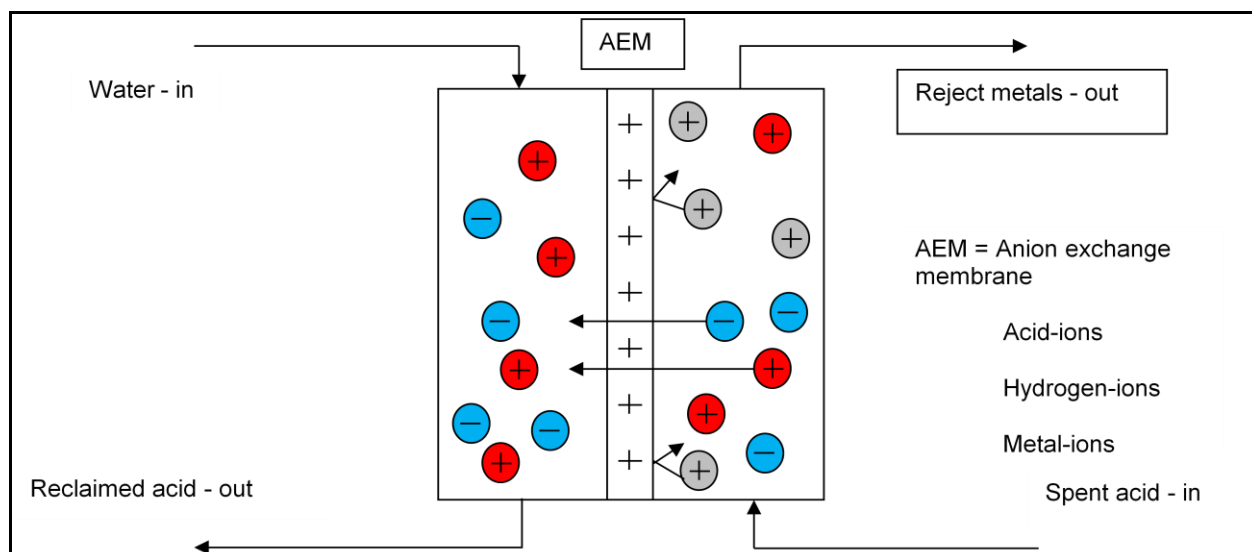
#### **2.2.3.6. Depth filtration**

Depth filtration involves the separation of a suspended particle or liquid droplet from its carrying fluid within the depth or thickness of the filter medium (Sutherland, 2008). Depth filtration has been successfully employed in biotechnology for the clarification of monoclonal antibody solutions, removal of particulates and contaminants from acidified protein solutions prior to chromatographic processing as well as the removal of DNA from mammalian cell cultures (Gottschalk, 2009). The filter material is constructed into matrices by random deposition of individual or continuous fibres. Pores are formed from the interstices among the fibres thus causing a wide band of particle retention. Varying the different filter media delivers a wide variety of properties for numerous applications. Polymeric properties can be chosen to ensure specific chemical, thermal, and mechanical stability or to introduce adsorptive

properties (Levy and Jornitz, 2006). Charged matrices remove colloidal or other oppositely charged particles for example positively charged depth filters can provide very high removal of negatively charged DNA, viruses and endotoxin. Depth filters are able to handle large process volumes, high cell densities, give fast and immediate separation of cell mass, are easy to use as disposable and scalable technology and have a low up – front capital investment (Prashad and Tarrach, 2006).

#### **2.2.3.7. Dialysis**

The term dialysis is more commonly associated to the most important medical use of membranes for the treatment of renal failure. This is called kidney dialysis or haemodialysis. Dialysis involves the movement of solutes and water across semi – permeable porous membranes by diffusion and convection. Diffusive clearance of a solute depends on its molecular weight, electrical charge, concentration gradient, flow rates and membrane characteristics. Smaller molecules such as urea are cleared well, whilst larger molecules such as albumin cannot pass through the membrane. Middle molecules such as  $\beta_2$  – microglobulin are removed across the membrane due to a hydrostatic pressure gradient (Sivalingam and Farrington, 2007). During diffusion dialysis mass transfer is driven by the potential of a concentration difference across pore – free ion exchange membranes. Anion exchange membranes can be used to recover strong acids from its metal salts while cation exchange membranes are used for strong bases. In the acid recovery system, negative ions such as  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , etc are permitted passage due to the anion exchange membrane while the metals are less likely to pass. The  $\text{H}^+$  ions, though positively charged, have a higher competition in diffusion than metal ions because of their smaller size, lower valence state and higher mobility (Luo et al., 2011). The dialysis process is applied for the recovery of free acids or bases from spent treatment bath solutions in surface finishing (Lin and Lo, 1998). Diffusion dialysis is becoming more prospective and popular because of its economical and clean nature, low installation and operating cost, operational simplicity and compatibility (Jung Oh et al., 2000).



**Figure 2-11: Acid recovery by diffusion – dialysis**

#### 2.2.4. Membrane structures and materials

The key components of a membrane system are the membrane, which provides the separation barrier, and the container or module, which governs the fluid mechanical environment adjacent to the membrane. Asymmetric membranes have a thin skin (0.1 to 1  $\mu\text{m}$ ) that provides separation with minimum hydraulic resistance. Reverse osmosis (RO), ultrafiltration (UF) and some cross flow filtration (CFF) membranes are asymmetric. An increasingly popular asymmetric membrane is the thin – film composite (TFC), which comprises a very thin surface film supported on a micro – porous sub – layer. CFF and dialysis membranes are likely to be symmetric, that is, they have no surface skin and little gradient in porosity. RO and some dialysis membranes have no ‘detectable’ pores, UF and dialysis membranes often have surprisingly low surface porosity and CFF membranes usually have high surface porosity. For the processing of fermentation broths and protein solutions, the fluxes of UF and CFF are more likely to be at the low end of known ranges. This is because the retaining species usually provide a significant resistance and cause fouling of the membranes. Fouling is a difficult problem which can be partially alleviated by the use of the more hydrophilic membranes. The criteria for selecting membrane materials include pH and temperature stability, as well as hydrophilic/hydrophobic character (Li et al., 2011).

Synthetic membranes are fabricated from a variety of materials, including both organic and inorganic materials such as metals, polymers and ceramics. Ceramics and metal membranes can be employed in separations where aggressive media such as acids and strong solvents are present. They are also suitable for high – temperature operations as they have excellent thermal stability. Polymeric membranes are less expensive and more versatile than inorganic membranes. They are typically formed by coating a thin polymer layer on a porous backing or support to create a combination that provides high permeability, selectivity, mechanical strength and chemical stability. Table 2-3 below lists commonly used polymers for commercial membranes.

**Table 2-3: Commonly used polymers for membrane separation processes (Wang and Zhou, 2013)**

| <b>Polymer</b>                   | <b>Membrane type</b> |
|----------------------------------|----------------------|
| Polyamide                        | RO, NF, UF, MF       |
| Cellulose acetate (CA)           | RO, UF, MF           |
| Polysulfone (PS)                 | UF, MF               |
| Polyether sulfone (PES)          | NF, UF, MF           |
| Polyvinylidene fluoride (PVDF)   | UF, MF               |
| Polyimide (PI)                   | NF                   |
| Polyetherimide (PEI)             | UF, MF, GS           |
| Polyethylene (PE)                | UF, MF               |
| Polypropylene (PP)               | UF, MF               |
| Polyacrylonitrile (PAN)          | UF, MF, PV           |
| Polyethylene terephthalate (PET) | MF                   |
| Polydimethylsiloxane (PDMS)      | NF, PV, GS           |

## 2.2.5. Membrane properties

### 2.2.5.1. Molecular weight cut – off or pore size

Pore size is determined by bubble point analysis, porosimetry, or microscopic analysis for pore size and pore size distribution. The pore size measured is not accurate however, as the membrane pores are interconnected or networked and are not cylindrical capillaries. Some membrane manufacturers frequently characterize their membranes using the 'cut – off' concept rather than pore size. The nominal molecular weight cut – off (MWCO) is a performance related parameter, defined as the lower limit of a solute molecular weight for which the rejection is 95 – 98 %. As the MWCO decreases, the mean pore diameter for most ultrafiltration membranes has been found to decrease. The MWCO may be sharp or diffuse, that is, there is a range of MWCO and in reality it is only a rough indication of the membrane's ability to remove a given compound as molecular shape, polarity, and interaction with the membrane affect rejection. Membrane surface characteristics may also influence the apparent size of particles retained.

### 2.2.5.2. Pure water permeability (PWP)

The rate of flow of water through a bed of thickness  $\Delta x$  is related to the driving pressure by the simple expression:

$$\frac{dV}{A.dt} = J = \frac{\Delta P}{\eta R_m} \quad \text{Equation 3}$$

Where  $J$  = linear fluid velocity (  $L / m^2 .h$  )

$V$  = volume (  $L$  )

$A$  = cross sectional area (  $m^2$  )

$t$  = time (  $h$  )

$\Delta P$  = pressure gradient (  $N / m^2$  )

$\eta$  = viscosity of the fluid (  $Ns / m^2$  )

$R_m$  = permeability of clean filter media

Water flux through a membrane is proportional to the pressure gradient across the medium and the permeability of the medium. In the situation of no fouling (clean membrane) and feed water completely free of any solutes, and assuming laminar flow through capillary tubes of radius  $r$ , the Hagen – Poiseuille law was obtained independently by Gotthilf Heinrich Ludwig Hagen and Jean Léonard Marie Poiseuille:

$$J = \frac{\varepsilon r^2}{8\eta\tau} \frac{\Delta P}{\Delta x} \quad \text{Equation 4}$$

Where  $\varepsilon$  = porosity (  $n\pi r^2 / \text{surface\_area}$  )

$n$  = number of pores

$r$  = pore radius (m)

$\eta$  = viscosity (Pa.s)

$\tau$  = tortuosity factor

$\Delta P$  = transmembrane pressure (TMP,  $N / m^2$ )

$\Delta x$  = membrane thickness (m)

Flux is therefore proportional to porosity, pore size and TMP.

### 2.2.5.3. Surface/pore charge (Zeta potential or isoelectric point)

Zeta potential ( $\zeta$ ) indicates the surface charge of a membrane and can be observed by measuring the streaming potential across a fluid shear plane at the surface. The isoelectric point is the pH at which the surface charge is zero. A streaming potential is generated when an ionic solution is forced to flow between two parallel membranes, and the electrodes detect the difference in streaming potential. Zeta potential can be derived by the Helmholtz – Smoluchowski equation:

$$\frac{\Delta\phi}{\Delta P} = \frac{\varepsilon\zeta}{\mu k} \quad \text{Equation 5}$$

Where  $\Delta\phi$  = streamlining potential (mV)

$\Delta P$  = forced pressure (Pa)

$\varepsilon$  = permittivity of the solution (s/m)

$\mu$  = viscosity (Pa.s)

$k$  = electrical conductivity of the solution (mS/m)

The surface charge implies different fouling tendencies. Generally, membrane materials carry a negative charge or are modified to have a negative charge because natural organic matter (NOM) in water is negatively charged at neutral pH, due to phenolic and carboxylic functional groups. A negatively charged membrane, therefore, prevents rapid deposition of NOM foulants on the membrane surface by charge repulsion.

#### 2.2.5.4. Roughness

With AFM, the three – dimensional structure of a membrane surface can be observed directly. The image is generated by probing or tapping on the membrane surface with a very fine tip. Roughness is calculated by the equation below:

$$R_a = \frac{\sum_{i=1}^N |Z_i| - Z_{cp}}{N} \quad \text{Equation 6}$$

Where  $R_a$  = arithmetic average of the deviations from the centre plane (nm)

$Z_i$  = current Z value (nm)

$Z_{cp}$  = Z value of the centre plane (nm)

$N$  = number of points within a give area

A change in roughness is associated with fouling. Generally, microfiltration membranes have higher roughness than ultrafiltration membranes. Roughness is reduced when foulants block pores and increases when foulants are deposited sporadically on a smooth membrane surface (Li et al., 2011).

## **2.2.6. Membrane flow geometry**

### **2.2.6.1. Dead – end filtration**

The conventional manner in which to operate a filter is called dead – end filtration. When the filter medium is a membrane, it usually has the appearance of a piece of paper with a shiny coating on one side. In dead – end membrane filtration, the fluid passes normal to and through the face of the membrane, and the particles or molecules retained by the membrane are held on its surface. Dead – end filtration requires only the energy necessary to force the fluid through the filter. In the simplest applications, gravity, a laboratory vacuum or a simple pump provide enough motive force to drive the application at an acceptable rate. The ideal energy requirement, if rate is not critical, is negligibly low. In dead – end filtration, essentially all of the fluid entering the filter, emerges as permeate, so the conversion is roughly 100% less the fluid entrained in the retained solids. By far the largest use of dead – end membrane filters is in the quantitative removal of particulates in the size range 0.1 – 10  $\mu\text{m}$  from aqueous liquids.

### **2.2.6.2. Cross – flow filtration**

In this type of operation, the fluid to be filtered is pumped across the membrane parallel to its surface. Only the small fraction of fluid, actually passing through the membrane, flows normal to the membrane. By maintaining a velocity across the membrane, the retained material is swept off the membrane surface. Cross – flow filtration is preferred when significant quantities of material will be retained by the membrane or when the solid retentate is compressible. In a cross – flow filter, far more of the feed passes by the membrane than passes through it, and conversion per pass is low. Recycling permits the ultimate conversion to be much higher.

## **2.2.7. Membrane configuration and modules**

Membrane configuration refers to the shape of the membrane. Membranes are usually supplied in three different configurations. These are flat sheets, hollow fibres or tubular membranes. Flat sheet membranes look like filter paper. They are available as filter discs or



rectangular sheets. Tubular membranes consist of a hollow tube of circular or other cross section, the wall of the tube functioning as the membrane. Hollow fibres are tube – like in appearance but have smaller diameters. The choice of membrane configuration is constrained by a number of factors. The ideal membrane module should have the following attributes:

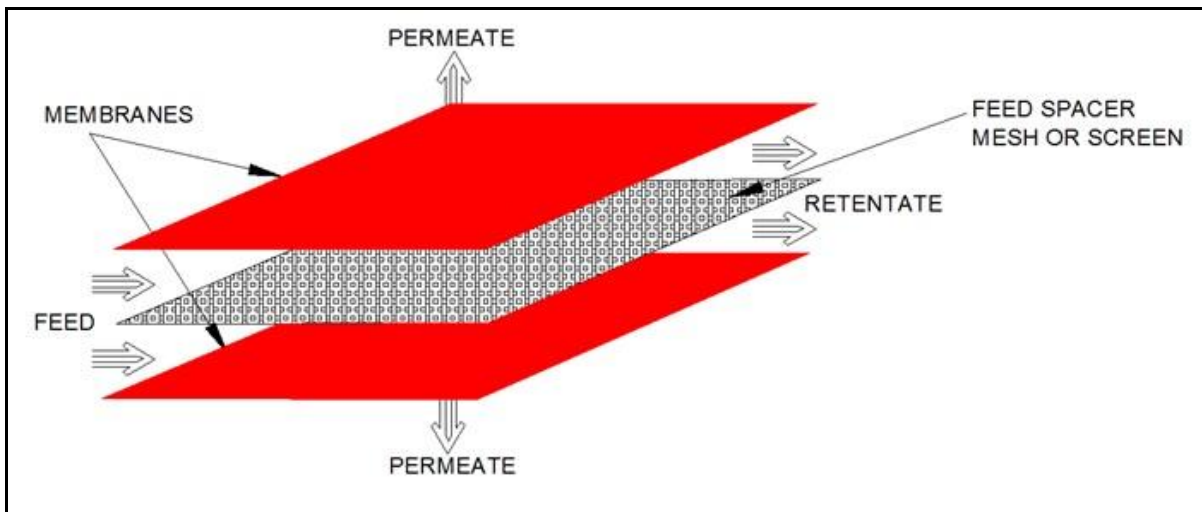
- High membrane area to module bulk volume ratio, that is, packing density
- High degree of turbulence for mass transfer promotion on the feed side
- Low energy expenditure per unit product volume
- Low cost per unit membrane area
- Design that facilitates cleaning
- Design that permits modularization (Henze, 2008)

Membrane module refers to the manner in which the membranes are packed to be installed in the system. A number of membrane modules are available and the most common types will be discussed.

#### **2.2.7.1. Plate – and – frame membrane module**

Plate – and – frame membrane modules were among the first to be introduced on a large scale. The design concept is very close to the flat membranes used in the laboratory. In this design, membranes are stacked one above another, with porous membrane support material and spacers between membranes forming the feed flow channel. This sandwich arrangement of spacer – membrane – support plates are stacked alternatively and clamped together between two endplates. The feed solution is channelled across the surface of the membrane by the feed side spacers. There are a variety of plate – and – frame designs on the market differing mainly in the design of the feed flow channels. In some modules, the membrane can be removed from the porous support plate, whilst in others, it is directly cast on a support structure. All plate – and – frame systems provide a large membrane area per unit volume. It is usually possible to dismantle and mechanically clean the membranes in a plate and frame module, but this is considerably more time consuming than the cleaning of a tubular system. The investment costs of a plate – and – frame unit depends on the specific module design. In general however, they

are somewhat lower than in tubular systems. Operating costs are also generally lower than in tubular systems (Ramakrishna et al., 2011).



**Figure 2-12: Plate and frame membrane module**

#### **2.2.7.2. Spiral wound membrane module**

The spiral wound membrane module uses flat sheet membranes. The major components of this membrane module are the membrane, the feed and permeate channels, spacers which keep the membrane leaves apart, the permeate tube and the membrane housing. Membrane sheets with the spacers in between are glued together on three sides to form a leaf and multiple leaves are rolled up around the permeate tube to create the feed and permeate channels of the spiral wound membrane module. A pressurized module housing holds the membrane leaves in place to prevent unwinding. Usually three or more modules are connected in series in the housing. The geometry of the spiral wound module is described by the number of leaves, the leaf length, width as well as the feed and permeate channel height. The feed solution flows in an axial direction parallel to the permeate tube through the feed channel. The permeate passes through the membrane and flows spirally along the curved permeate channel until it is collected in the permeate tube. The performance of spiral wound membrane modules are affected by leaf geometry, spacers, fouling propensity and cleaning ability as well as operating conditions (Schwinge et al., 2004).

### **2.2.7.3. Stirred cell membrane module**

A stirred cell membrane module is a stirred tank with a membrane disc fitted at the bottom. Permeate collection is facilitated by a grooved plate on which the membrane sits. The tank is filled with feed and then pressurized by nitrogen or compressed air. The feed could also be continuously fed from a reservoir via a pump. The tank is well mixed using a stirrer. Stirred cell modules are generally operated in dead – end mode. They are useful for small scale manufacturing and research applications. This type of module provides uniform conditions near the membrane surface and is therefore useful for process development and optimization work.

### **2.2.7.4. Tubular membrane module**

A tubular membrane module consists of several tubular membranes which are arranged as tubes. The membrane is cast on the inside of a porous support tube which is often housed in a perforated stainless steel pipe. Individual modules contain a cluster of tubes in series held within a stainless steel permeate shroud. The tubes are generally 10 – 25 mm in diameter and 1 – 6 m in length. The feed stream is pumped into the lumen, which is the inside of the tubular membranes from one end and the retentate is collected from the other end. The permeate passes through the membrane and is collected on the shell side. Tubular membranes are used for all types of pressure driven separations. Tubular modules are widely used where it is advantageous to have a turbulent flow regime, for example, in the concentration of high solids content feeds. Advantages include low fouling, relatively easy cleaning, easy handling of suspended solids and viscous liquids and the ability to replace or plug a damaged membrane. Disadvantages include high capital cost, low packing density, high pumping costs and high dead volume (Ghosh, 2006).

### **2.2.7.5. Hollow fibre membrane module**

The hollow fibre membrane module consists of fibres with an O.D of 25 – 250  $\mu\text{m}$  and a wall thickness of 5 – 50  $\mu\text{m}$ . The fibre wall has a structure of the asymmetric membrane, the active skin layer being placed to the feed side. A bundle of hollow fibres are mounted in a pressure

vessel, and the open end of the U – shaped fibres is potted into a head plate. Feed under pressure flows over the outside surface of the fibres. Permeate, then flows out through the base of the fibres and is collected as product. In a hollow fibre module, the permeate flow per unit area of membrane is low, and therefore, the concentration polarization is not high at the membrane surface. The result is that the hollow fibre units operate in a non – turbulent or laminar flow regime. Typically, a single hollow fibre permeator can be operated at up to 50 percent recovery and meet the minimum reject flow required. Hollow fibre membranes can be melt – spun, wet – spun or formed by interfacial polymerization. The major advantages of hollow fibre membrane modules are as follows:

- A large number of fibres can be packed into a cartridge, thereby rendering high area/volume ratio and low cost
- Hollow fibre is a flexible membrane. It can carry out the filtration in two ways, either inside – out or outside – in
- Tangential flow along the membrane surface limits membrane fouling
- Hollow fibre membranes can be designed for circulation, dead – end and single pass operations (Nath, 2008).

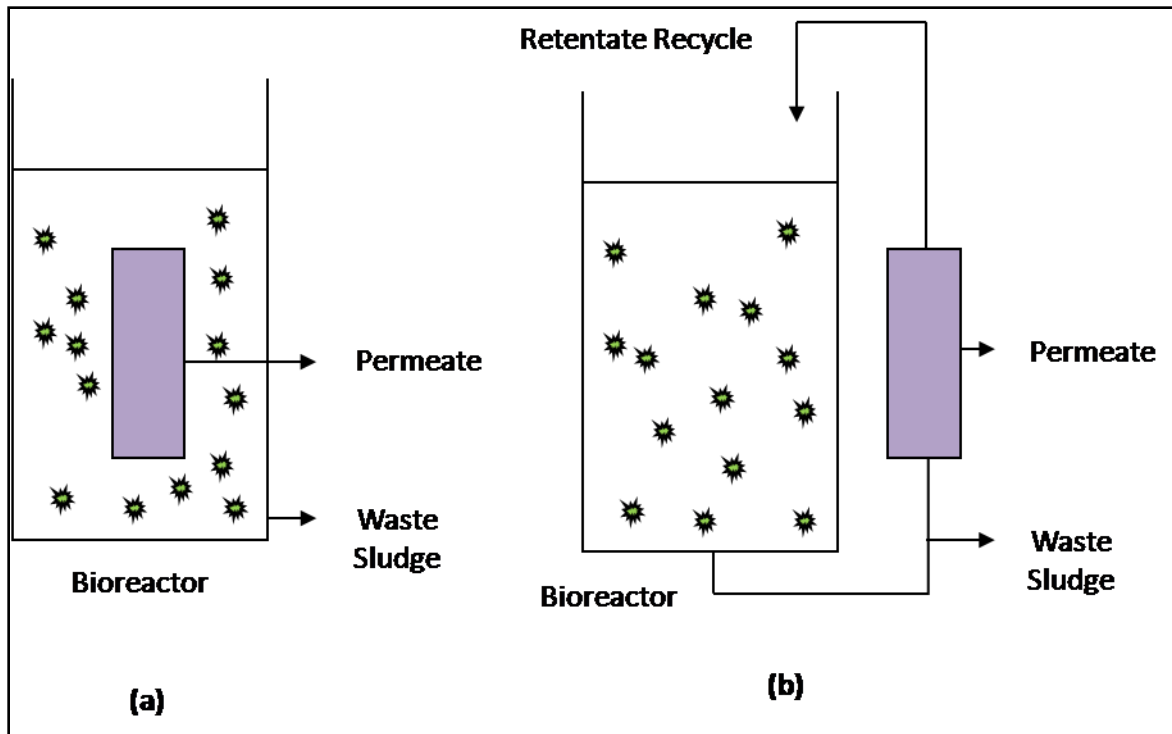
#### **2.2.8. Membrane bioreactors**

A chemical reactor is an equipment unit in a chemical process where chemical transformations or reactions take place to generate a desirable product at a specified production rate, using given chemistry (Mann, 2009). A bioreactor then, is a reactor containing enzymes or cells that is used to effect specific chemical reactions in the production of some economically desirable product (Smith et al., 2000). Dorr – Oliver developed the first membrane bioreactors in the late 1960's for sewage treatment aboard ships. This system established the principle of coupling an activated sludge process with a membrane to concentrate the biomass simultaneously with obtaining a clarified, disinfected product (Judd, 2010). Bioreactors supply a homogenous environment by constantly stirring the contents and give the cells a controlled environment by

ensuring the same temperature, pH and oxygen levels. Bioreactors may be run in batch, fed – batch or continuous mode.

#### **2.2.8.1. Immersed versus non – immersed membranes in bioreactors**

In a membrane bioreactor, filtration may occur either within the bioreactor or externally through recirculation. When the membrane is within the bioreactor it is submerged or immersed, whereas when the membrane is external to the bioreactor, it is referred to as a side stream system, as in Figure 2-13 below. The contents of the bioreactor are subject to a pressure drop across the membrane which is driven by either the hydraulic head or a pump. Sterilization of the submerged membrane is easier and both energy and space is reduced. Submerged reactors ensure constant reaction conditions, rule out additional shear and have less cell loss because of reduced dead volume (Carstensen et al., 2012). Aeration within the bioreactor provides oxygen transfer for the growth of the micro – organisms as well as mixing of the reactor contents. A coarse bubble diffuser is often used in the submerged configuration. The oxygen transfer is not as efficient as the non – immersed configuration, but the rising bubbles provide a turbulent cross – flow velocity over the surface of the membrane. This aids in maintaining the flux through the membrane by reducing the build up of material at the membrane surface and thereby increases the operational cycle of the system. Cleaning of the membrane is less frequent and rigorous compared to the side stream system. Aeration is usually provided through a fine bubble diffuser in the side stream system, which offers much more efficient oxygen transfer. The cross – flow velocity used in these systems is usually higher, and the operational flux of the system is higher as the system is driven by a differential head. Fouling of the membrane in this configuration is more pronounced and therefore more rigorous cleaning is required to restore the operational flux (Till and Mallia, 2001).



**Figure 2-13: Submerged (a) and side stream (b) membrane bioreactor configurations**

### 2.2.8.2. Film bioreactors

A biofilm is a collection of micro – organisms surrounded by the slime they secrete, attached to either an inert or living surface (Singh and Kapoor, 2010). Biofilms exist wherever surfaces contact water. Filamentous fungi, when grown in submerged culture, exhibit different morphological forms such as dispersed mycelial filaments or interwoven mycelial masses called pellets. Biofilms increase in depth while pellets increase in density. The hyphal arrangement in biofilms reflects a cellular process with different metabolic responses to that of pellets. Cell adhesion may be considered as the initial step necessary to trigger signalling and differential gene expression mechanisms, absent in conventional submerged fermentation. This may explain higher enzyme productivity in certain fungal biofilms (Gutiérrez-Correa et al., 2012).

Fixed film bioreactors are used for treating biodegradable contaminants in air and water. Biofilms are supported on inert material such as plastic, stone, sand, wood and ceramics (Bishop and Brenner, 1993). The filamentous fungi, *P. chrysosporium* has previously been immobilized in a capillary membrane bioreactor, referred to as a membrane gradostat reactor,

for the production of lignin peroxidases and manganese peroxidases and studies reported that the organism was able to grow ideally, forming a biofilm and secreting the desired enzyme without decline (Solomon and Petersen, 2002). Studies have also shown that the enzyme activity increased by seven times in an up scaled membrane gradostat reactor (Govender et al., 2003). Membrane aerated film reactors have use in wastewater treatment, where oxygen diffuses through a gas permeable membrane into the biofilm in which oxidation of pollutants takes place (Casey et al., 1999).

### **2.2.8.3. Applications of membrane bioreactors**

#### **2.2.8.3.1. Wastewater treatment**

In wastewater treatment, bacterial digestion of waste organic material is combined with the separation of the treated effluent from residual suspended solids. The membranes may be submerged or set up as an external loop and are commonly arranged as flat sheets or hollow fibre modules. Traditional municipal wastewater treatment involves preliminary and primary treatment comprising of screens, rit chambers and primary clarifiers, a secondary treatment phase consisting of biological reactors and secondary clarifiers and lastly a tertiary disinfection phase (Riffat, 2012). The membrane bioreactor replaces the entire secondary phase which involves sludge settlement, and can eliminate some parts of the tertiary phase as ultrafiltration membranes remove pathogens such as protozoa, bacteria and most viruses. A major advantage of this process is that it can operate at much higher solids concentration than a conventional activated sludge plant due to the limitations of settling. Longer retention times in the membrane bioreactor permit the destruction of molecules which are difficult to biodegrade such as detergents. Nitrogen and phosphorous contents can also be significantly reduced with proper design (Sutherland, 2010). The membrane bioreactor offers other advantages including; excellent quality of treated water, small footprint size of the treatment plant, reduced sludge production and better process reliability (Visvanathan et al., 2000).

#### **2.2.8.3.2. Chemical fermentation**

Kubota has successfully developed a methane fermentation system coupled with submerged membranes. Raw materials such as food waste are fed to the system. It is then stored in a solubilization tank. Thermophilic digestion takes place in a methane fermentation tank. Submerged membrane units in a sub – compartment concentrate the anaerobic sludge, after which it is recirculated to the methane fermenter or pumped to a sludge treatment line. Methanogenic bacteria are retained by the membrane while dissolved ammonia, which is a methane fermentation inhibitor, are filtered out in the permeate. The biogas generated consists of methane and carbon dioxide and is utilized either at a power generation facility or boiler, thus recovering energy (Kanai et al., 2010).

In the fermentation of ethanol, high concentrations of the product itself, inhibit further ethanol production as it becomes toxic to the organisms. To increase the productivity of the fermentation process, systems with cell recycle have come into use recently incorporating continuous ultrafiltration and microfiltration, removing the ethanol as it is formed (Kargupta et al., 1998).

Other valuable fermentation products which have shown increased productivity using either side stream or submerged membranes with cell recycle were lactic acid (Zhao and Chen, 2009, Ramchandran et al., 2012), L(+) – tartaric acid (Willaert and De Vuyst, 2006), acetone – butanol – ethanol solvents (Ennis and Maddox, 1989), oligosaccharides (Foda and Lopez-Leiva, 2000, Kuroiwa et al., 2009) and 2 – ethylhexyl palmitate (Tan et al., 2006).

#### **2.2.8.3.3. Food fermentation**

Membrane bioreactors have been used in the food industry in such applications as the production of wine, natural sweeteners, preservatives (Mostafa, 2001), acidifiers (Enzminger and Asenjo, 1986) and vitamins (Miyano et al., 2000). Wine is traditionally made from grape juice containing a high concentration of sugar. This is converted to ethanol and leads to high



osmotic stress for the micro – organism, *Saccharomyces cerevisiae*, resulting in poor growth and thus poor productivity. The membrane bioreactor provides a means to continuously remove the product which inhibits yeast production and thus increases cell density in the fermentation vessel. Productivity was observed to increase by 28 times in a double vessel membrane bioreactor than traditional batch fermentation (Takaya et al., 2002). Xylitol is a natural sweetener and can replace sucrose on a weight to weight basis. It promotes oral health and caries prevention and is an important sugar substitute for diabetes patients as insulin is not required for its metabolism. Using a submerged membrane bioreactor with cell – recycle fermentation of *Candida tropicalis*, xylitol productivity was reported to increase by 3.4 times compared to batch fermentation (Kwon et al., 2006).

#### **2.2.8.3.4. Enzyme production**

In the production of enzymes, like all other fermentation processes, the removal of the cells from the broth is required in the purification process. Filtration and centrifugation techniques are generally used for this purpose, but are labour intensive, as well as time and energy consuming. External loop membranes have been used in the clarification of fermentation broth. In previous studies, performed by Kiy et al. (1996) and Kao et al. (2007), such membranes were used resulting in yield of enzymes with high extracellular activities and increased productivity.

Lysosomal enzymes were produced by the fermentation of the ciliated protozoan, *Tetrahymena thermophila* in a bench – top fermenter. Cell – free broth was harvested by means of 2 metre polypropylene hollow – fibres. Fresh medium was added at the same rate as exhausted broth was removed. An increase in biomass by 20 times and enzyme activity by 40 to 50 times was observed. This increase was due to the continuous nutrient supply to the cells and the dilution of growth – and secretion – inhibiting metabolites via the perfusion module. Both these factors led to high cell densities and the corresponding high extracellular enzyme activities (Kiy et al., 1996).

Chitinase which is an enzyme used in the hydrolysis of chitin found in shell – fish waste was produced in a membrane bioreactor by *Paenibacillus* spp., CHE – N1. Cells were cultured batch – wise for days, following which, the operation was switched to membrane mode, as the fermentation broth passed through the membrane and the permeate collected and analysed. De – ionised water was continuously supplied to the bioreactor. At regular intervals, liquid was removed from the membrane filtration unit and fresh media of the same volume was added to supplement the broth. Results showed that enzyme activity could be maintained for a longer period of time and the overall total chitinase productivity was increased (Kao et al., 2007).

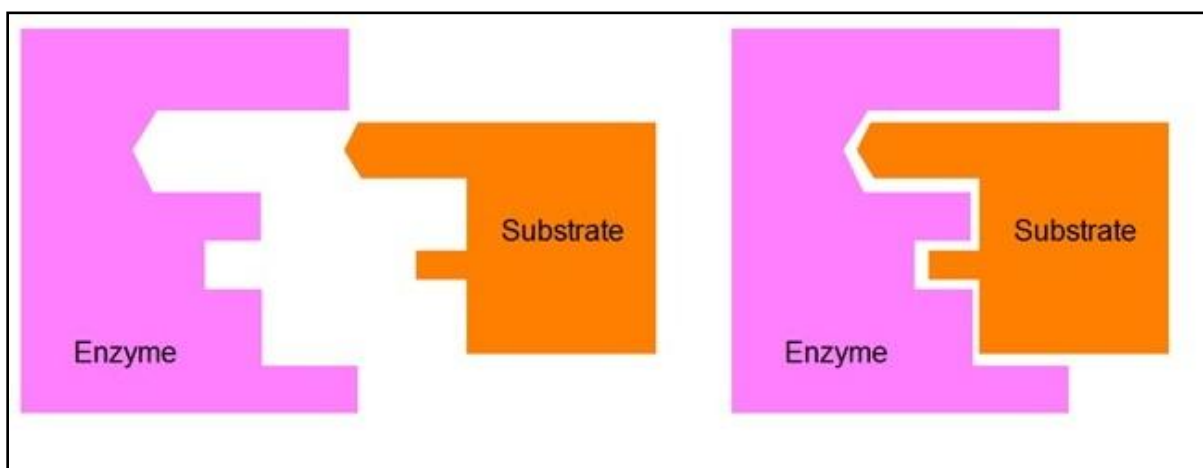
### **2.3. Enzymes as catalysts**

Catalysis is the speeding up of a reaction. Catalytic agents may be organic or inorganic. Enzymes, colloidal protein complexes, are organic catalysts. Enzymes act by forming transitory chemical combinations with one or more of the substrates. A substrate is the substance altered by the enzyme. Once the catalyzed reaction is complete, the enzyme separates from the substrate. The first proposal for a general mechanism of enzymatic action was developed by E. Fischer in 1894 and it assumes that an enzyme and its substrate mechanistically interact in a lock and key fashion (Faber, 2011). Enzymes act exceedingly rapidly and efficiently. Enzymes are specific, that is, an enzyme can react only with certain particular substrates that have a corresponding stereochemical structure.

Enzymes are protein complexes and therefore are sensitive to factors that affect proteins in general, such as temperature, concentration of heavy metals, hydrogen and hydroxyl ions, corrosive agents, variations in pH, hydrostatic and osmotic pressures as well as radiation. The nomenclature of enzymes is characterised by adding the suffix “ase” to the substrate that the enzyme acts upon. For example, the enzymes that hydrolyse lipids are known as lipases.

Enzymes can typically be gathered into groups based on their overall functions, which are, hydrolysing, transferring, oxidizing and reducing, ligation, isomerization, as well as adding and

removing chemical groups (Kulkarni and Deshpande, 2007). Each species of living cells has a genetically determined natural endowment with certain functioning enzymes. These are constantly present in the cells and are referred to as “constitutive enzymes”. In addition, many cells contain genes for the synthesis of enzymes which do not ordinarily appear. These enzymes are expressed when an inducing substrate or related substance enters the cell. In the presence of the inducer, the enzyme which is specific for the inducer substrate is synthesized. These enzymes are referred to as “inducible enzymes”. The cell however does not synthesize enzymes which are not necessary, but only those that may be needed from time to time (Bhatia, 2009). Enzymes are produced industrially by micro – organisms as they serve many useful functions as discussed previously.



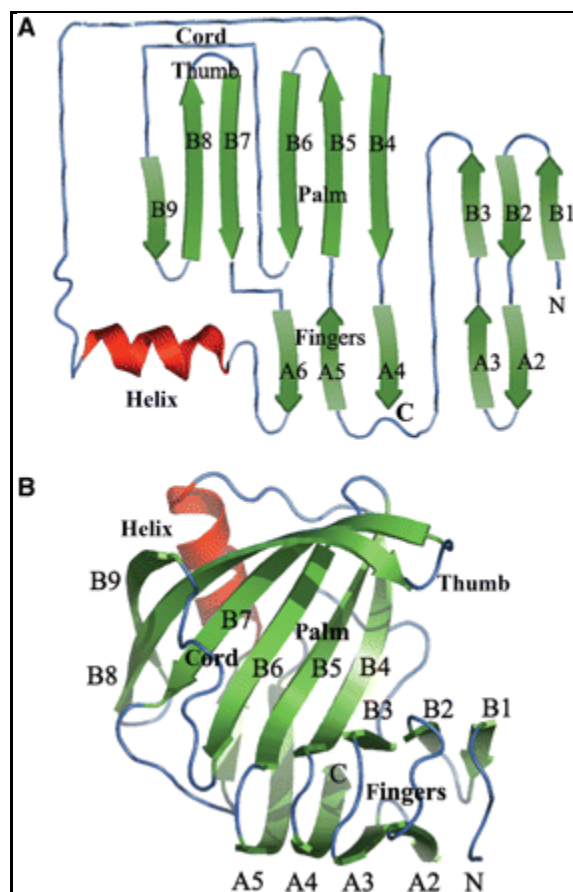
**Figure 2-14: Schematic representation of enzyme – substrate ‘lock and key’ mechanism**

### **2.3.1. Xylanase**

Xylanase, the enzyme to be produced in this study by submerged fermentation, is one that catalyzes the hydrolysis reaction of xylan, a major component of plant tissue. Xylanase depolymerizes the xylan molecules into monomeric units that are used by micro – organisms as a primary carbon source. Xylanase has numerous industrial applications and can be biologically produced and harvested from certain microbial species.

### **2.3.1.1. Classification and structure**

Xylanases are glucosidases, that is, they function as catalysts for the endohydrolysis of the 1,4 –  $\beta$  – D – xylosidic linkages in xylan. Their official name is endo – 1,4 –  $\beta$  – xylanase. Classification of these enzymes is based on primary structure comparisons of the catalytic domains and enzymes are grouped into families of related sequences. Enzymes within a particular family have a similar three – dimensional structure and similar molecular mechanism. Of interest in this study is a xylanase belonging to family 11. Family 11 enzymes are characterized by a high  $pI$ , that is, the isoelectric point or  $pH$  where the protein has no net charge, low molecular weight, a double displacement catalytic mechanism, two glutamates acting as the catalytic residues and a  $\beta$  – jelly roll fold structure. Xylanases may be extremophiles, as is the case with this xylanase which is active at very high temperatures (Collins et al., 2005). Family 11 xylanases are constructed with two  $\beta$  – sheets and an  $\alpha$  – helix forming a so – called  $\beta$  – sandwich structure. The different parts of the enzyme are named after the right hand (Törrönen et al., 1994), thus containing regions named fingers, thumb, palm, cord and  $\alpha$  – helix as shown in Figure 2-15 below.

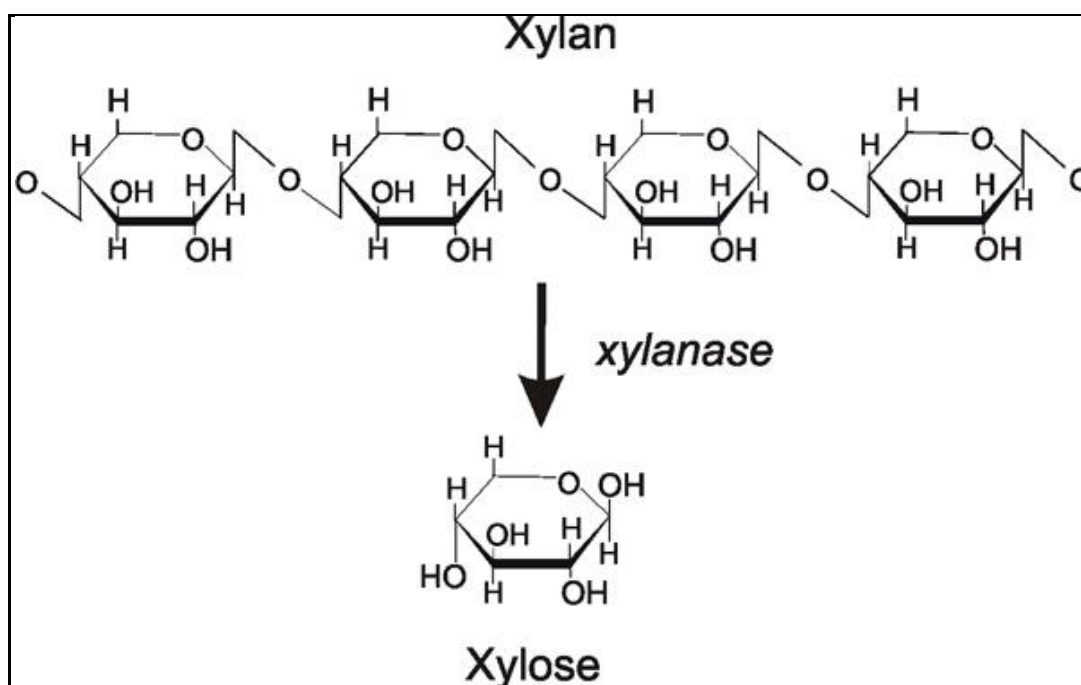


**Figure 2-15: (A) Topology diagram and (B) a schematic 3D structure of the family 11 xylanases (Purmonen et al., 2007).**

#### 2.3.1.2. Substrate and mechanism

Xylan is a major heteropolysaccharide which may represent up to 30 percent of the dry weight of the cell walls in hard and softwoods as well as in annual plants such as maize and sugar cane (Prade, 1996). It is a major reserve of reduced carbon on earth. Xylans make up a group of complex structural polymers referred to as hemicelluloses. Xylans are linear or branched polysaccharides. In its simplest form, xylans are linear homopolymers that contain only D – xylose monomers linked through  $\beta$  – 1,4 – glycosyl bonds. Hemicelluloses are situated between the lignin and the collection of cellulose fibres in plants. They are covalently linked to the lignin while producing a coat around the underlying cellulose via hydrogen bonding (Uffen, 1997). Complete degradation of the xylan molecule requires the action of several enzymes. Xylanase

hydrolyzes 1,4 –  $\beta$  – D – xylosidic bonds within the  $\beta$  – (1, 4) – linked D – xylosyl backbone of xylan randomly and liberate  $\beta$  – anomeric xylo oligosaccharides or XOS (Belkacemi and Hamoudi, 2003). Xylose is a primary carbon source that may be substituted for glucose and is thus very valuable. In recent years the bioconversion of xylan has received much attention because of its importance in the efficient conversion of lignocellulosic biomass to bioethanol and chemicals, such as xylitol and xylonic acid (Cuyvers et al., 2011).



**Figure 2-16: Xylan degradation by xylanase (Held, 2012)**

### **2.3.1.3. Applications of xylanase**

#### **2.3.1.3.1. Supplementation of animal feed**

Wheat is an important component of poultry feed. However, the nutrient composition and nutritive quality of wheat vary widely, compared to other cereal grains. The apparent metabolisable energy (AME) of wheat fluctuates significantly. Evidence suggests that the responsible factors for this is that the water soluble pentosan component, accounting for 50 to 80% of the wheat non – starch polysaccharides, consists mainly of arabinoxylans. The non –

starch polysaccharides are of high molecular weight and dissolve in the intestine, leading to an increase in the viscosity of the gut contents (Silversides and Bedford, 1999). High viscosity leads to reduced growth rate, depress nutrient utilisation and performance. Supplementation of the feed with exogenous xylanases, have shown to lower the viscosity of intestinal contents and improve digestibility of starch, protein and fat and the AME in broilers fed on diets containing wheat (Hew et al., 1998). Studies have also been carried out on weanling pigs, concluding that supplementation of wheat – based diets with xylanases increased the apparent ileal digestibilities of organic matter (OM), crude fibre (CF), natural detergent fibre (NDF), crude protein (CP) and amino acids (AA) with the exception of a few (Diebold et al., 2004).

#### **2.3.1.3.2. Production of biofuel**

Butanol or ethanol is produced by the conversion of abundant and renewable cellulosic biomass using either non – food crops or waste from food or woody crops. Lignocellulose from perennial bioenergy crops like poplar and switchgrass consists of crystalline cellulose embedded in a hemicellulose and lignin matrix. The major component of hemicelluloses which has been targeted as feedstocks for the production of cellulosic ethanol is methylglucuronoxylans. These are found in woody biomass and consist of a  $\beta$  – 1,4 – linked xylan backbone with 4 – O – methylglucuronic acid residues linked to xylose units. Cellulose and hemicellulose components can be converted into fermentable sugars for the production of cellulosic ethanol by enzymatic hydrolysis. For complete hydrolysis of plant cell walls, the hemicellulose coating must be degraded to expose the cellulose fraction (Chatterjee et al., 2010). The processing of cellulose and hemicellulose to release fermentable sugars requires thermochemical pre – treatment to dissociate the hemicellulose from the cellulose and lignin. Acid or alkaline treatments at high temperatures have been used, but have limitations such as the formation of inhibitors like furfural. The hemicellulose fraction may be enzymatically processed with hemicellulases such as xylanase and fermented to ethanol. Maximum yields were achieved when compared to several other methods evaluated (Kim et al., 2011).

#### **2.3.1.3.3. Food production processes**

Enzymes have been used in food processing since the eighteenth century when chymosin from the stomachs of calves was used in cheese manufacturing. Xylanases are used in the food industry for clarification of juices and wines, and extraction of coffee, plant oils and starches. In the baking industry, xylanases can replace the usual additives such as emulsifiers, oxidants and barley or wheat malts. The factors evaluated are the yield expressed as specific volume of the final product, extent of water retention, shelf life of the bakery product and the amount of dough kneading. Quality assessment is performed by considering the volume and appearance as well as crispness and aroma. The increased specific loaf volume caused by xylanase is attributed to the redistribution of water between pentosan and gluten fractions. An increase in gluten volumes results in better dough rise (Romanowska et al., 2006). The xylanase hydrolyses the water – insoluble pentosans, mainly arabinoxylans, leading to the improvement of rheological properties. The higher release of fermentable sugars in xylanase supplemented doughs results in a higher rate of carbon dioxide evolution by yeast and thus the rise or expansion of the dough volume is remarkably higher than dough which is not supplemented with xylanase (Shah et al., 2006). Xylitol is a five carbon sugar alcohol which is used as a sweetener and is produced by a chemical reaction under alkaline conditions of the xylose derived from wood hydrolyzate. Xylitol is consumed in food products such as chewing gum, candy, soft drinks and ice cream (Dhiman et al., 2008).

#### **2.3.1.3.4. Pulp and paper processing**

Pulp is made by cooking plant raw materials at high temperature and pressure, consuming vast amounts of energy and chemicals. Wood chips are cooked in a liquor of sodium hydroxide and sodium sulphide, conferring a dark colour to the pulp. Oxygen is used in the pre – bleaching process, removing some of the impurities. The pulp is then a yellow colour. The removal of light absorbing substances called chromophores, result in the attainment of a pure white colour. This process is carried out in stages with the use of ozone, chlorine dioxide, sodium hydroxide, oxygen and hydrogen peroxide (Polizeli et al., 2005). Biobleaching with xylanase has



proven to be an environmentally friendly bleaching technology. The bond between lignin and hemicelluloses is primarily between lignin and xylan which can be removed by xylanase. Once this layer is removed, the lignin layer is easily available for degradation by the action of ligninolytic enzymes (Ziaie-Shirkolaee et al., 2008). Xylanase is suitable for the biobleaching of pulp due to its stability at high temperatures and alkaline pH (Khucharoenphaisan and Sinma, 2010).

#### **2.3.4. *Thermomyces lanuginosus* as a producer of xylanase**

Xylanases are produced by fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans, insects and seeds. Bacterial xylanases are produced at lower activity levels than in fungi and are also restricted to the intracellular or periplasmic fractions (Polizeli et al., 2005). Filamentous fungi are interesting as they secrete xylanase into the media, eliminating the need for cell disruption. In addition to xylanases, fungi typically produce several auxiliary enzymes which are necessary for debranching of the substituted xylans (Haltrich et al., 1996). *Thermomyces lanuginosus* is a thermophilic fungus which has attracted attention due to its production of thermostable enzymes, especially the xylanase belonging to family 11 of glycosyl hydrolases. By definition a thermophilic fungus is one that thrives at temperatures up to 60°C and fails to grow below 20°C (Hudson, 1991). Strains have been reported to occur in dry and waterlogged grassland, loamy garden soil and aquatic sediments, but are more specifically associated with organic substrates such as culms, roots and leaves of grasses, composts of various plant materials and the dung of various birds and mammals (Singh et al., 2003). The production of xylanase by *T. lanuginosus* is not accompanied by the production of cellulases which is a desirable trait in the pulping industry as cellulose in pulp will not be degraded, and thus poor quality pulp is avoided (Xiong et al., 2004). The strain *Thermomyces lanuginosus* DSM 5826 was first isolated from a pile of jute waste in a jute factory in Bangladesh in which jute fibres were treated with an oil emulsion. The temperature in the pile of jute was 65°C to 70°C. The strain was then deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen under DSM 5826. *Thermomyces lanuginosus* DSM 5826 is particularly suitable for producing xylanase with high activities in a medium containing corn cobs (Wizani et al., 1993).

### 3. METHODOLOGY

---

This chapter details the apparatus and materials used, experimental set – up and procedure and analytical methods employed to develop and optimize the membrane reactor for xylanase production. The work entailed growing and maintaining the fungal culture, *Thermomyces lanuginosus* DSM 5826, optimizing the production conditions by submerged flask fermentation, performing fed – batch and semi – continuous submerged flask fermentation, partially characterizing the enzyme and finally developing a system to perform semi – continuous membrane fermentation. Hence, this chapter outlines the five different procedures that were performed during this study:

- a) Growing and maintaining the fungal culture
- b) Determining optimum conditions for the production of xylanase
- c) Producing xylanase in fed – batch and semi – continuous operation in flasks
- d) Partial characterization of the enzyme
- e) Development of a membrane fermenter system for xylanase production

All production experiments were performed in triplicate.

#### 3.1. Maintenance of culture

##### 3.1.1. Equipment and material

- 1 L glass bottle
- Analytical balance (RADWAG, AS22/C/2)
- Spatula
- Weighing boat
- Potato dextrose agar (70139 Fluka Potato Dextrose Agar, Sigma – Aldrich)
- Autoclave (Huxley HL – 341 vertical steam sterilizer, Polychem Supplies)
- Petri plate
- Laminar flow cabinet/hood (LASAIR III 310 C, Vivid Air Laboratory Equipment and Filtration Supplies)

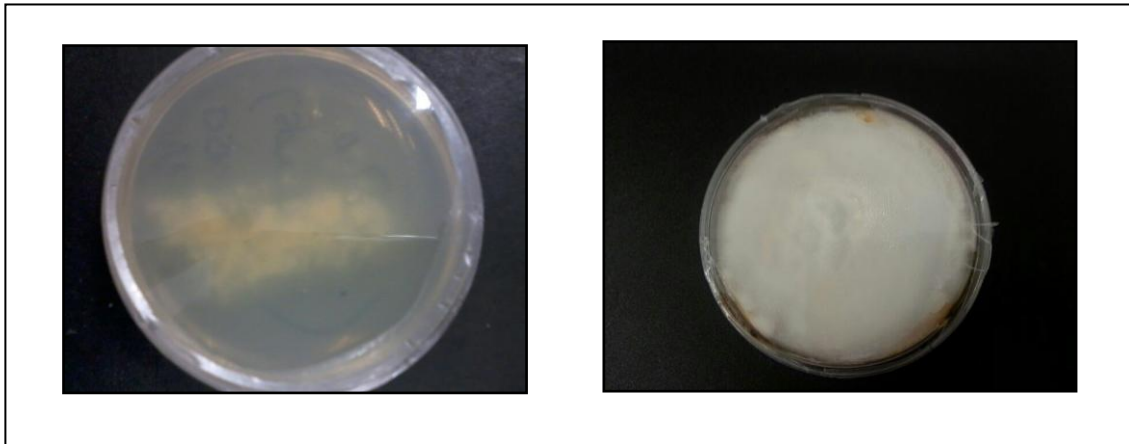
- *Thermomyces lanuginosus* DSM 5826 (Durban University of Technology, Department of Biotechnology and Food Technology culture collection)
- Laboratory scalpel
- Inoculation loop
- Sodium chloride (NaCl) (99.0 – 100.5 %, Merck)
- Tween 80 (TWEEN® 80 Oleic acid, ≥ 58.0 % (balance primarily linoleic, palmitic, and stearic acids), Sigma – Aldrich)
- Incubator (Infors HT Ecotron, Polychem Supplies)
- De – ionized water

### 3.1.2. Methodology

All work was done in a laminar flow hood which provides an aseptic work area while allowing the containment of infectious splashes or aerosols generated by many microbiological procedures. This is done by maintaining constant, unidirectional flow of HEPA (high – efficiency particulate absorption) – filtered air over the work area.

The fungal culture *Thermomyces lanuginosus* was obtained from the Department of Biotechnology at DUT. This culture is part of the German Type Collection (DSM 5826). *T. lanuginosus* DSM 5826 was grown on potato dextrose agar (PDA) and periodically sub – cultured. PDA was prepared by dissolving 39 g in 1 L of de – ionized water and autoclaving at 121 °C for 20 minutes. The purpose of autoclaving is to sterilize the desired substance by subjecting it to high pressure saturated steam.

The agar was cooled to touch and poured onto 10 cm petri plates. Once the agar had solidified, fungal plugs, approximately 1 cm × 1 cm were placed on the agar and cultivated in a 50 °C incubator for 14 days or until sporulation was observed. Plates were stored at 4 °C for further use.



**Figure 3-1: Thermomyces lanuginosus agar plates during growth**

For use as inoculum, the fungal spores were scraped in an aqueous solution of 0.9 % sodium chloride (NaCl) and 0.05 % Tween 80.

## **3.2. Production of xylanase by shake flask submerged fermentation**

### **3.2.1. Equipment and material**

- 250 mL Erlenmeyer flasks
- 200 mL glass bottles
- 50 mL measuring cylinder
- Spatula
- Weighing boats
- Analytical balance (RADWAG, AS22/C/2)
- Oven (Scientific, Series 9000)
- Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (> 99 %, Merck)
- Beechwood xylan (> 90 % (HPLC) Cell wall polysaccharide Xylan from Beechwood, Sigma Life Sciences)
- Oatspelt xylan (Xylan from oat spelts 10 % arabinose and ~ 15 % glucose residues, Sigma – Aldrich)
- Corn cobs

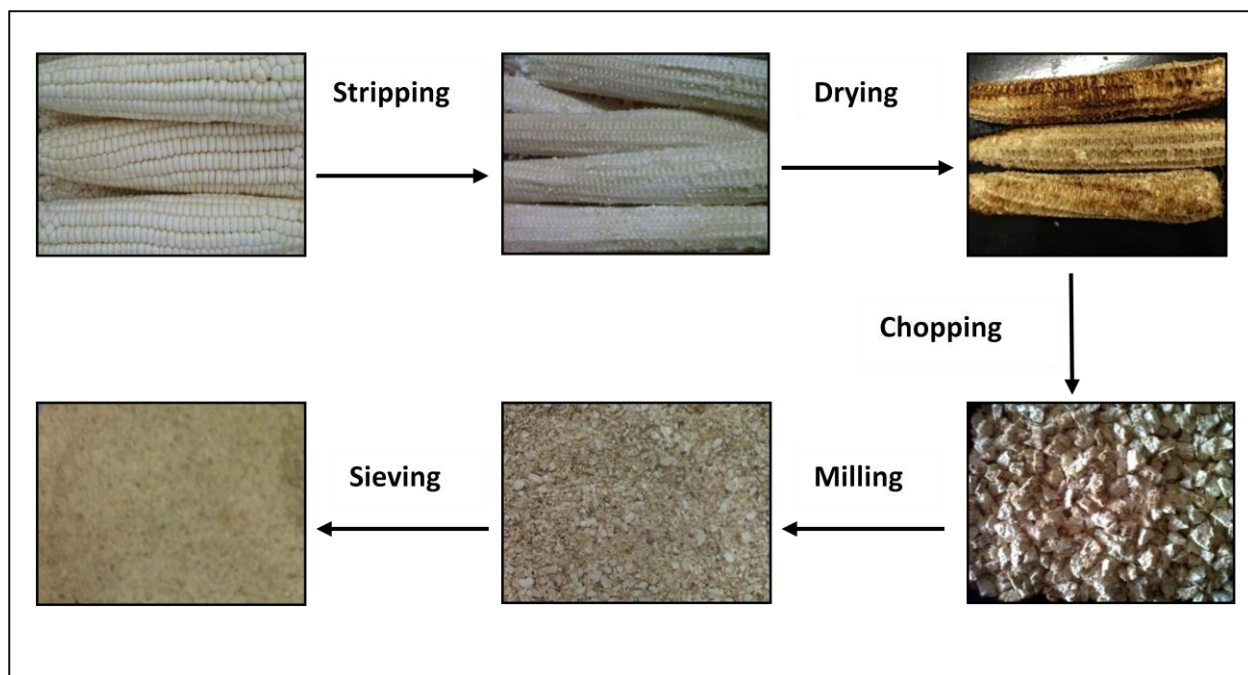
- Wheat bran (Snowflake, Premier Foods)
- Yeast extract (BIOLAB)
- Peptone (Fermtech®)
- Urea (Urea Ultrapure, Separations)
- Ammonium nitrate (99.5 %, Merck)
- Sodium nitrate (min 96 %, Merck)
- Sodium hydroxide ( $\leq 100$  %, Merck)
- pH meter (Orion 2 Star pH bench top, Thermo Scientific)
- Stirrer hotplate (Stuart heat – stir CB 162)
- Stirrer bar
- Pipette
- Autoclave (Huxley HL – 341 vertical steam sterilizer, Polychem Supplies)
- Shaking incubator (Infors HT Ecotron, Polychem Supplies)
- Centrifuge (Eppendorf, 581R)
- 15 mL centrifuge tubes
- De – ionized water
- Food processor (Waring Commercial Laboratory Blender)
- Sieve (1 mm )

### **3.2.2. Methodology**

#### **3.2.2.1. Evaluation of the effect of carbon source**

Five different carbon sources were evaluated. The carbon sources used in the experiment included Beechwood Xylan (Sigma Aldrich), Oatspelt Xylan (Sigma Aldrich), wheat bran, coarse corn (mielie) cobs (2 – 3 mm) and fine corn cobs (< 1 mm). The corn cobs were purchased from a local supplier and processed before use. The kernels were removed from the maize and the cobs were dried in an 80 °C oven. The cobs were then chopped and milled using a food processor. The coarser particles were separated from the fine particles using a sieve. The preparation of the corn cobs is illustrated in the flow diagram below. The two fractions of cob

(large and small) were then autoclaved at 121 °C for 20 minutes and stored at 4 °C for future use. All shake flask fermentations were performed in triplicate in 250 mL Erlenmeyer flasks containing 100 mL of growth medium. The growth medium consisted of the carbon source (1.5 % w/v), yeast extract (1.5 % w/v) and potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (0.5 % w/v) in de – ionized water, concentrations obtained from literature. The pH was adjusted to 6.5 using a 1 M sodium hydroxide ( $\text{NaOH}$ ) solution while stirring on a magnetic stirrer hotplate, before autoclaving at 121 °C for 20 minutes. Upon cooling, the flasks were inoculated with a 1 mL spore solution. The inoculated flasks were incubated at 50 °C in a shaking incubator at a speed of 150 rpm for 7 days. Sampling was carried out every 24 hours. The sampled broth from each flask was centrifuged at 4000 rpm for 15 minutes. The supernatants were used to perform the xylanase assay. Note that all subsequent flask experiments used coarse corn cobs as carbon source due to the high cost of commercial xylan.



**Figure 3-2: Processing of corn cobs**

#### **3.2.2.2. Evaluation of the effect of nitrogen source**

Five different nitrogen sources were evaluated. The various nitrogen sources included yeast extract, peptone, urea, ammonium nitrate and sodium nitrate. The growth medium consisted of the nitrogen source (1.5 % w/v), coarse corn cobs (1.5 % w/v and  $\text{KH}_2\text{PO}_4$  (0.5 % w/v). The preparation of the growth medium and fermentation were carried out as in Section 3.2.2.1 above. Samples were withdrawn every 24 hours for 7 days, centrifuged at 4000 rpm and stored at 4 °C until analyzed.

#### **3.2.2.3. Initial pH optimization**

The initial pH of the growth medium consisting of coarse corn cobs (1.5 % w/v), yeast extract (1.5 % w/v) and  $\text{KH}_2\text{PO}_4$  (0.5 % w/v) was adjusted to five different pH values ranging from 5 to 7 in increments of 0.5. The preparation of the growth medium and fermentation were carried out as in Section 3.2.2.1 above. Samples were withdrawn every 24 hours for 7 days, centrifuged at 4000 rpm and stored at 4 °C until analyzed.

#### **3.2.2.4. Rotational speed optimization**

The effect of the agitation on the xylanase activity was determined by performing the fermentation in shaking incubators of varied rotational speed settings. The growth medium consisting of coarse corn cobs (1.5 % w/v), yeast extract (1.5 % w/v) and  $\text{KH}_2\text{PO}_4$  (0.5 %) was prepared as in Section 3.2.2.1 above, and placed in shaking incubators with rotational speeds of 120 rpm, 150 rpm and 180 rpm. Samples were withdrawn every 24 hours for 7 days, centrifuged at 4000 rpm and stored at 4 °C until analyzed.

#### **3.2.2.5. Inoculum size optimization**

To determine the effect of the inoculum size on the enzyme activity various amounts of inoculum was added to the shake flasks containing the nutrient medium. The inoculum size ranged from 0.5 to 2 mL and increased in increments of 0.5 mL. The nutrient medium was

prepared as in Section 3.2.2.1 above, placed in a 50 °C incubator and fermentation was allowed to proceed for 7 days. Samples were withdrawn every 24 hours, centrifuged at 4000 rpm and stored at 4 °C until analyzed.

#### **3.2.2.6. Production temperature optimization**

Fermentation was carried out at different temperatures in order to establish the optimal production temperature. The nutrient medium containing coarse corn cobs as carbon source was prepared as in Section 3.2.2.1 above. The flasks were placed in incubators with the temperature ranging from 40 to 60 °C in increments of 10 °C and at 65 °C as this is the highest temperature setting on the incubator. Fermentation was allowed to proceed for 7 days with samples being withdrawn every 24 hours, centrifuged at 4000 rpm and stored at 4 °C until analyzed.

#### **3.2.2.7. Fed – batch operation**

In order to determine the effect of fed – batch operation on the enzyme activity and productivity, shake flask experiments were conducted in this mode. The flasks were initially charged with 100 mL of the nutrient medium containing coarse corn cobs, yeast extract and  $\text{KH}_2\text{PO}_4$ , in the same concentrations as in section 3.2.2.1 above. Fermentation was allowed to proceed for 6 days with sampling done every 24 hours. On the sixth day, following sampling, 50 mL of fresh nutrient medium was added to the flask. Nutrient medium of the same initial concentration was prepared in a 200 mL bottle and stored at 4 °C until required. The medium was brought up to temperature by placing it in a 50 °C water bath for 30 minutes prior to addition. Fermentation was allowed to proceed for a further 8 days with sampling every 24 hours. The samples were centrifuged at 4000 rpm and stored at 4 °C until analyzed.

#### **3.2.2.8. Semi – continuous operation**

Semi – continuous operation implies that a volume of broth is removed from the flask at regular intervals and replenished with fresh nutrient medium. This experiment was carried out by



preparing 100 mL of nutrient medium and inoculating as previously done and allowing fermentation to proceed for 6 days. On day 6 and day 12, 50 mL of broth was removed and replaced with 50 mL of fresh nutrient medium. Nutrient medium of the same initial concentration was prepared in a 200 mL bottle and stored at 4 °C until required. The medium was brought up to temperature by placing it in a 50 °C water bath for 30 minutes prior to addition. Samples were taken every other day for 21 days. On day 6 and day 12, samples were taken before and after the addition of fresh nutrient medium.

### **3.3. Partial characterization of xylanase enzyme**

Enzymes act on a specific reactant which is the substrate. They are characterized by greater activity, specificity and susceptibility to the influence of pH, temperature and other environmental changes. Enzymes are globular proteins with three – dimensional shape with molecular masses ranging from 12 kDa to 1000 kDa. The purpose of conducting characterization experiments is to better understand enzyme function. This section describes how the optimal temperature and pH for enzyme action is determined, as well as how the size and substrate of the enzyme is confirmed.

#### **3.3.1. Equipment and materials**

- As for xylanase assay
- Tris (TRIZMA® Base, (titration) crystalline, Sigma – Aldrich)
- HCl (Hydrochloric acid 32 %, Merck)
- De – ionized water
- Acrylamide (Acrylamide/Bis – Acrylamide, 30 % SOLUTION, Sigma – Aldrich)
- SDS (BioXtra, ≥ 99.0 % (GC), Sigma – Aldrich)
- AMPS (≥ 97.5 % (GC), Sigma – Aldrich)
- TEMED (BIORAD)
- Glycine (>99.0 % (GC), Sigma – Aldrich)
- Mini – Protein Tetra Cell (BIORAD)

- Power supply (PowerPac™ Basic Power Supply, 300 V, BIORAD)
- Page Ruler Unstained Broad range Protein Marker
- B – Mercaptoethanol ( $\geq 99.0\%$ , Sigma – Aldrich)
- Bromophenol blue (Sigma – Aldrich)
- SDS (BioUltra,  $\geq 99.0\%$  (GC), Sigma – Aldrich)
- Coomassie Brilliant Blue R250 (Thermo Scientific)
- Sea – saw rocker (Stuart Sea – Saw Rocker SSL4, LASEC SA)
- Acetic acid ( $>99.0\%$ , Merck)
- Ethanol (Ethanol Absolute 99.5 %, Merck)

### **3.3.2. Methodology**

#### **3.3.2.1. Temperature optima**

Temperature optima were determined by performing the xylanase assay at various incubation temperatures. The substrate was prepared in sodium citrate buffer of pH 6.5. The method for the assay remained unchanged except that the incubation temperature ranged from 40 to 90 °C, increasing in increments of 10 °C.

#### **3.3.2.2. pH optima**

pH optima were performed using buffers of various pH values in the xylanase assay. For each assay, the pH value was changed by using sodium citrate buffers ranging from 5 to 7, increasing in increments of 0.5. For each assay, the substrate solution and standards were prepared in a buffer of specific pH. The methodology for the assay remained unchanged. The incubation temperature for the assay was 50 °C.

#### **3.3.2.3. SDS PAGE**

SDS (Sodium dodecyl sulphate) PAGE (polyacrylamide gel electrophoresis) is a technique employed to determine the molecular weight of a protein. Proteins are separated according to

their electrophoretic mobility, which is a function of the length of a polypeptide chain and its charge. SDS is an anionic detergent applied to the protein sample to linearize it and impart a negative charge to linearized proteins. An electric field is applied across the gel, causing negatively charged proteins to migrate across the gel towards the positive electrode. The SDS PAGE analysis was performed using the Laemmli system (Laemmli, 1970) which uses tris – glycine gels. The stacking gel helps focus the proteins into sharp bands at the beginning of the electrophoretic run while the resolving or separating gel is used to separate the proteins based on their molecular weight.

#### **3.3.2.3.1. Gel preparation**

The short plate and spacer plate of the casting stand were cleaned with ethanol and placed in the casting frame with a rubber seal at the bottom and clamped. Leaks were checked for by pouring a small amount of water into the holder. The water was then discarded. Separating gel (12 %) was prepared and pipette between the inner and outer plates, after which it was overlayed with de – ionized water. The gel was allowed to solidify and excess water was removed. Stacking gel was prepared and loaded over the separating gel. The comb was inserted to form wells into which the samples could be loaded.

#### **3.3.2.3.2. Gel electrophoresis and staining**

The gel was then inserted into the green gasket. The gel and the buffer dam on the other side formed the inner buffer chamber. The frame was then slid into the support and the levers were closed to lock the frame in place. The assembled support and frame were lowered into the outer buffer chamber. Running buffer (1 x) was poured into the inner chamber to check for a tight seal. 3 µL of Unstained Broad Range Protein Ladder (Fermentas) was loaded into a well. The sample was prepared by adding 5 µL of SDS dye ((β – Mercaptoethanol (5 %), Bromophenol blue (0.02 %), SDS (10 %), Tris – Cl (250 mM, pH 6.8)) to 20 µL of the sample, followed by boiling for 5 minutes. The sample was then loaded into the wells. The tank was then filled with the Running buffer. The Mini – Protein Tetra Cell was connected to the PowerPac Basic. It was set

at 25 mA for 30 minutes. The gel was removed from between the plates and stained in Coomassie Brilliant Blue R250 for 5 minutes, left shaking on a sea – saw rocker. The gel was then destained with a solution of acetic acid and ethanol. Bands were observed and the positions recorded.

**Table 3-1: Preparation of 12 % separating gel**

| Reagent                  | Volume   |
|--------------------------|----------|
| 1.5 M Tris – HCl pH 8.85 | 1.875 mL |
| De – ionized water       | 2.4 mL   |
| 30 % Acrylamide          | 3.0 mL   |
| 10 % SDS                 | 75 µL    |
| 10 % APS (100 mg/mL)     | 45 µL    |
| TEMED                    | 11 µL    |

**Table 3-2: Preparation of stacking gel**

| Reagent                 | Volume  |
|-------------------------|---------|
| 0.5 M Tris – HCl pH 6.8 | 0.5 mL  |
| De – ionized water      | 1.18 mL |
| 30 % Acrylamide         | 355 µL  |
| 10 % SDS                | 20 µL   |
| 10 % APS (100 mg/mL)    | 18 µL   |
| TEMED                   | 3.2 µL  |

**Table 3-3: Preparation of 10 x running buffer (1 L)**

| Reagent | Mass  |
|---------|-------|
| Tris    | 30 g  |
| Glycine | 144 g |
| SDS     | 10 g  |

#### **3.3.2.4. Xymogram**

Zymography denotes the visualization of enzymatic activity by substrate conversion. The biochemical reaction is measured by detection methods specific for either the appearance of the reaction product or the disappearance of the substrate. Zymography techniques can thus be developed for any hydrolase acting on any biological substrate (Vandooren et al., 2013), in this case the hydrolase being xylanase and the substrate xylan. The zymogram analysis was performed based on the overlay method by Royer and Nakas (1990). The proteins were separated as in the method for SDS PAGE though under native conditions, that is, gels were run in non – denaturing conditions so that the analyte’s natural structure is maintained. The gel was run in the absence of SDS and the sample was not heat treated prior to loading. An overlay gel (2 % agarose, 0.5 % xylan) was prepared in 0.2 M citrate buffer, pH 6.5. This was placed over the native gel and the gel sandwich was incubated at 50 °C for 30 minutes. The gels were separated and the overlay gel was developed in 95 % ethanol until the background turned white. Xylanase activity was visualized by translucent zones of hydrolysis on the gel.

### **3.4. Translation to membrane fermenter system**

In this section, the equipment and procedures used to develop and evaluate a coupled membrane fermenter system are described. A brief description of the initial designs and challenges are also presented in this section.

#### **3.4.1. Equipment and materials**

- 1.5 L cylindrical glass vessel (150 mm diameter, 300 mm height)
- 5 mm OD silicone tubing
- 5 mm PVC nipples
- Megabond glue
- Q – bond glue and sand
- Woven fabric sheets
- 60 mL syringes
- 20 mL syringes
- 20 micron sterile filters
- 20 cm air pipes (5 mm diameter)
- Plastic mesh
- Centrifuge (Eppendorf, 581R)

#### **3.4.2. Methodology**

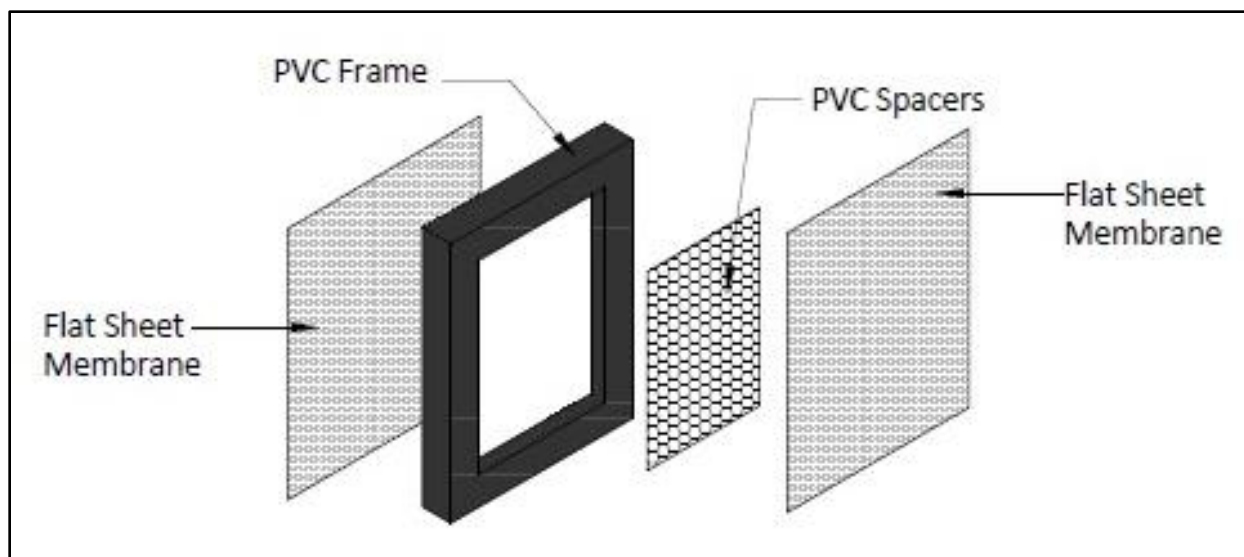
##### **3.4.2.1. Description of apparatus**

The final membrane fermenter apparatus consisted of a 1.5 L glass vessel into which a framed 75 mm × 100 mm engineering PVC, membrane was inserted. A 20 cm length air pipe was coiled around the bottom of the membrane to provide a means of aeration and of scouring in order to prevent fouling of the membrane surface. The membrane was fitted with an outlet at the bottom to allow for permeate collection. Silicone tubing of 5 mm outer diameter was used for the withdrawal of permeate as well as biomass – containing – broth removal. A 60 mL syringe

was used to withdraw permeate from the membrane within the vessel, while a 20 mL syringe was used for broth withdrawal. The air supply tubing was fitted with a 20 micron sterile filter. A similar fermenter vessel for batch operation was prepared with tubing for sample withdrawal, and aeration with a 20 micron sterile filter attached.

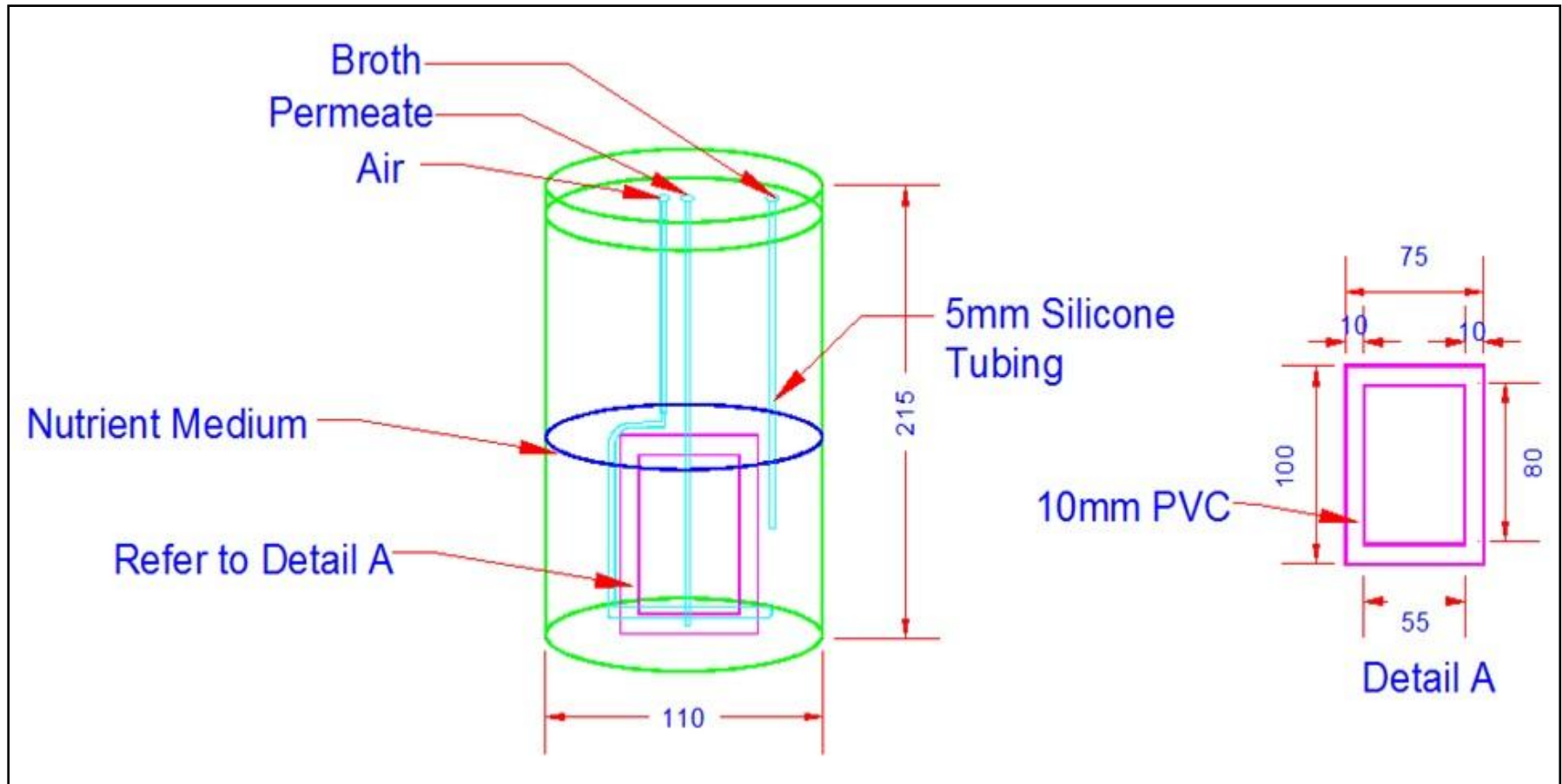
#### **3.4.2.2. Fabrication of apparatus**

PVC sheets (Maizey Plastics) of 10 mm thickness were cut to ensure the exterior dimensions were 75 mm × 100 mm. To allow for a frame of 10 mm sides, the inside was cut, leaving the working dimension of the membrane at 55 mm × 80 mm. The PVC was sterilized using ethanol, as autoclaving distorted the shape due to the high temperature and pressure. 5 mm connection nipples were attached to the bottom of the PVC for the attachment of the silicone tubing as well as onto the lid of the vessel. The woven fabric membrane was supplied by the Department of Chemical Engineering at the Durban University of Technology. Two sheets, 75 mm by 100 mm were cut and sterilized before being glued to the PVC. Plastic grids or mesh, served as spacers, and were inserted between the fabrics prior to being glued. The membrane was then sealed to prevent leakage through the sides of the PVC – fabric contact area. The air pipe was sterilized and wound around the bottom of the membrane. The pipe was connected to autoclaved silicone tubing at the lid of the vessel and then attached to the 20 micron sterile filter. The silicone tubing was also attached to the membrane on one end and to the lid of the vessel on the other. Tubing was attached to nipples on the outside of the vessel lid, which was then fitted to the syringes.



**Figure 3-3: Membrane module fabrication**





**Figure 3-4: Schematic of membrane fermenter system**

### **3.4.2.3. Membrane fermenter optimization**

#### **3.4.2.3.1. Troubleshooting and modification of procedure**

Numerous challenges were faced and addressed during the initial trial phase of the experimentation. Three experiments were conducted concurrently to determine the effect of semi – continuous membrane fermentation on enzyme activity, micro – organism growth and productivity. The experiments were conducted in three fermenter vessels operation in different mode viz,

- Batch mode
- Semi – continuous mode with beechwood xylan nutrient replenishment
- Semi – continuous mode with de – ionized water replenishment

The experimental procedure was repeated and refined until suitable results could be obtained. The hindrances included:

- Melting of the membrane module
- Permeation of spores on day 1
- Leaks in the membrane module and vessel lid
- Inconsistent sampling
- Movement of the membrane module

#### **a) Melting of the membrane module**

The membrane module could not be inserted into the fermentation vessels prior to autoclaving as the high temperature caused the glue holding the membrane to the PVC to melt and the woven fabric to come apart. The Q – bond used to attach the nipples to the lid of the vessels also could not endure the temperature (121 °C) of the autoclave. Upon cooling, the fermenter vessels were inoculated with a 10 mL spore solution. The inoculated vessels were closed and incubated at 50 °C in a shaking incubator at a speed of 150 rpm for 2 days, following which the membrane module was inserted.

#### **b) Permeation of spores on day one**

It was found that inserting the membrane into the vessel following inoculation, resulted in immediate filtration causing the module to fill up. Thus the sample taken after 24 hours was jeopardized and spores were still small enough to pass through the membrane, which was undesirable for the experiment. Initial trials proved that 48 hours was adequate for germination of the fungal spores and enzyme production to occur. Therefore after 48 hours, the membrane module with the air – pipe coiled around its base was inserted into the two vessels marked for semi – continuous operation. The vessels were supplied with air manually several times a day due to difficulties connecting an air – pump to the system which was shaking in a closed incubator.

#### **c) Leaks in the membrane module and vessel lid**

It was found that the membrane fabric detached from the PVC while in the incubator. The edges on subsequent modules were reinforced to a greater degree with the Megabond glue to ensure there was no possibility of seepage through leaks on the sides. Due to the rotation of the vessel in the incubator, the broth swirled vigorously in the vessel. The lids of the vessels, though closed, had minor seepages which contaminated the incubator. The lids were adequately sealed with high quality insulation tape for future experiments.

#### **d) Inconsistent sampling**

For beechwood xylan nutrient medium replenishment, 100 mL of permeate was withdrawn from the vessel and replenished with 100 mL of fresh nutrient medium for the first 4 days. From day 5 to 9 permeate removal was only 40 mL per day, and was replenished with nutrient medium of the same volume. For de – ionized water replenishment, 100 mL of permeate was withdrawn from the vessel and replenished with 100 mL of de – ionized water for the first 4 days. From day 5 to 9 permeate removal was 60 mL per day, and was replenished with de – ionized water of the same volume. The reduced permeate volume was due to a number of factors including fouling, kinks in permeate tubing and irregular sampling pressure. The air – pipes were introduced in subsequent experiments and coiled around the base of the membrane module. Air was supplied as a means to scour the membrane as well

as to provide the micro – organism with oxygen. The nipples attaching the permeate withdrawal line to the membrane module as initially attached to the bottom of the module. It was observed that the tubing kinked over time, resulting in stagnant flow of permeate while sampling. The module was modified so that a T – piece nipple was attached to the side of the base, allowing for better flow of permeate. Sampling was initially irregular and poorly controlled. The syringes were locked in an “open” position when sampling to allow for simpler, less strenuous sampling until the required volume of permeate was removed. These three modifications aided the increase in permeate withdrawal in subsequent experiments.

#### **e) Movement of the membrane module**

The membrane module was initially inserted into the fermenter vessel without any supports to keep it in place. The membrane bobbed freely and appeared to be not fully submerged. An L – bracket was attached to the top of the membrane module and held onto the lid of the vessel to keep the module upright and lowered into the broth as it should be.

15 mL of broth were sampled from all three fermenter vessels. The sampled broth from each flask was centrifuged at 4000 rpm for 15 minutes. The supernatants were used to perform the xylanase assay.

#### **3.4.2.3.2. Effect of nutrient manipulation on activity and productivity**

After successful trials were conducted over a 9 day period, it was decided that further trials be conducted over a longer period of time. This was to gauge the ability of the micro – organism to produce xylanase over a significant time frame and maintain its biomass. The protein content and pH of the system would also be measured at this stage. For this set of experiments the three modes of operation were:

- Batch mode
- Semi – continuous mode with beechwood xylan nutrient replenishment
- Semi – continuous mode with alternating beechwood xylan and de – ionized water replenishment

For all three modes of experiments, 1 L of nutrient medium consisting of beechwood xylan (1 % w/v), yeast extract (1 % w/v) and potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (0.33 % w/v) was prepared in 1.5 L fermenter vessels. The pH was adjusted to 6.5 with a 1 M solution of sodium hydroxide and the vessels were autoclaved at 121 °C for 20 minutes. Upon cooling, the fermenter vessels were inoculated with a 10 mL spore solution. The inoculated vessels were sealed and incubated at 50 °C in a shaking incubator at a rotational speed of 150 rpm. After 48 hours, the membrane and air – pipe were inserted into the vessels marked for membrane filtration. 10 mL broth samples were withdrawn daily from the all three fermenter vessels. The samples were centrifuged at 4000 rpm for 30 minutes. The pellets were dried in an oven at 80 °C for dry weight analysis, while the supernatants were stored at 4 °C until required for analysis. Permeate was withdrawn daily from the membrane fermenter vessels. Air was supplied during sampling to scour the membrane and provide oxygen to the micro – organism. In one vessel, the volume of permeate removed was replenished with an equal volume of beechwood xylan medium of same concentrations as the initial medium. In the second vessel, the volume of permeate removed was replenished with either an equal volume of de – ionized water or beechwood xylan nutrient medium, alternating after every two days. This exercise sought to investigate the effect of “starving” the micro – organism for two days in an effort to trigger it to produce more xylanase. Medium for replenishment were brought up to 50 °C prior to being introduced to the vessels to prevent a delay in acclimatization. Permeate for both experiments were stored at 4 °C until required for analysis.

#### **3.4.2.3.3. Optimization of nutrient medium dilution rate**

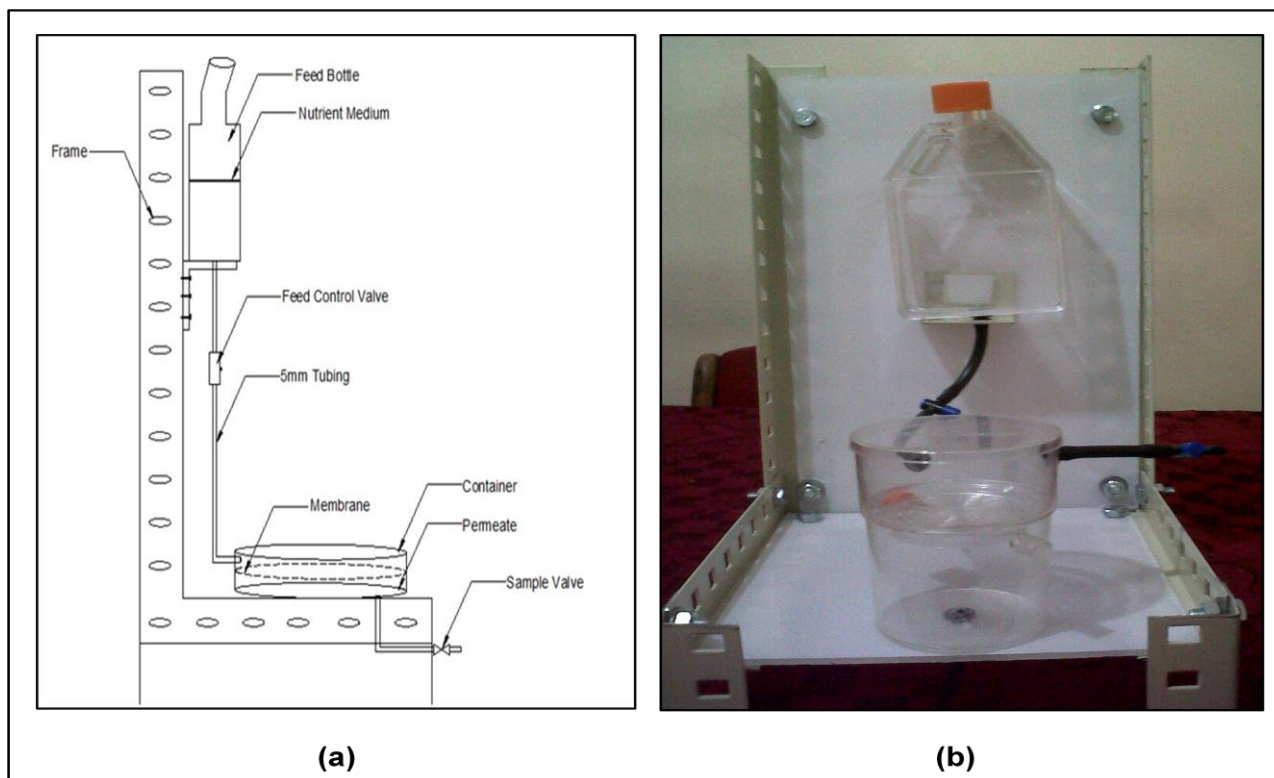
Dilution rate is the ratio between the volume of nutrient medium added to the vessel and the final volume in the vessel. The effect of dilution rate on the productivity of the system was determined by varying the volume of permeate removed. This experiment was conducted using only beechwood xylan which was prepared in similar fashion as in section 2.5.2.3.3 above in three fermenter vessels. Following inoculation and membrane module insertion after 48 hours, permeate was removed in volumes of 40, 80 and 120 mL daily from the three membrane fermenters and replenished with equal volumes of beechwood xylan nutrient medium. Permeate was stored at 4° C until required for xylanase and protein analysis.

#### **3.4.4. Prior concepts for the membrane fermenter system**

Two concepts for membrane filtration were initially developed for fungal production of xylanase. The first was a film fermenter borne from the knowledge that fungal organisms develop as biofilms in nature, on trees etc. The second concept was a tank fermenter which was aimed at serving as a scaled up version of the flask experiments performed. The development and short – comings of these concepts will be described here.

##### **3.4.4.1. Film fermenter**

The feed vessel and membrane vessels were mounted on a steel frame which was fabricated by cutting two L pieces to the required dimensions and bolting them into place using a Perspex sheet as the base. The woven fabric membrane was attached to a lip created on the inside of a 250 mL filter vessel. The feed vessel was mounted above the membrane vessel and connected via 5 mm plastic tubing and the feed was controlled by a feed valve before the membrane container. Permeate was collected via a sample valve below the membrane vessel. *Thermomyces* spores were inoculated onto the surface of the membrane and allowed time to become immobilized. Beechwood xylan medium was prepared as in the previous section and allowed to drip onto the membrane surface. The apparatus was placed in a 50 °C incubator and left rotating at very low rotational speeds. The experiment aimed at using gravity as the means for permeate to flow through the membrane. Unfortunately due to the small surface area of the membrane, this force was counted insufficient and very little to no permeate was collected. The xylanase activity measured was also low and this concept was disregarded for further experimentation.



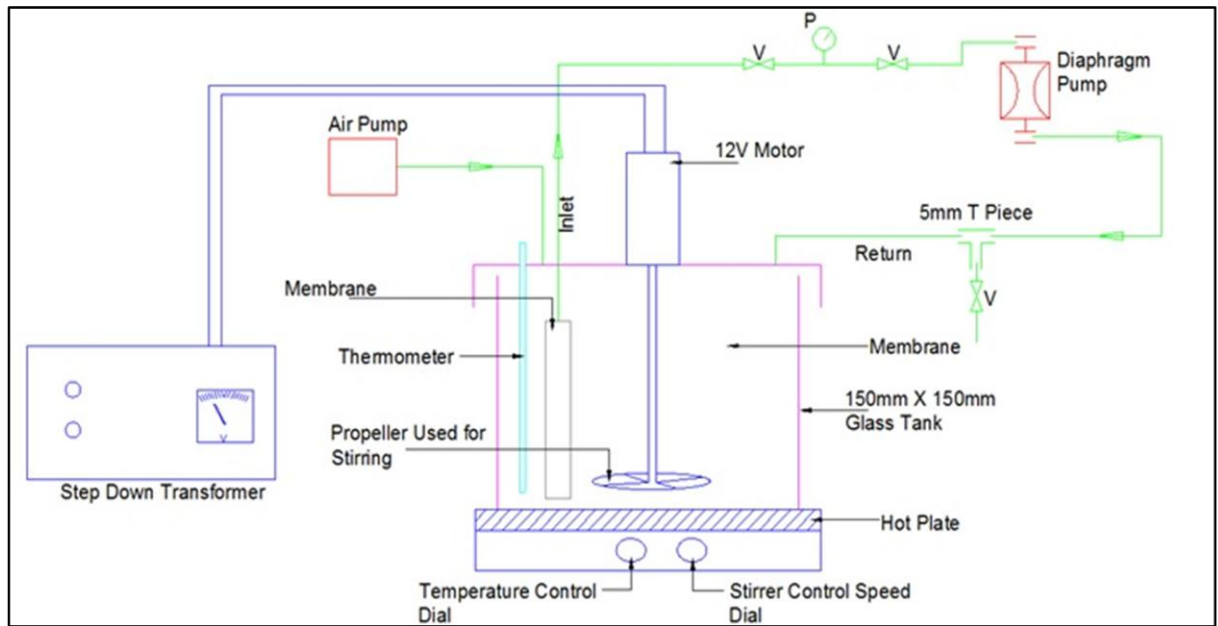
**Figure 3-5: Film fermenter (a) Diagrammatic representation and (b) Fabricated concept**

#### 3.4.4.2. Tank fermenter

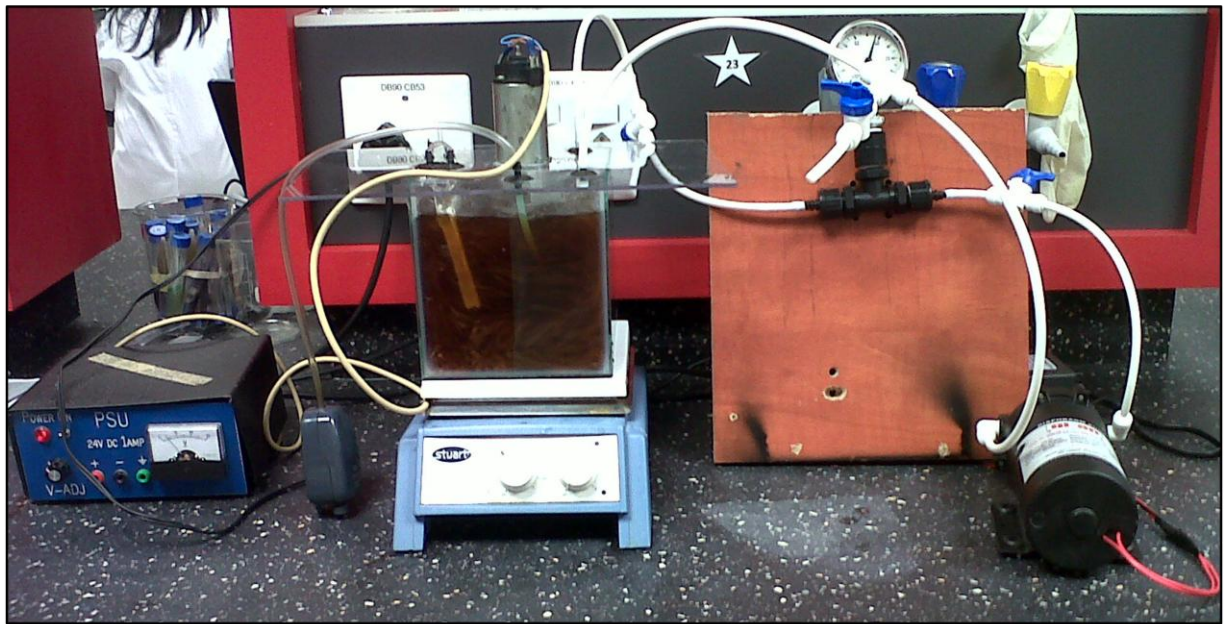
The tank fermenter aimed at solving the lack of suction pressure as was the disadvantage of the film fermenter concept above. A peristaltic pump was connected to the membrane module which was inserted into a glass tank (150 mm × 150 mm × 150 mm). The tank was placed on a magnetic stirrer hotplate to achieve the required production temperature of 50 °C. 3 L of nutrient medium consisting of coarse corn cobs (1.5 % w/v), yeast extract (1.5 % w/v) and potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (0.5 % w/v) was prepared, the pH of which was adjusted to 6.5 prior to autoclaving at 121 °C for 20 minutes. A 1 % inoculum was introduced to the tank and a magnetic stirrer served to agitate the nutrient medium. A thermometer was used to measure the temperature. The first obstacle arose when the magnetic stirrer bar became ineffective as it often became lodged in the corn cobs rendering it immobile and causing the corn cobs to settle. Coarse corn cobs were used instead of beechwood xylan due to the large working volume of the tank and the cost implications thereof. A propeller mixer was then built and fitted into the tank to keep the corn cobs suspended. A stepper motor was mounted to the perspex lid and the metal shaft was lowered

into the tank. A 4 mm hole was drilled on one end of the Perspex rod and the metal blade was fixed to it using a 5mm self – tapping screw to form a propeller. The propeller was then fixed to the stepper motor shaft using the 8 mm PVC tubing. A bracket was constructed using the 3 mm metal sheet and mounted to the lid to form a guide to ensure the propeller spun straight. The step down transformer was then connected to the stepper motor and the voltage was set at 3 volts. This was done to ensure the motor did not spin too fast creating a vortex in the tank. The membrane was mounted to the Perspex lid using an L bracket to ensure it is always kept away from the propeller to avoid damage to the membrane. Little or no xylanase activity was recorded using this concept. *Thermomyces lanuginosus* growth was retarded by the propeller blades which may have caused excessive shearing of the mycelia. Poor temperature control and a non – sterile environment may have also contributed greatly to the poor growth of the micro – organism. It was at this stage that a simple system that could be maintained inside a shaking incubator which offered a controlled temperature, rotational speed and sterility was decided upon, resulting in the membrane fermenter system fabricated for the remainder of the research.





(a)



(b)

**Figure 3-6: Tank fermenter (a) Diagrammatic representation and (b) Fabricated concept**

### **3.5. Analytical methods**

In this section, the various analytical techniques used are outlined

#### **3.5.1. Determination of xylanase activity**

##### **3.5.1.1. Equipment and materials**

- Test tubes
- Test tube racks
- Micro – pipettes (25  $\mu$ L – 5 mL)
- Water bath (Julabo 9000, LABOTEC)
- Vortex (Vortex Geni2, Scientific Industries, LASEC SA)
- Beechwood xylan
- Sodium hydroxide
- Citric acid (99.5 %, Merck)
- Stirrer hotplate
- Stirrer bar
- Micro – centrifuge tubes
- Cuvettes
- Spectrophotometer (Libra S21, Biochrom)
- 3,5 – dinitrosalicylic acid (98 %, Sigma – Aldrich)
- Sodium potassium tartrate (99 – 101 %, Merck)
- De – ionized water
- Xylose standard (D(+)) – Xylose for biochemistry, purity HPLC > 99.0 %)
- Bradford reagent (Bio – Rad protein assay)
- Bovine serum albumin (Lyophilized powder,  $\geq$  96 % (agarose gel electrophoresis), Sigma – Aldrich)

##### **3.5.1.2. Methodology**

Xylanase activity was assayed according to the method of Bailey *et al*, (1992) using a 1 % (w/v) substrate solution of Beechwood Xylan. 100  $\mu$ L of appropriately diluted enzyme sample was

added to 900  $\mu$ L of substrate solution and incubated at 50 °C for 5 minutes. The DNS method was used to estimate the concentration of reducing sugars released by the enzyme. 1.5 mL of DNS was added to the reaction mixture and boiled for 15 minutes. Upon cooling, the absorbance of the sample was read at a wavelength of 540 nm. The spectrophotometer was zeroed using a reagent blank. The absorbance of enzyme blanks was subtracted from that of the sample and the xylanase activity was calculated based on a xylose standard curve. One international unit of xylanase corresponds to the amount of enzyme required to release 1 micromole of reducing sugar (xylose) in 1 minute, according to the International Union of Biochemistry.

#### **3.5.1.2.1. Substrate solution preparation**

The beechwood substrate solution was prepared by dissolving beechwood xylan (1 % w/v) in a reduced volume of sodium citrate buffer of pH 6.5. The mixture was heated while stirring until boiling was achieved. The heat was turned off while the mixture was allowed to stir overnight. The mixture was brought up to volume the following day with sodium citrate buffer. Substrate solution was stored at 4 °C until used and was consumed within 7 days of preparation.

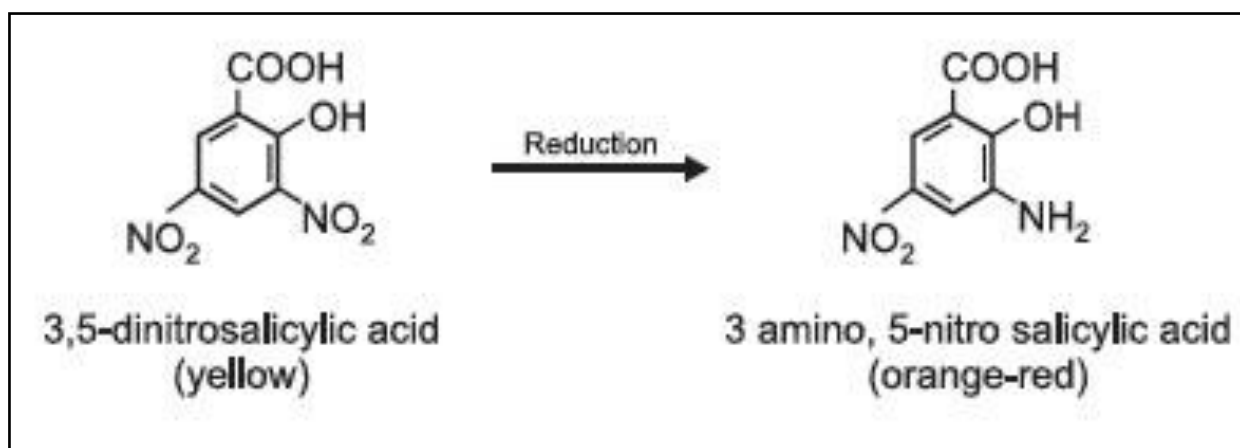
#### **3.5.1.2.2. Sodium citrate buffer preparation**

Sodium citrate buffer was prepared by dissolving 10.3 g of citric acid in 700 mL of de – ionized water. The pH was adjusted to 6.5 by the addition of 1 M sodium hydroxide. The volume was brought up to 1 L with de – ionized water and the pH re – adjusted if necessary. The buffer was then autoclaved at 121 °C for 20 minutes to prevent microbial growth.

#### **3.5.1.2.3. DNS reagent preparation**

A reducing sugar is one that has an aldehyde group or is capable of forming one through isomerism. The aldehyde group allows it to act as a reducing agent. When the reducing sugar reacts with the DNS it converts the DNS to 3 – amino – 5 – nitrosalicylic acid as in Figure 20 below, which is the reduced form of DNS. The formation of 3 – amino – 5 – nitrosalicylic acid results in a change in the amount of light absorbed, at wavelength 540 nm. The absorbance

measured using a spectrophotometer is directly proportional to the amount of reducing sugar. Xylose, with its free carbonyl group, is a reducing sugar. DNS reagent was prepared by dissolving 10 g of 3,5 – dinitrosalicylic acid in 600 mL of de – ionized water and reacting with 15 g NaOH. 300 g of Potassium sodium tartrate was added incrementally while heated and stirring continuously. The solution was brought up to a volume of 1 L thereafter.



**Figure 3-7: Reduction of 3,5 – dinitrosalicylic acid (Goel, 2007)**

#### **3.5.1.2.4. Reagent blank preparation**

This was prepared by incubating 900  $\mu$ L substrate solution at 50 °C for 5 minutes, after which 1.5 mL DNS was added, followed by 100  $\mu$ L of sodium citrate buffer. The mixture was boiled for 15 minutes and cooled to room temperature in a cold water bath. This was used to zero the spectrophotometer.

#### **3.5.1.2.5. Enzyme blank preparation**

The enzyme blanks were prepared to eliminate the effect of any reducing sugars that may have been present in the sample prior to the reaction. For this, 900  $\mu$ L of substrate solution was incubated at 50 °C. 1.5 mL of DNS was then added, followed by 100  $\mu$ L of the enzyme sample. The mixture was boiled for 15 minutes, cooled to room temperature in a cold water bath and the absorbance read at 540 nm.

#### **3.5.1.2.6. Standard curve preparation**

A 10  $\mu\text{mol.mL}^{-1}$  xylose standard stock solution was prepared by dissolving 150 mg of xylose in 100 mL of sodium citrate buffer. Standards ranging from 0 to 10  $\mu\text{mol.mL}^{-1}$  in increments of 2  $\mu\text{mol.mL}^{-1}$  were prepared by appropriate dilution of the stock solution. In separate test tubes, 900  $\mu\text{L}$  of the enzyme sample was incubated at 50 °C, after which 1.5 mL of DNS and 100  $\mu\text{L}$  of the standards were added. The test tubes were then placed in a 100 °C water bath for 15 minutes. Upon cooling in a cold water bath, the absorbance of each standard was read at 540 nm. The standard curve was generated by plotting the absorbance against the concentration of the standard.

### **3.5.2. Total protein analysis**

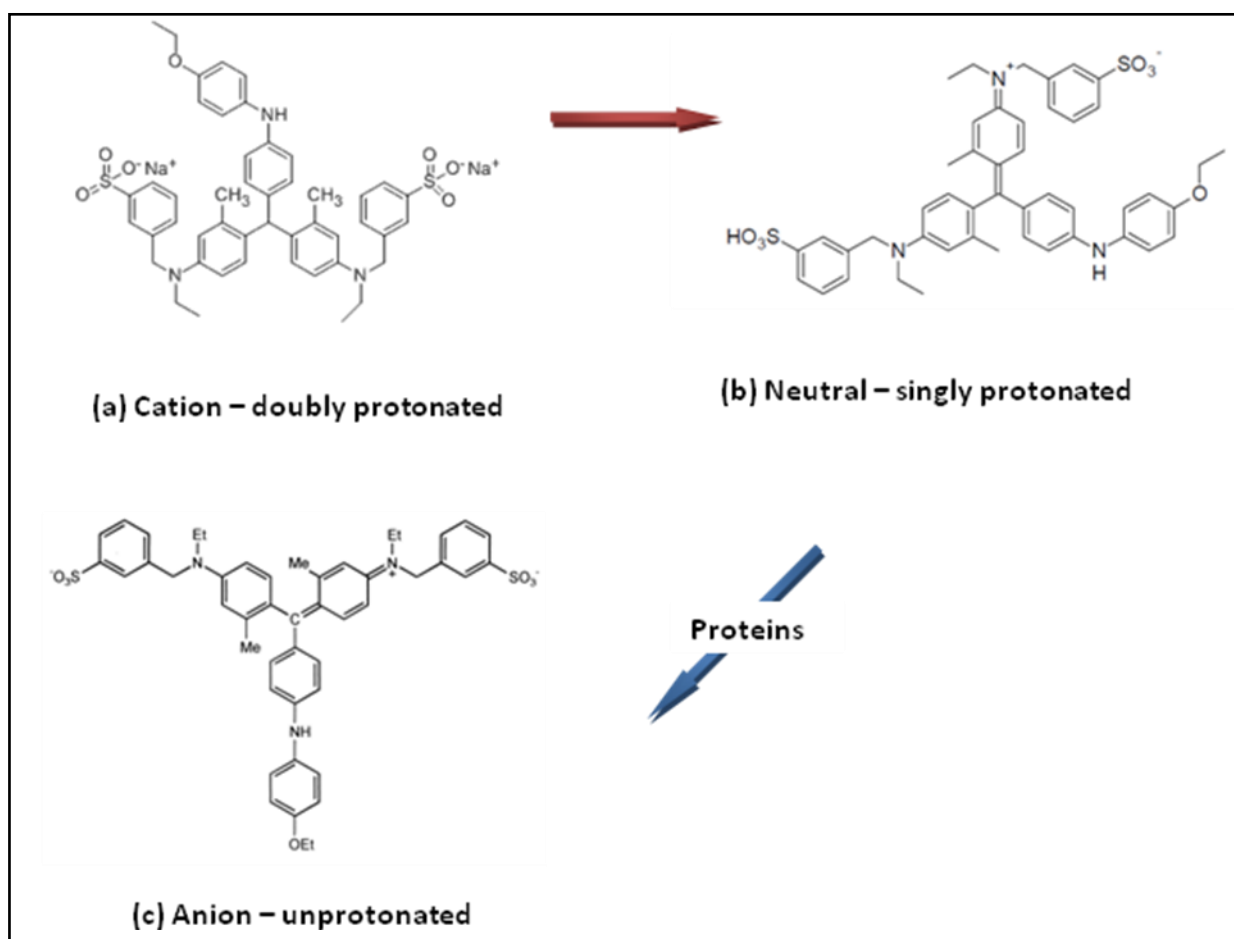
#### **3.5.2.1. Equipment and materials**

- Test tubes
- Test tube racks
- Micro – pipettes (25  $\mu\text{L}$  – 5 mL)
- Vortex (Vortex Geni2, Scientific Industries, LASEC SA)
- Micro – centrifuge tubes
- Cuvettes
- Spectrophotometer (Libra S21, Biochrom)
- De – ionized water
- Xylose standard (D(+)-Xylose for biochemistry, purity HPLC > 99.0 %)
- Bradford reagent (Bio – Rad protein assay)
- Bovine serum albumin (Lyophilized powder,  $\geq 96$  % (agarose gel electrophoresis), Sigma – Aldrich)

#### **3.5.2.2. Methodology**

The total protein was analyzed in order to determine the effectiveness of the membrane in retaining larger protein molecules. This analysis was performed as per the Bradford Protein Assay (Bradford, 1976). Bradford used the binding of dyes to proteins to estimate the

concentration of protein. It is the most widely used method due to its simplicity, high sensitivity towards most proteins and rapid colour development. The Bradford reagent (Biorad Protein Assay Solution) uses Coomassie dye (brilliant blue G – 250) which binds to protein molecules at acidic pH, producing a colour change from red – brown to blue measured at 595 nm (Walker, 1996) as illustrated in Figure 3-8 below. For this assay 30 µL of enzyme sample were incubated at room temperature with 5 mL of Bradford reagent for 5 minutes. The absorbance was then read at 595 nm and the total protein was calculated based on the protein standard curve prepared.



**Figure 3-8: Coomassie dye changes from a red cationic form (a) (Congdon et al., 1993) to a blue anionic form (c) (Kilkowski and Gross, 1999) in the presence of proteins. The intermediate neutral form (b) is green (Katrahalli et al., 2010)**

#### **3.5.2.2.1. Preparation of the standard curve**

The ideal protein to be used in any protein assay is a purified preparation of the protein being assayed. In the absence of such an absolute reference protein, another protein must be selected as a relative standard. One of the most common protein standards used for protein assays is Bovine Serum Albumin (BSA). A concentration series of the standard protein solution (BSA) was prepared in the range 0.148 to 1.332  $\mu\text{g} \cdot \text{mL}^{-1}$ . A calibration curve was generated by plotting the absorbance of the standards at 595 nm against the concentration of the standards.

### **3.5.3. Dry weight analysis**

#### **3.5.3.1. Equipment and materials**

- Micro – pipettes (25  $\mu\text{L}$  – 5 mL)
- Micro – centrifuge tubes
- Centrifuge (Eppendorf, 581R)
- Oven (Scientific, Series 9000)
- Analytical balance (RADWAG, AS22/C/2)

#### **3.5.3.2. Methodology**

The biomass was determined by performing dry weight analysis. 5 mL samples of the broth were withdrawn from the membrane fermenter. Following centrifugation at 4000 rpm for 30 minutes, the supernatant was removed and stored for further analysis at 4 °C. The pellet was dried in an 80 °C oven until all liquid was evaporated. The difference between the mass of an empty centrifuge vial and that containing the pellet gave the dry weight of the sample.

## **4. RESULTS AND DISCUSION**

---

The results and relevant discussion are presented in a sequence that corresponds with the development of the final membrane fermentation system i.e.

- a) Experiments conducted in shake flasks to determine operating feed medium and conditions.
- b) Experiments to characterize the enzyme.
- c) Experiments conducted in the coupled membrane fermenter system.

### **4.1. Experiments conducted in shake flasks**

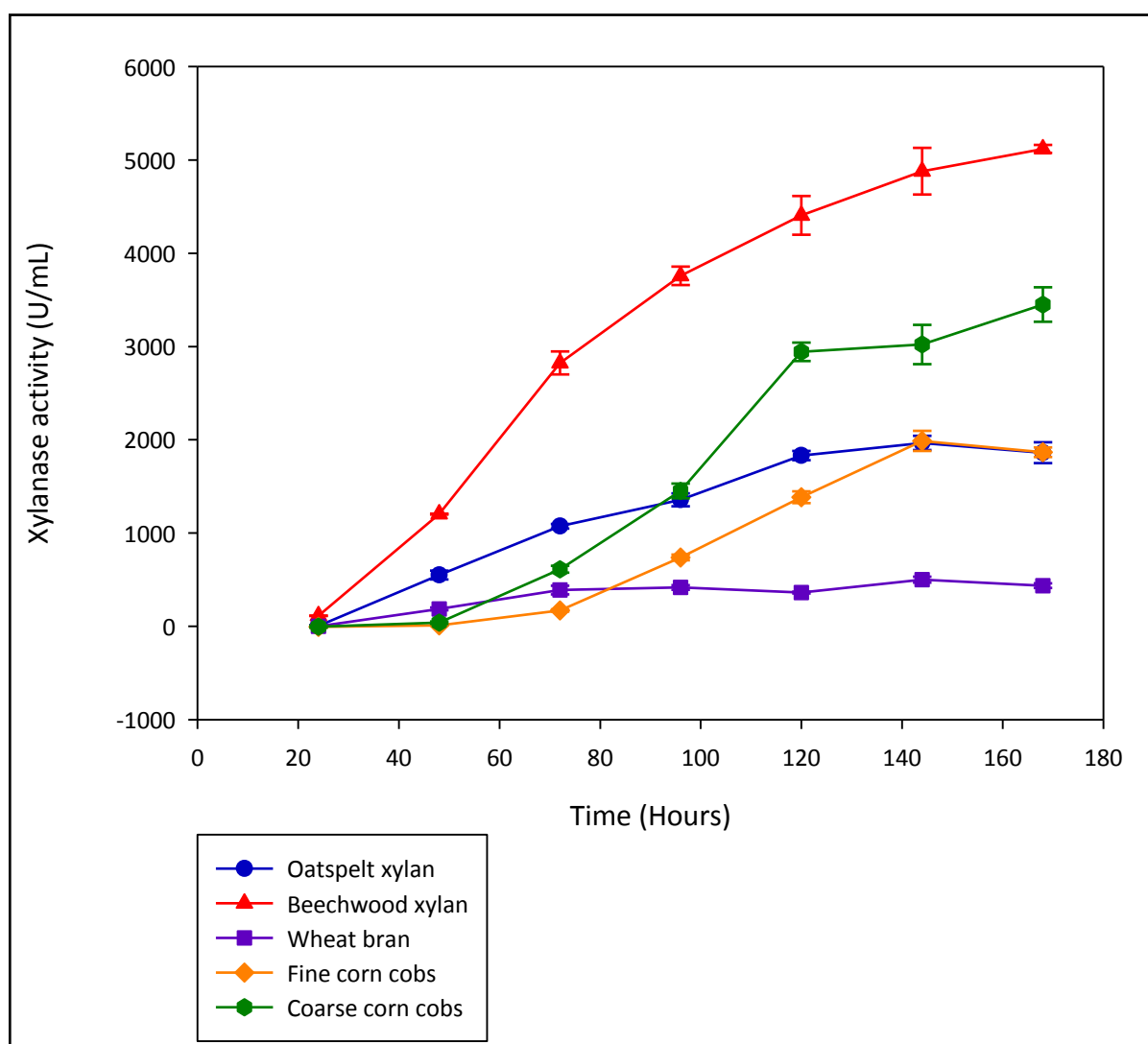
It is essential to design a medium which is economical on an industrial scale and which is capable of giving high yields of the desired product. The media should contain all the essential constituents required for the growth of the micro – organism. It is always preferable to have these constituents in the same proportion as in the cell. For this reason the nutritional sources which gave the best xylanase production were analyzed. The ideal physical and environmental conditions for xylanase production were also determined. The experiments conducted in shake flasks included the optimization of the carbon source, nitrogen source, initial pH, size of the inoculum, production temperature and rotation speed. Fermentation in shake flasks were thereafter conducted in fed – batch and semi – continuous mode to determine the effects of these modes of operation on xylanase production.

#### **4.1.1. Effect of various carbon sources on xylanase activity**

Each species of living cells has a genetically determined set of certain functioning enzymes called constitutive enzymes. Many cells possess genes for the synthesis of enzymes that do not ordinarily appear. These genes are expressed when an inducing substrate or molecules derived from the enzyme system enters the cell. The cell does not synthesize all its potential enzymes but only those which are necessary (Machve, 2009). The choice of an appropriate substrate is of great importance for the successful production of xylanase. The substrate not only serves as the carbon source but also provides the necessary inducing compounds for the organism, preferentially for an extended period of time which can then result in an increased



overall productivity of the fermentation process. The most economically important and most widely used carbon sources are the carbohydrates. They are commonly found and most are economically priced. The five carbohydrates selected for this experiment were fine and coarse corn cobs which is a waste product, wheat bran which is economical and readily available, i.e., not seasonal, and two commercial purified xylan sources.



**Figure 4-1: Effect of various carbon sources on xylanase activity**

Figure 4-1 indicates that beechwood xylan induced a maximum xylanase activity of  $5118.60 \pm 42.76 \text{ U.mL}^{-1}$  followed by coarse corn cobs, fine corn cobs, oatspelt xylan and wheat bran each inducing activity of  $3451.16 \pm 184.18 \text{ U.mL}^{-1}$ ,  $1866.28 \pm 50.98 \text{ U.mL}^{-1}$ ,  $1860.47 \pm 111.82 \text{ U.mL}^{-1}$  and  $436.05 \pm 24.67 \text{ U.mL}^{-1}$  respectively after 168 hours.

The trends observed, indicate that the commercial purified xylan sources triggered a rapid response since the xylan is more readily available for hydrolysis. It must at this juncture also be remembered that beechwood xylan was used as the substrate in the enzyme assay and the enzyme might be more specific to the substrate that the micro – organism was grown on. This could also explain why enzyme activity achieved using Oatspelt xylan was less than half of that achieved using the beechwood xylan.

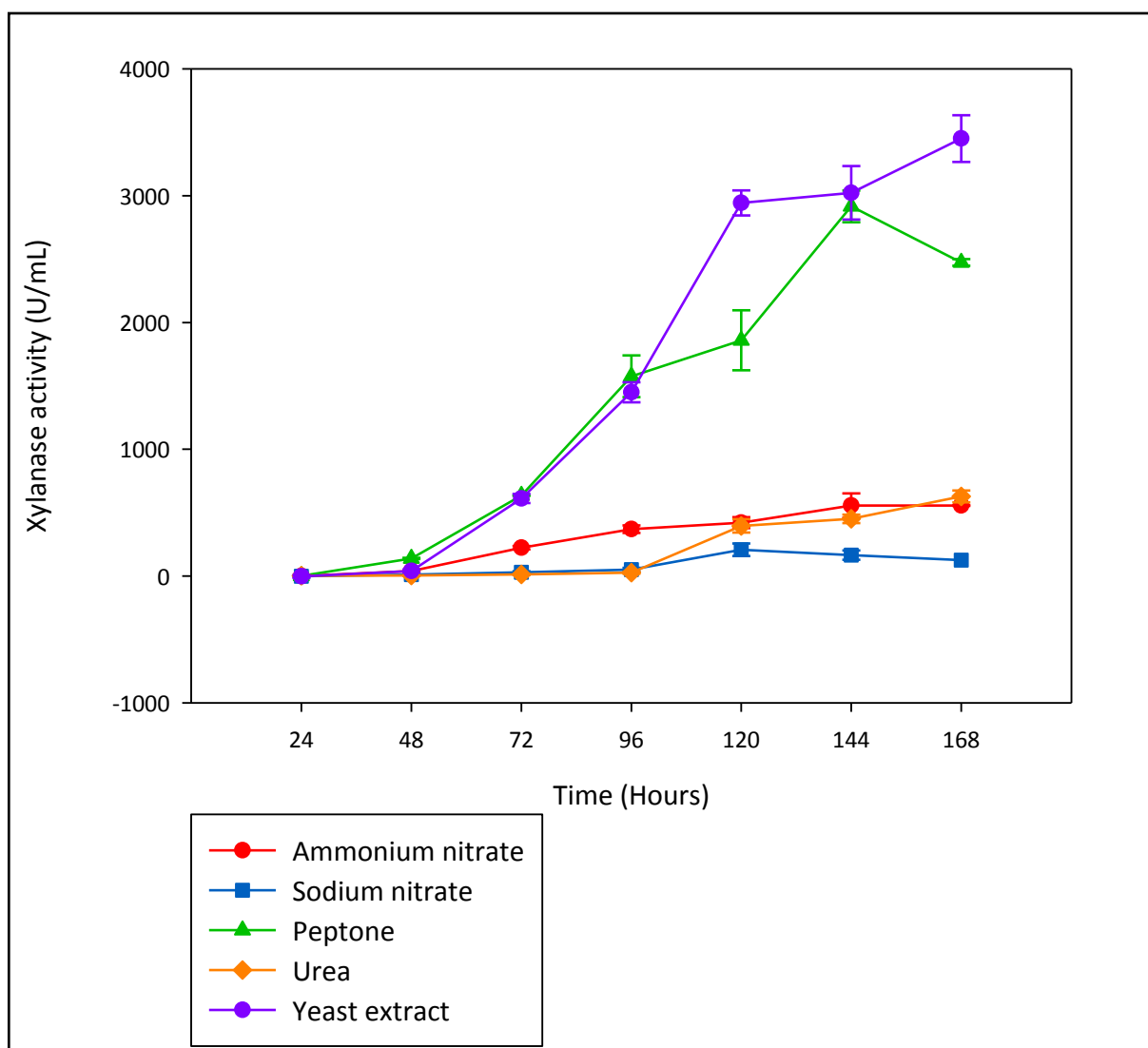
Though stagnant for the first 48 hours, the production of xylanase on the coarse corn cobs increased steadily after 72 hours. It can be seen that pre – treatment of the corn cobs has a negative on the xylanase activity achieved. Here a larger particle size is favoured over the finely milled cob with an almost 2 – fold decrease in activity when the latter was used. This suggests that a slower release of soluble sugars due to a reduced available surface area of the substrate can be beneficial for xylanase production (Haltrich et al., 1996). It has also been suggested that the coarse corn cobs provide a surface for the dispersed mycelial filaments to adhere to and thus creates a support system for fungal growth (Purkharthofer and Steiner, 1995). The coarse corn cob is therefore advantageous in many respects including its economical viability, high enzyme activities and that further pre – treatment is not required.

The size of the substrate particles affects the extent and the rate of microbial growth, air penetration and CO<sub>2</sub> removal. Small particles, with high surface – to – volume ratios are preferred because they present a relatively large surface area for microbial action. However particles that are found in shapes that pack tightly together such as flat flakes are undesirable because close packing reduces inter – particle voids that are essential for aeration (Robinson et al., 1999). This could provide an explanation to the lower xylanase activities achieved using wheat bran as a carbon source. Another observation was that the wheat bran adhered to sides of the flasks and the amount of carbon source available in the medium for fungal growth was reduced.

#### **4.1.2. Effect of various nitrogen sources on xylanase activity**

The assimilation of nitrogen into macromolecules is essential for growth in all biological systems. Living cells all contain simple compounds and complex macromolecules of which

nitrogen is a major element. Nitrogen is converted to ammonia and glutamate from both inorganic and organic sources, although ammonia is the preferred source. Good nitrogen sources yields relatively higher growth rates than poor nitrogen sources. The organism selects the best nitrogen sources by a mechanism called nitrogen catabolite repression. Nitrogen regulation is the mechanism designed to prevent or reduce the unnecessary divergence of the cell's synthetic capacity to the formation of enzymes and permeases for the utilization of compounds that are non – preferred sources of glutamate and glutamine when a preferred nitrogen source is available (Khajure et al., 2011). 5 different nitrogen sources were used to determine their effect on xylanase production.



**Figure 4-2: Effect of various nitrogen sources on xylanase activity**

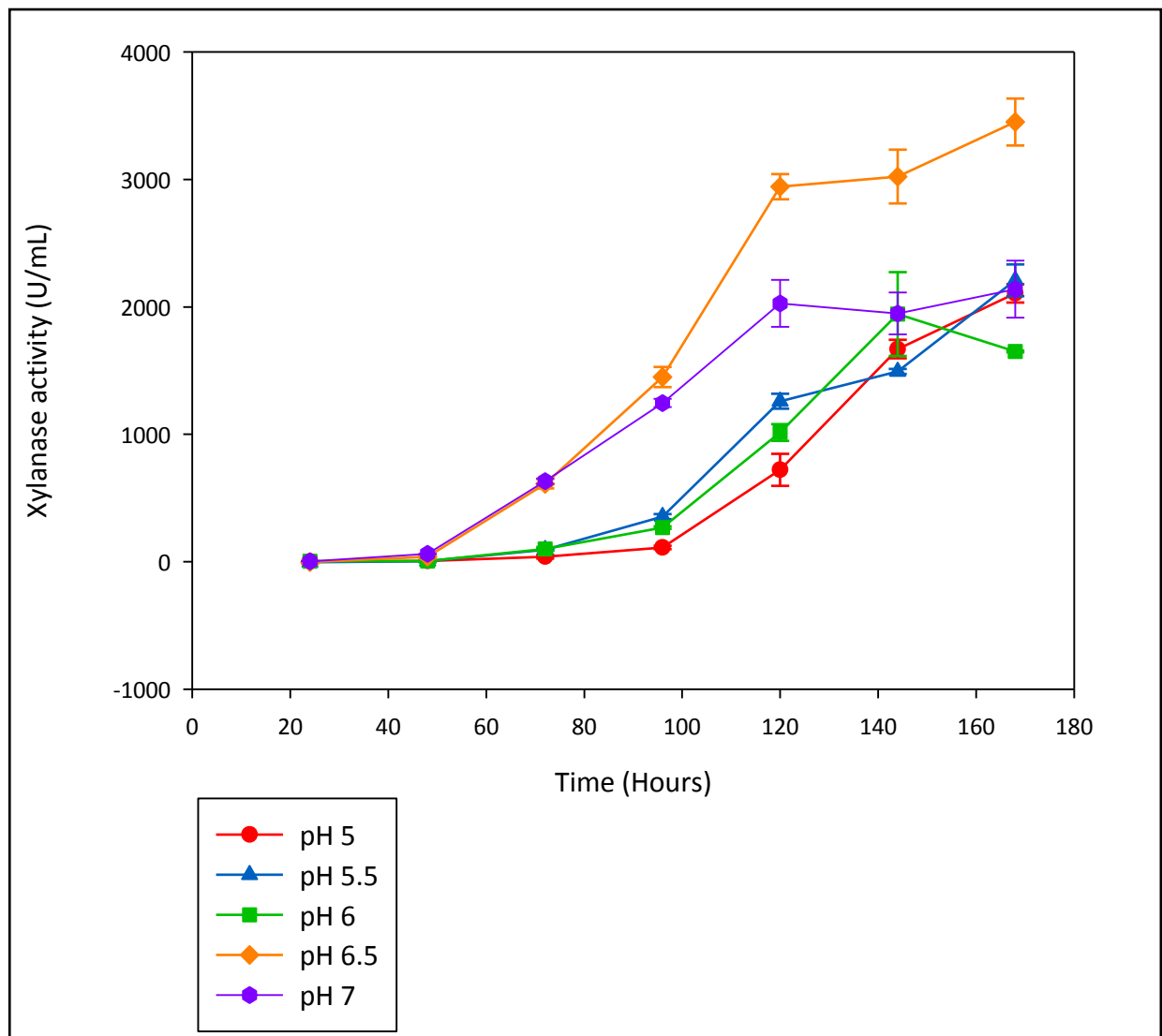
Figure 4-2 indicates that yeast extract produced a maximum xylanase activity of  $3451.16 \pm 184.17 \text{ U.mL}^{-1}$  followed by peptone, urea, ammonium nitrate and sodium nitrate each contributing to production of xylanase with activities of  $2474.42 \pm 26.31 \text{ U.mL}^{-1}$ ,  $627.91 \pm 46.04 \text{ U.mL}^{-1}$ ,  $555.81 \pm 3.1 \text{ U.mL}^{-1}$  and  $125.58 \text{ U.mL}^{-1}$  respectively after 168 hours.

The graph indicates that the inorganic nitrogen sources resulted in lower xylanase production compared to complex nitrogen sources. It has been reported that ammonium has a propensity to inhibit protein formation and fundamentally impedes numerous cellular functions. This phenomenon has been found to occur in a number of fungal strains, including *Aspergillus*, and *Fusarium spp* (Gaffney et al., 2009).

It has been suggested that organic nitrogen sources are better growth stimulators than inorganic nitrogen sources. The reason for this might be that the fungus hydrolyzes the organic nitrogen and in the process releases the mineral content of the organic nitrogen source as well as other growth factors that may be contained in them, which may be easily utilized during cellular metabolism thus promoting growth and enzyme production (Juwon and Emmanuel, 2012).

#### **4.1.3. Effect of varying the initial pH on xylanase activity**

The pH of the medium is a very important but often neglected environmental factor. It can affect growth by influencing the activity of enzymes in the membrane or cell wall. The pH affects solubility; proteins will coagulate and precipitate at their isoelectric point. In small scale experiments it is common to use NaOH for pH control (Todaro and Vogel, 2014). The pH range for the shake flask experiments were 5 – 7, increasing in increments of 0.5.



**Figure 4-3: Effect of varying the initial pH on xylanase activity**

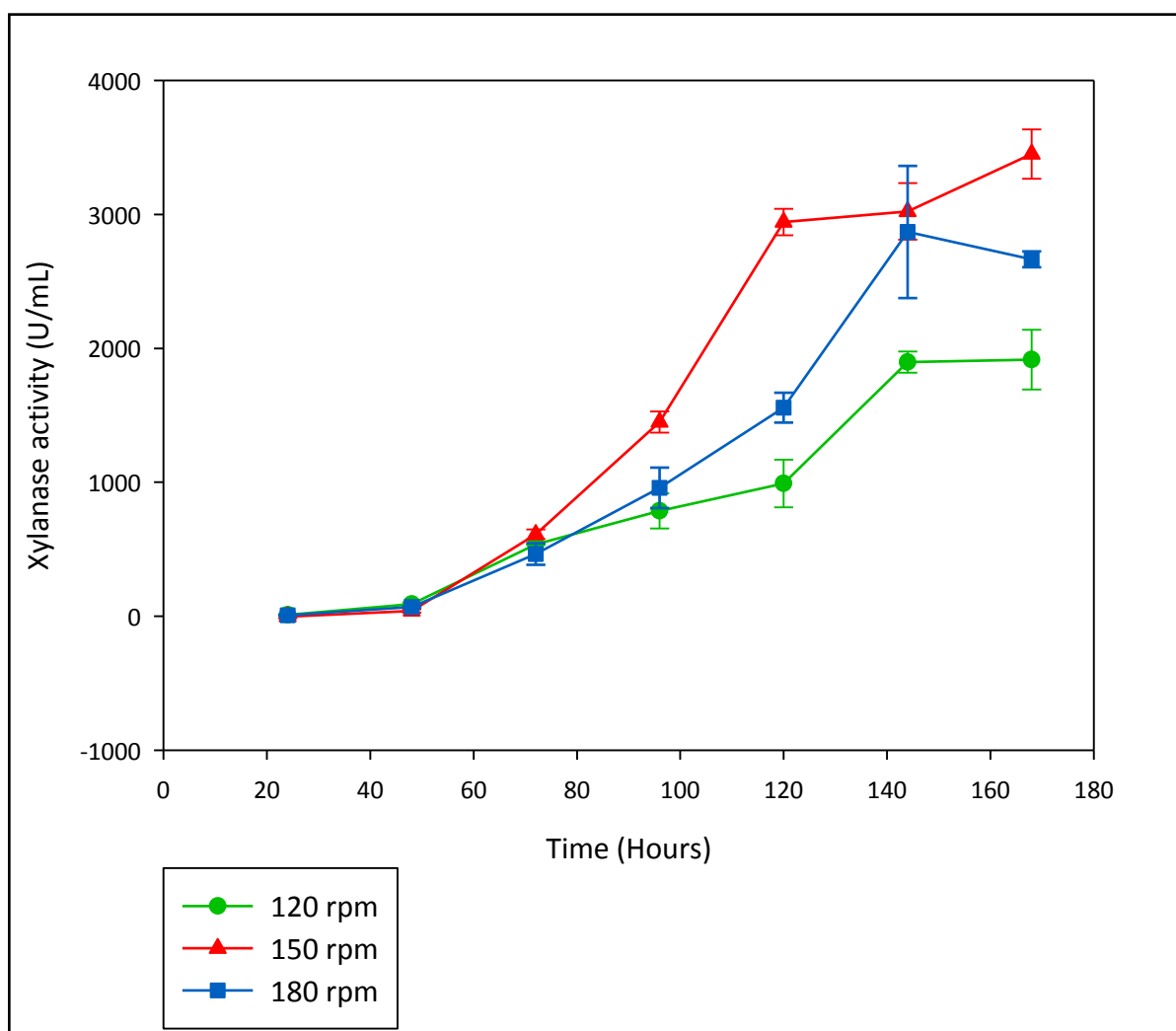
Figure 4-3 indicates that when the initial pH was adjusted to 6.5 a maximum xylanase activity of  $3451.16 \pm 184.17 \text{ U.mL}^{-1}$  was achieved after 7 days. An initial pH of 5, 5.5, 6 and 7 produced xylanase with activities of  $2106.98 \pm 72.361 \text{ U.mL}^{-1}$ ,  $2209.30 \pm 124.98 \text{ U.mL}^{-1}$ ,  $1651.16 \pm 6.50 \text{ U.mL}^{-1}$  and  $2139.53 \pm 223.64 \text{ U.mL}^{-1}$  respectively after 168 hours.

The plot above indicates that *Thermomyces lanuginosus* DSM 5826 is able to grow in the range 5 – 7 but maximal enzyme production occurs near neutral pH. This is consistent with the patented preparation of xylanase by Wizani et al. (1993) who stated the optimal initial pH range to be between 6.0 and 7.0 for *Thermomyces lanuginosus* DMS 5826. It has been stated

that most fungi prefer a neutral pH value but are tolerant of acidity (Dion et al., 2007) as is confirmed by the results achieved.

#### 4.1.4. Effect of varying the rotational speed on xylanase activity

Suitable mixing in a fermenter brings about the transfer of energy, nutrients, substrate and metabolite. Stirring is essential for the dispersion of air, homogenization to equalize the temperature and the concentration of nutrients throughout the fermenter, to suspend the micro – organism and nutrients and to disperse immiscible liquids (Pumphrey and Julien, 1996). However, continuous excessive agitation damages the surface hyphae (Robinson et al., 1999). Fermentation was conducted in a shaking incubator at 3 different rotational speeds to determine the optimal speed for xylanase production.



**Figure 4-4: Effect of varying the rotational speed on xylanase activity**

The rotational speed was limited to 3 variations due to laboratory constraints. Figure 4-4 shows an optimal xylanase activity of  $3451.16 \pm 184.17 \text{ U.mL}^{-1}$  achieved at a rotational speed of 150 rpm. Fermentation allowed to proceed at rotational speeds of 120 and 180 rpm achieved xylanase activities of  $1916.28 \pm 223.64 \text{ U.mL}^{-1}$  and  $2665.12 \pm 59.20 \text{ U.mL}^{-1}$  respectively after 168 hours.

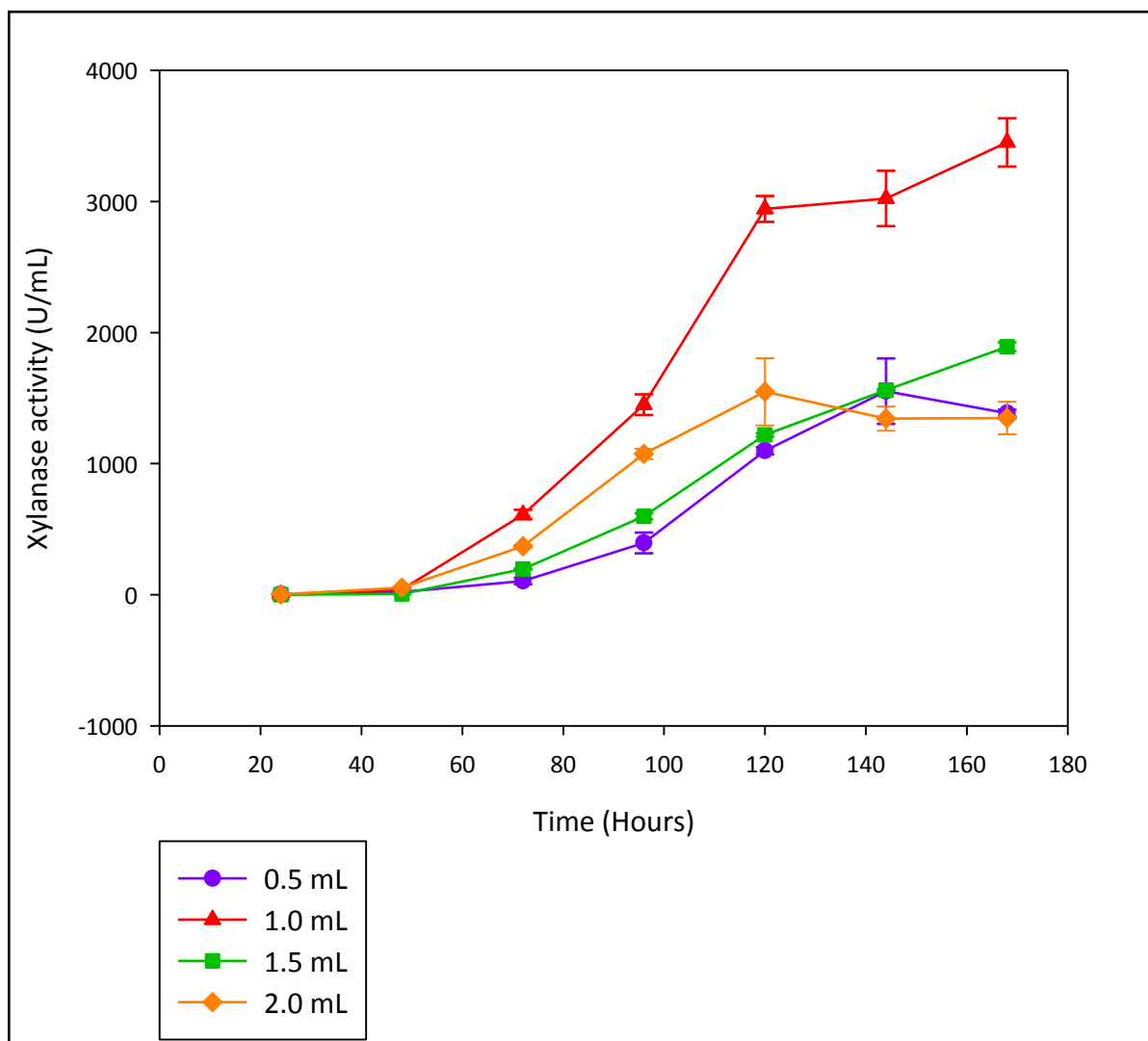
Changes in the characteristics of the nutrient solution occur during the fermentation which when coupled with the mycelial growth affect the viscosity of the solution. The lower xylanase activity at 120 rpm can be ascribed to reduced oxygen transfer as well as poor heat and mass transfer (Papagianni, 2004) as the viscosity increased significantly after 72 hours and could not be overcome by the slower agitation. The decrease in xylanase activity at a higher rotational speed is thought to be due to mycelial fragmentation or shearing which is known to interrupt growth and thus enzyme activity (Kumar et al., 2009).

#### **4.1.5. Effect of varying the inoculum size on xylanase activity**

The inoculum size was varied between 0.5 mL and 2.0 mL of spore solution. The optimum amount of fungal spore solution to start a culture is an important aspect in generating sufficient bio – mass and hence xylanase.

Figure 4-5 illustrates that 1.0 mL of inoculum resulted in the maximum xylanase activity of  $3451.16 \pm 184.17 \text{ U.mL}^{-1}$ . Xylanase activities of  $1386.05 \pm 26.31 \text{ U.mL}^{-1}$ ,  $1893.02 \pm 32.89 \text{ U.mL}^{-1}$  and  $1348.84 \pm 124.98 \text{ U.mL}^{-1}$  were achieved after 168 hours when inoculated with volumes of 0.5 mL, 1.5 mL and 2.0 mL respectively.

Okafor (2007) states the inoculum is usually 5 – 20 % of the final volume of the fermentation medium in large scale production so that the production time is shortened. The results presented here indicate however, that a 1 % inoculum size was favoured.



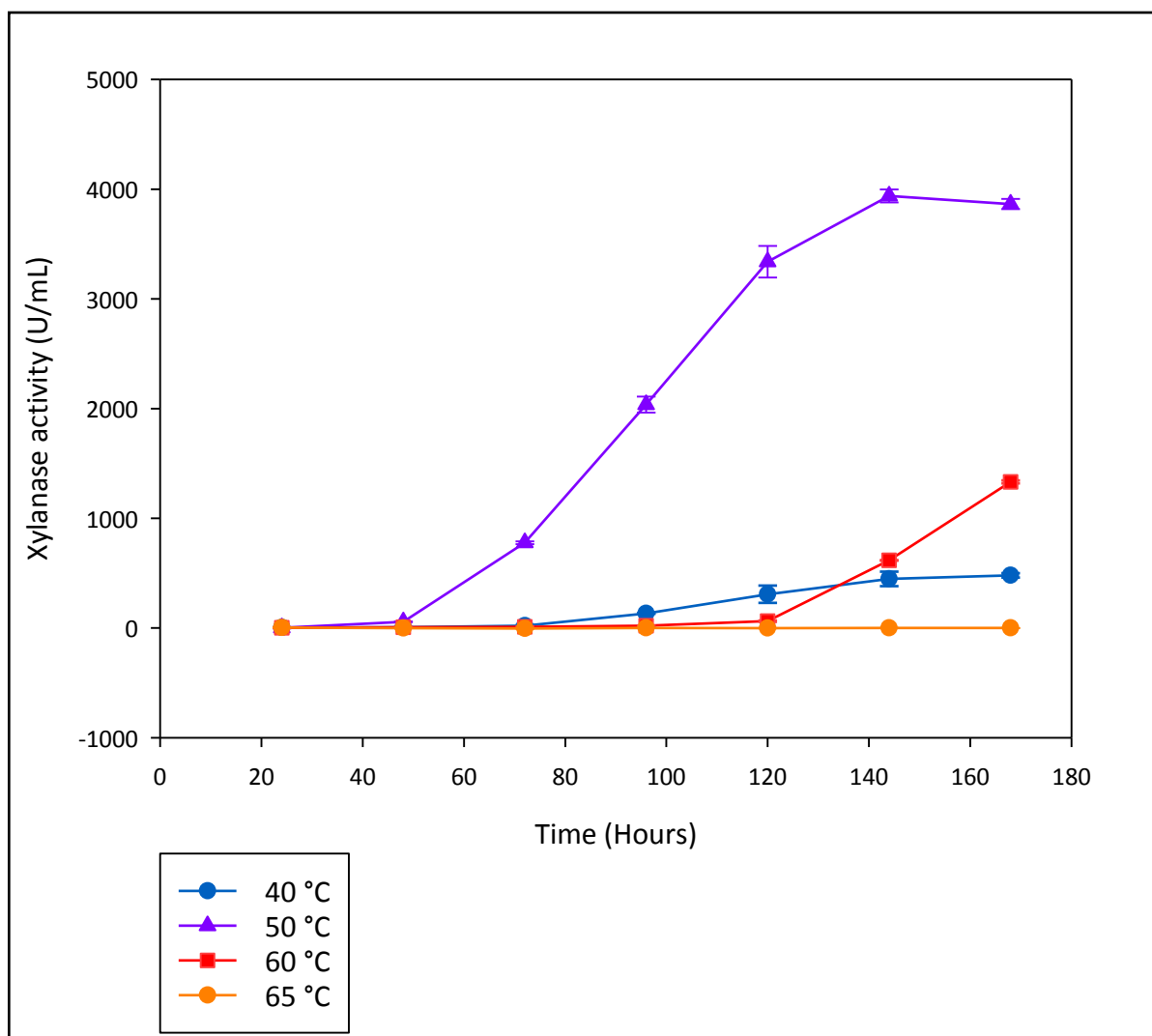
**Figure 4-5: Effect of varying the inoculum size on xylanase activity**

Lower inoculum volumes might not accommodate mycelial expansion and subsequent product formation reiterated by the results obtained when a 0.5 mL inoculum was used. Increased inoculum volumes typically improve growth related activities but after a certain point they serve to restrict gaseous exchange, reduce heat removal, and increase the demand for nutrients from the substrate. Furthermore, a high inoculum volume can increase the incidence of bacterial contamination (Gaffney et al., 2009). The activities achieved using a 1.5 mL and 2.0 mL inoculum volumes are consistent with these assumptions.



#### 4.1.6. Effect of varying production temperatures on xylanase activity

The thermophilic nature of *Thermomyces lanuginosus* is of interest since this species can grow and survive at high temperatures. An experiment to determine the best temperature for growth and hence xylanase production was conducted, the results of which are presented here.



**Figure 4-6: Effect of varying production temperatures on xylanase activity**

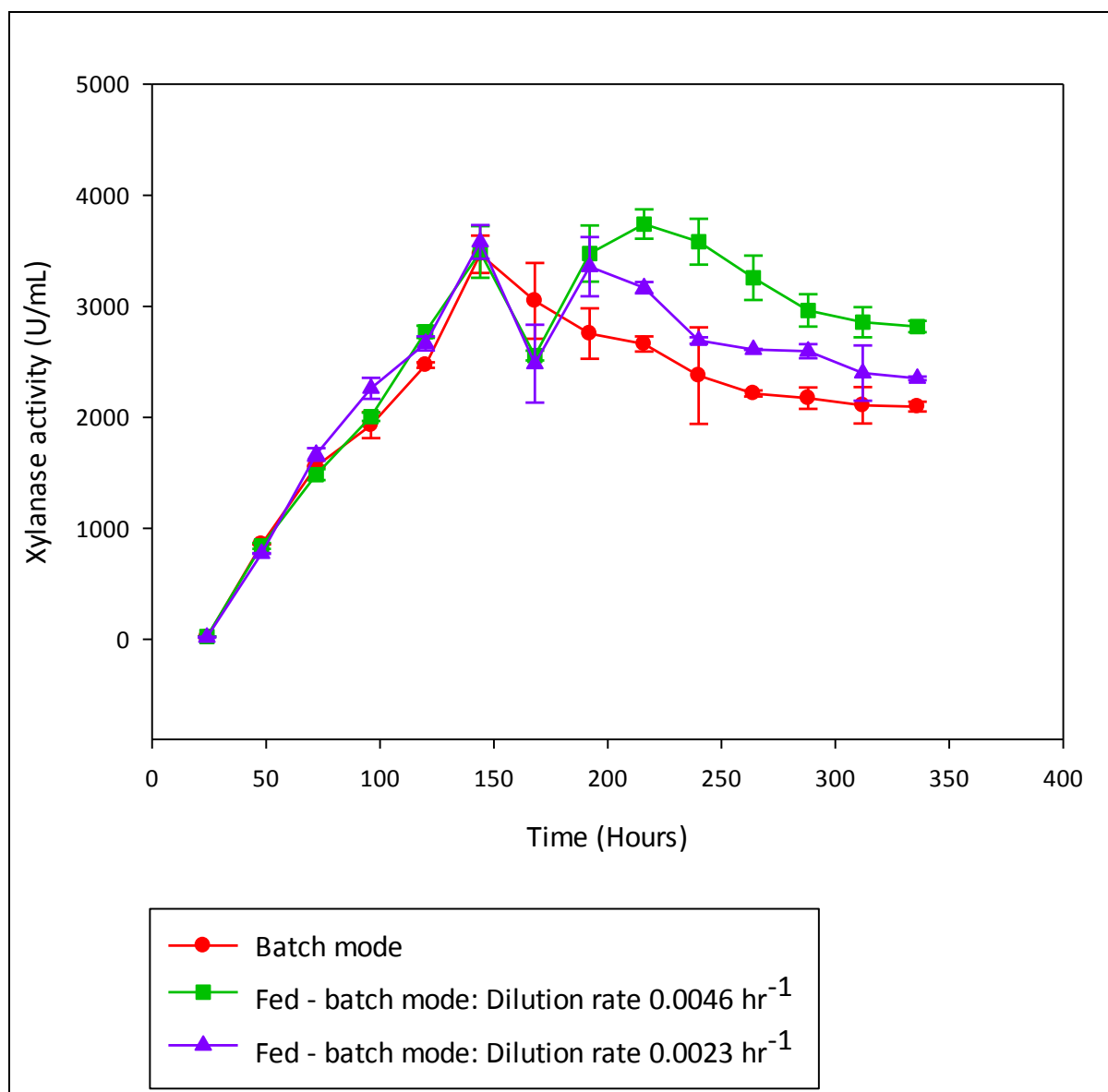
Production temperatures were selected in increments of 10 from 40 °C and cut – off at 65 °C as this was the incubator constraint. From Figure 4-6 the thermophilic nature of *Thermomyces lanuginosus* DSM 5826 is apparent. Fermentation at 50 °C appears to be ideal, as the maximum xylanase activity of  $3451.16 \pm 184.17 \text{ U.mL}^{-1}$  was recorded at this

temperature. This is similar to another report by Azad et al. (2013) where an optimal production temperature for xylanase from *T. lanuginosus* was recorded at 50 °C as also stated by (Singh et al., 2003). Production at 40 and 60 °C achieved xylanase activities of  $479.07 \pm 19.73 \text{ U.mL}^{-1}$  and  $1330.47 \pm 13.48 \text{ U.mL}^{-1}$  respectively after 168 hours while no production was achieved at 65 °C.

According to Papagianni (2004) studies of temperature effects on growth and metabolite production are few with respect to filamentous fungi. Although temperature is an environmental parameter that is simple to control, changes in temperature produce simultaneous changes in other culture variables. From the graph above, it is evident that an increase in temperature from 40 to 50 °C enhanced the growth and xylanase activity while a further increase proved ineffective. This may be attributed to the fact that dissolved oxygen tension is temperature dependant and varies inversely with increasing temperature. At 60 and 65 °C the oxygen supply to the cells may have been inadequate.

#### **4.1.7. Effect of operating in fed – batch mode**

In the conventional batch process as in the previous experiments, all of the substrate was added at the beginning of the fermentation. An enhancement of the closed batch process is the fed – batch fermentation. In a fed – batch system, a fresh aliquot of the medium is added continuously or periodically without the removal of the culture fluid. The most important advantage of this system is the addition of rapidly used carbon, nitrogen and phosphate sources. In this experiment volumes of 50 mL and 100 mL of nutrient medium were added to 100 mL of broth after 6 days, while fermentation was allowed to proceed in another flask without any further nutrient medium addition.



**Figure 4-7: Effect of fed – batch operation on xylanase activity**

The maximum xylanase activity achieved during batch operation was  $3467.66 \pm 168.86 \text{ U.mL}^{-1}$  after 144 hours. Thereafter activity decreased constantly to  $2097.01 \pm 44.33 \text{ U.mL}^{-1}$  after 336 hours. When 50 mL of nutrient medium was added on day 6, equating to a dilution rate of  $0.0023 \text{ hr}^{-1}$  xylanase activity dropped to  $2483.58 \pm 350.39 \text{ U.mL}^{-1}$  after 168 hours from a maximum of  $3580.60 \pm 151.98 \text{ U.mL}^{-1}$  after 144 hours. After another 24 hours however, enzyme activity reached  $3357.00 \pm 265.96 \text{ U.mL}^{-1}$  before continuing to decrease steadily to  $2351.00 \pm 16.89 \text{ U.mL}^{-1}$  at the end of the fermentation duration. These results are lower than that achieved when 100 mL of nutrient medium were added after day 6.

The  $0.0046 \text{ hr}^{-1}$  dilution rate appears to be more favourable for microbial growth and hence enzyme production. While the xylanase activities are similar after 144 hours due to similar conditions, activity decreased to  $2557.01 \pm 44.33 \text{ U.mL}^{-1}$  after 168 hours from  $3489.55 \pm 232.18 \text{ U.mL}^{-1}$  after 144 hours. This rapid decrease in both fed – batch systems are attributed to the large volume of liquid added thus rendering the concentration of enzymes more dilute. Following this decrease, enzyme activity stabilized rapidly and reached a maximum of  $3741.12 \pm 132.98 \text{ U.mL}^{-1}$  after 216 hours. At the end of the fermentation duration of 336 hours, enzyme activity was  $2818.06 \pm 50.67 \text{ U.mL}^{-1}$ .

The cessation of growth in the batch system after 144 hours may be due to the exhaustion of a nutrient component or the accumulation of a toxic product. Since the growth of the biomass is limited by the concentration of the nutrients, the addition of fresh nutrient would result in the rapid consumption of the nutrients. This is observed in the two fed – batch systems where growth was extended by the aliquots of fresh nutrient medium after 168 hours. At the end of the fermentation period, a 34 % increase in enzyme activities was achieved in fed – batch operation with a  $0.0046 \text{ hr}^{-1}$  dilution rate compared to batch operation. This increase does not appear to be too significant compared to a study by Persson et al. (1991) who recorded three to four fold increases in xylanase production by *Trichoderma*. It is possible that the total biomass may have increased in the fed – batch vessels but due to the increase in the volume by the added nutrient, the concentration of the biomass did not increase significantly, fed – batch processes are thus said to be in quasi – steady – state. The conditions in the flask were maintained resulting in the levelling off of the activity.

Batch processing and fed – batch processing are difficult to control. Environmental constraints such as oxygen consumption which begin at low rates at the start of fermentation but increase during the exponential growth phase play a major role in the maintenance of the growth rate. This increased oxygen consumption could be a possible contributor to the decrease in activity after the medium was added resulting in an increased volume in the same flask. Although a 200 mL flask was used, the head space may have been too little to accommodate the requirements of the biomass. Fed – batch is generally superior to batch processing and is especially beneficial when changing nutrient concentrations affect the

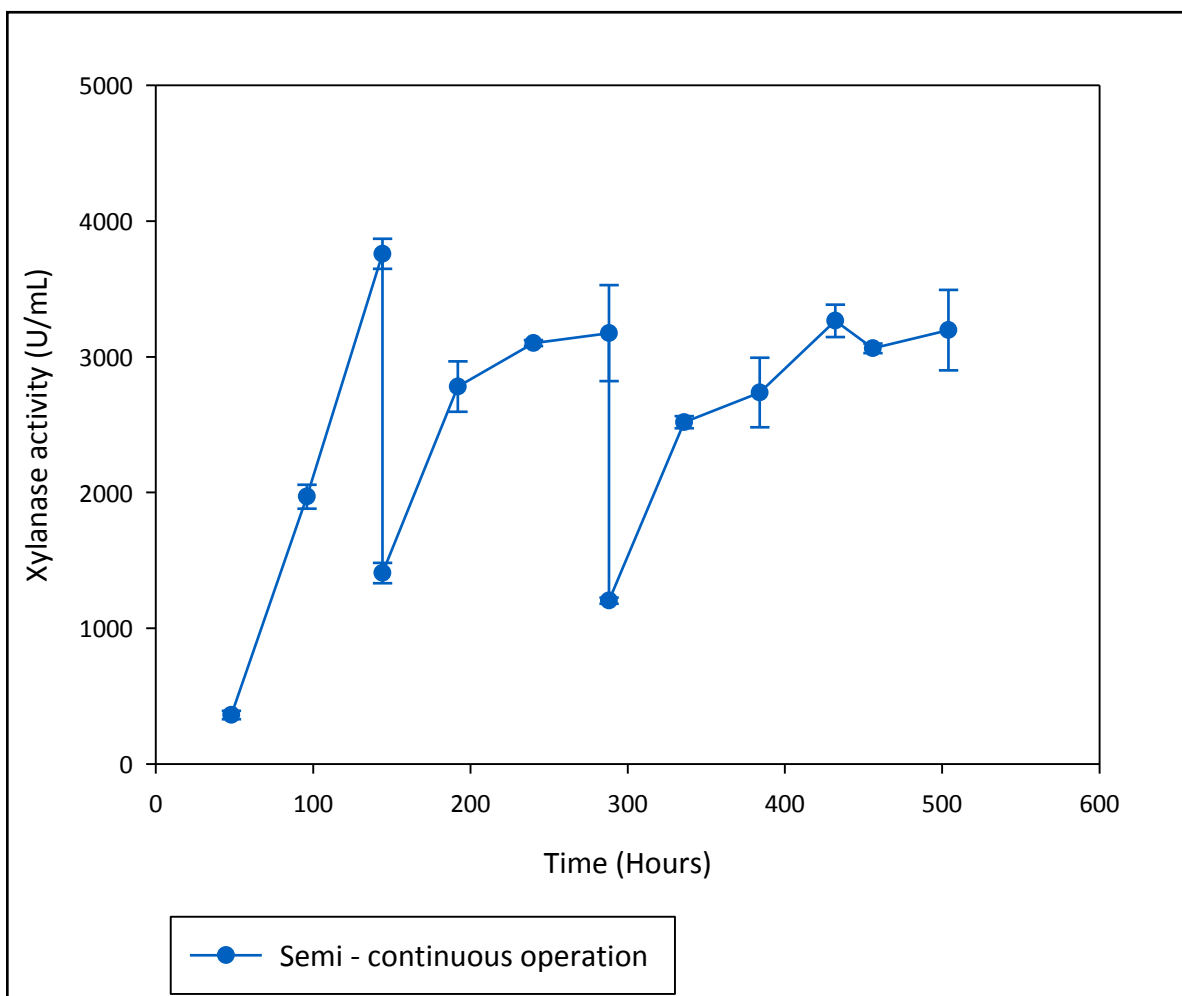
productivity and yield of the desired product. The toxic effects of metabolic products are lowered by the dilution of the broth upon nutrient addition. This could provide an explanation for the system with the higher dilution rate resulting in higher enzyme activity at the end of the fermentation.

#### **4.1.8. Effect of operating in semi – continuous mode**

In continuous fermentation or in this case semi – continuous fermentation, sterile nutrient medium is added to the fermenter while an equivalent amount of converted nutrient solution with micro – organisms, that is, broth, is simultaneously removed from the system. An experiment was conducted whereby a 50 mL of broth was removed from the shake flasks after 144 and 288 hours and replaced with fresh nutrient medium of equal volume. The effect on xylanase activity was investigated.

The addition of nutrients increases the volume of the medium in the flask. In traditional continuous fermentation, the increased volume would have drained off as an overflow, which is collected and used for the recovery of products. In this case, a volume of liquid (50mL) was siphoned out of the flask and replaced by an equal volume of fresh medium. At this stage, the medium was diluted both in terms of biomass and product. This is evident by the sudden decline in xylanase activity from  $3759.38 \pm 110.49 \text{ U.mL}^{-1}$  to  $1406.25 \pm 175.13 \text{ U.mL}^{-1}$  noted after 144 hours when the aliquot of broth was taken and medium replenished. At this point the concentration of biomass in the flask decreased while the concentration of the substrate increased.

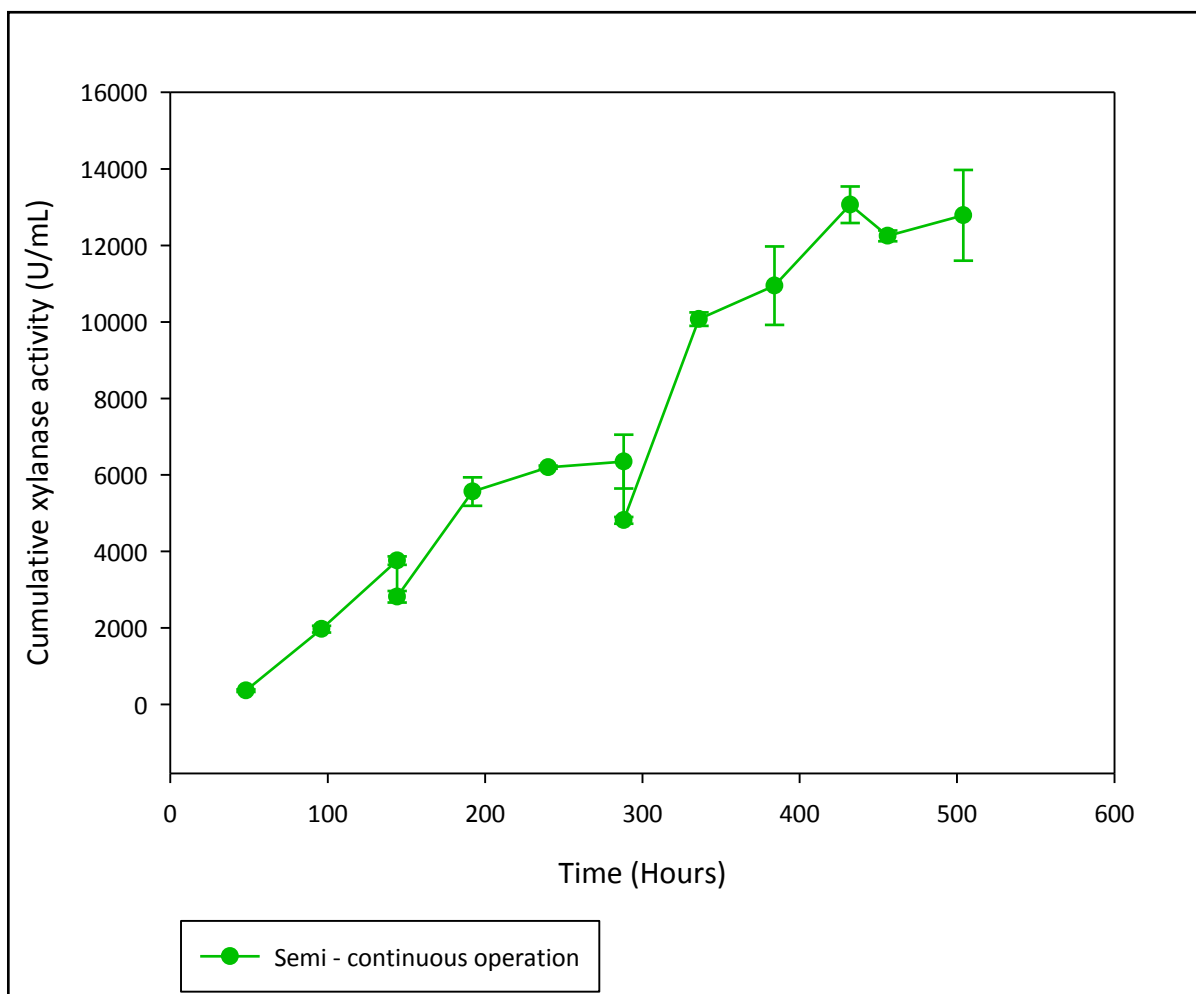
New growth was stimulated by the added nutrient medium and the growth rate was significant, indicated by the doubling of the xylanase activity. The system reached a new steady state as observed by the levelling off of the graph between 192 and 288 hours. At this point a second aliquot of broth was removed and substituted with nutrient medium. A similar cycle of dilution in product was noted at 288 hours when this process occurred. Here again a decrease in biomass concentration by removal in the broth gave rise to an increase in substrate concentration, an increased growth rate observed at 336 hours and a steady state was re – established.



**Figure 4-8: Effect of semi – continuous operation on xylanase activity**

Fermentation proceeded until 504 hours when it was terminated at an enzyme activity of  $3196.88 \pm 296.10 \text{ U.mL}^{-1}$ . Figure 4-8 thus proves that a semi – continuous system is a nutrient limited self – balancing culture system which may be maintained in a steady state and offers much control of the growth rate of biomass and thus the production of xylanase to sustain this growth rate.

A cumulative plot of these results is shown in Figure 4-9. Here the xylanase produced in broth and in the aliquot removed is added together taking into account the dilution factor when fresh nutrient was added. The graph indicates that semi – continuous fermentation results in an overall increase in the amount of xylanase produced per volume, confirming that this mode is better suited to achieve higher productivity over a longer period of time.



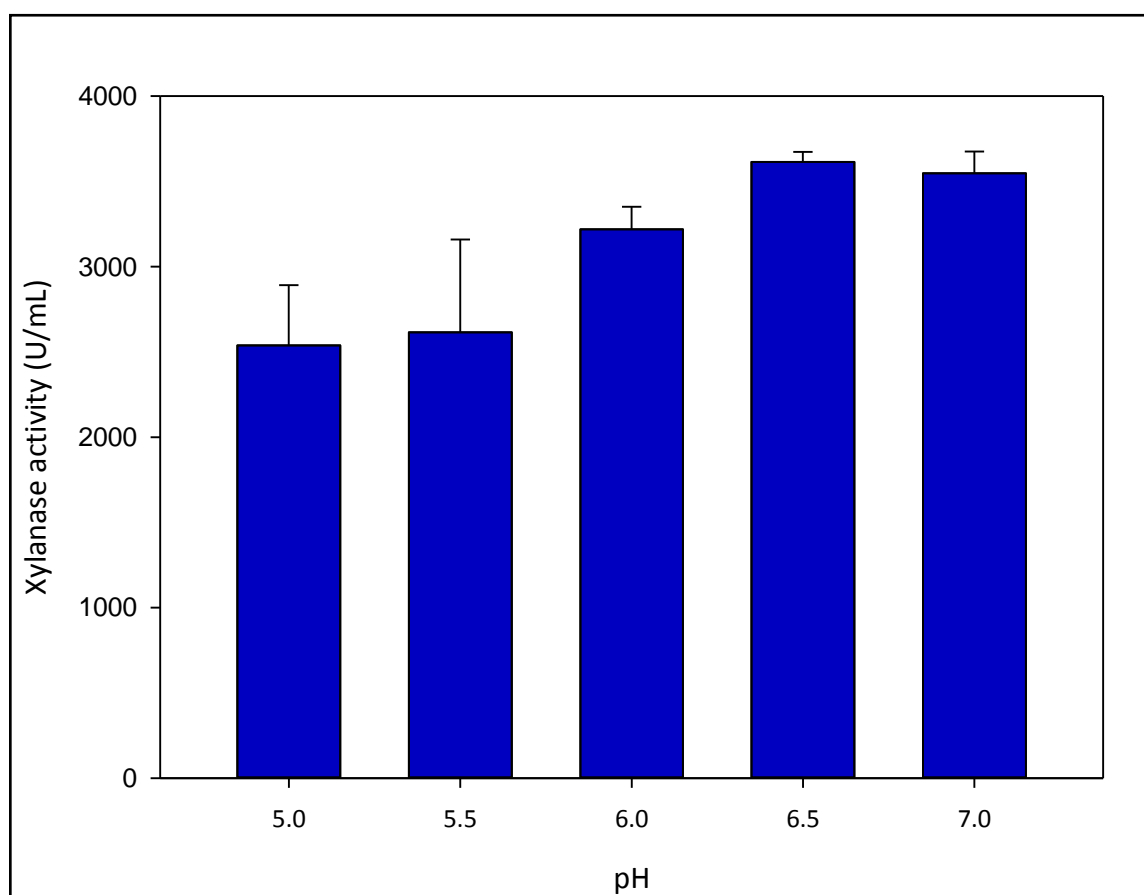
**Figure 4-9: Cumulative effect of semi – continuous operation on xylanase activity**

## **4.2. Experiments conducted to partially characterize the enzyme**

Characterization in biological terms is the attributing of distinguishable traits to an organism or system. In this section, the results of pH optima, temperature optima, molecular weight and substrate specificity of xylanase from *Thermomyces lanuginosus* DSM 5828 are presented and discussed. These characteristics are essential in determining the environment that xylanase is active in and processes that it is suitable for.

### **4.2.1. pH optima**

pH optima is the determination of the best suited pH for enzyme activity as obtained by the pH of the buffer solution used in the enzyme assay.



**Figure 4-10: pH optima of xylanase from *T. lanuginosus* DSM 5826**

Figure 4-10 shows that the optimal pH for xylanase from *T. lanuginosus* at 50 °C is 6.5 resulting in an activity of  $3614.58 \pm 58.93$ . These results corroborate with studies performed by Gomes et al. (1993) and Maharaj et al. (2011) who reported optima at pH 6.5 while Cesar and Mrša (1996) reported maximum activity around pH 7.0.

Enzymes are very sensitive to variations in pH or pOH (measure of the molar concentration of hydrogen and hydroxide ions respectively). Different enzymes and different organisms have different requirements but optima generally range from pH 4.5 to pH 8.5. The optimal for most cells are near 7.0 or neutrality (Tripathi, 2006). The isoelectric point of xylanase has been found to be between 3.7 and 4.1 (Singh et al., 2003), thus a greater pH than 4.1 is required to prevent the enzyme from coagulating and precipitating out of solution.

Large changes in pH can reversibly alter the configuration of the enzyme and even larger changes may ultimately destroy the enzyme (Nauman, 2008). It is logical then that since the



production pH of the enzyme was 6.5, assaying with a buffer at the same pH would result in a minimal pH change when determining enzyme activity and thus promote the least disturbance to the enzyme environment.

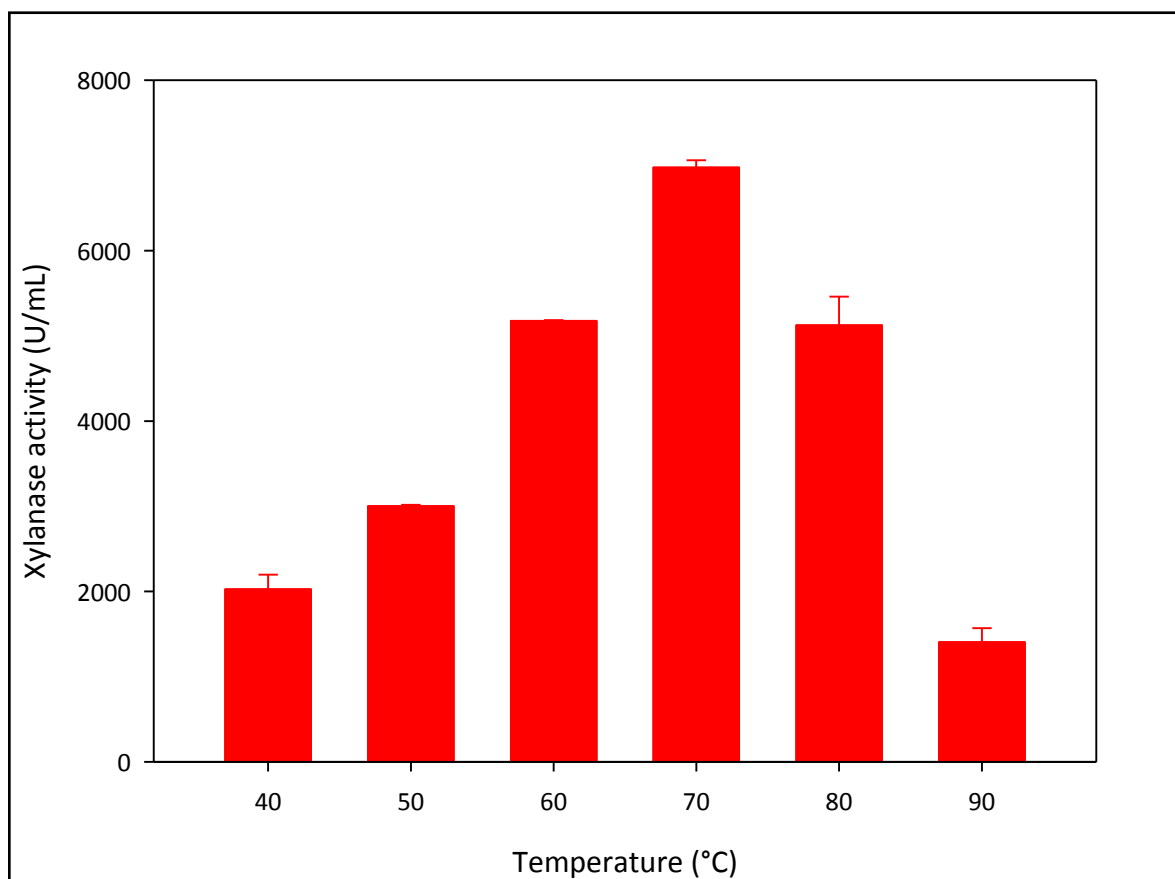
#### **4.2.2. Temperature optima**

Temperature optima experiments are performed to determine the best suited temperature for enzyme activity as obtained by the temperature of the water bath during the enzyme assay. Each enzyme has an optimal temperature at which it functions best. This varies with enzymes as well as organisms. Extreme limits range from -2 to 70 °C (Bhatia, 2009).

Figure 4-11 below indicates that the optimal temperature for the action of xylanase from *Thermomyces lanuginosus* DSM 5826 is 70 °C which is much higher than the optimal production temperature of 50 °C. These results correspond to those obtained by Cesar and Mrša (1996) who reported optimal activity between 60 and 70 °C while Wizani et al. (1993) reported an optimal temperature of 65 °C. At temperatures between 40 and 70 °C the enzyme is not destroyed, but are merely slowed down though not to a point where enzyme action is inhibited. Further experiments below 40 °C would determine at which temperature the enzyme is inactive. This is in accordance with the Arrhenius equation which relates the rate of a reaction to temperature.

Enzyme catalyzed reactions are thus also increased with an increase in temperature (Tripathi, 2006). This increase in the rate of reaction results from the acceleration of diffusion and other chemical processes of thermostable enzymes. This characteristic of thermophilic xylanases makes large scale fermentation more technically and economically feasible (Azad et al., 2013). Enzyme activity remained high at 80 °C but dropped significantly at 90 °C. Even the most resistant enzymes are denatured irreversibly at extreme high temperature as they are protein in nature. Proteins in solution fold into elaborate but characteristic shapes to increase like – to – like interactions. The folded state is the native or natural state and is the state in which enzymes have their catalytic activity. At high temperatures, random thermal forces disrupt the folded chain and destroy the catalytic sites. Very high temperatures cause

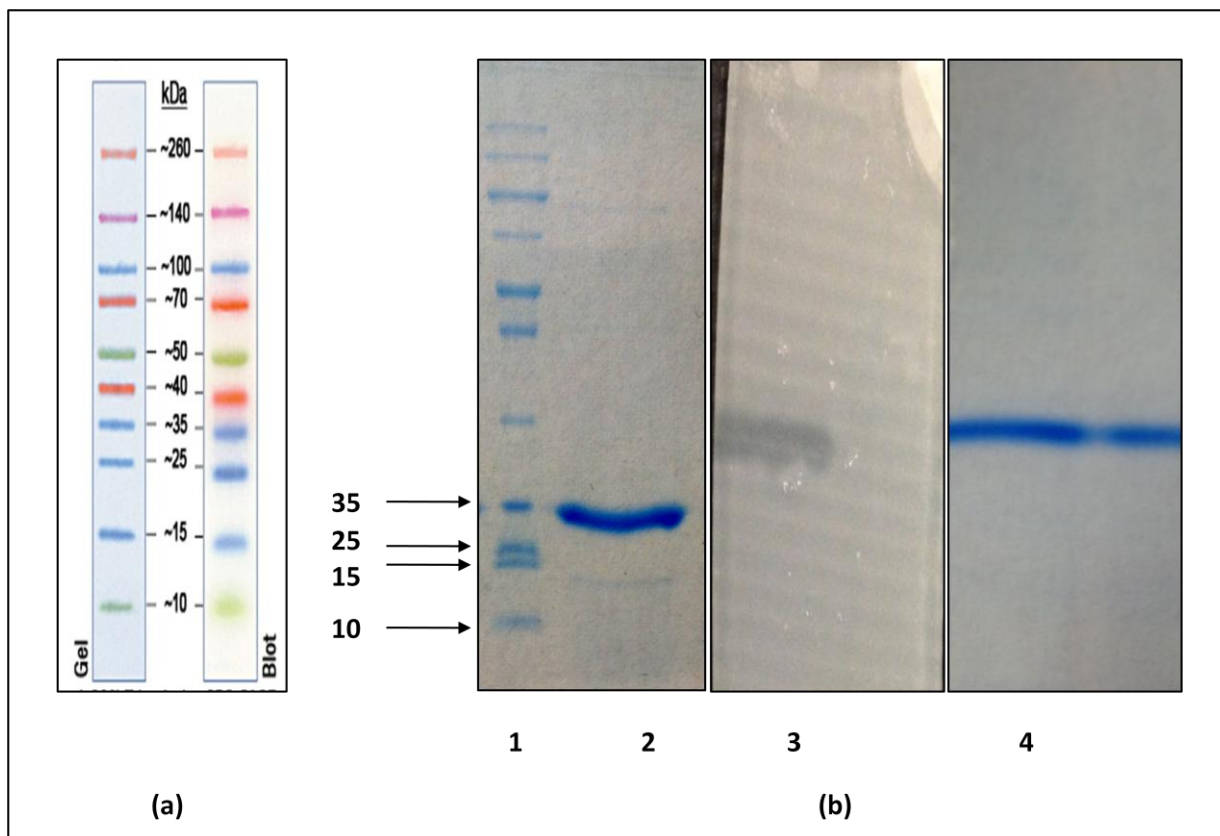
coagulation or other structural and chemical changes which lead to irreversible deactivation and the proteins are denatured (Nauman, 2008).



**Figure 4-11: Temperature optima of xylanase from *T. lanuginosus* DSM 5826**

#### **4.2.3. SDS PAGE and zymogram analysis**

SDS PAGE analysis was conducted to determine the approximate molecular weight of the enzyme. A zymogram was prepared to qualitatively validate the presence of xylanase in the samples. The images from these experiments are presented here.

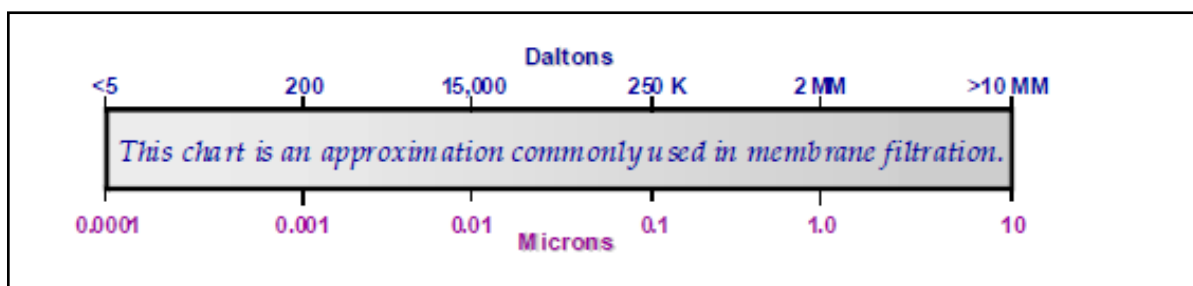


**Figure 4-12: SDS PAGE and zymogram of xylanase from *Thermomyces lanuginosus* DSM 5826 (a) Spectra Multicolour Broad Range Protein Ladder, (b) Analysis of xylanase sample, Lane 1 – Protein marker, Lane 2 – Xylanase sample, Lane 3 – Zymogram for xylanase revealed with ethanol, Lane 4 – Protein staining**

The SDS PAGE analysis illustrated that the molecular weight of xylanase from *T. lanuginosus* DSM 5826 is between 25 and 35 kDa as seen in lane 2 of Figure 4-12(b). The band appears prominent due to the high concentration of xylanase in the sample. In other studies molecular weights of 24 kDa (Maharaj et al., 2011), 24.7 kDa (Singh et al., 2000) and 25.5 (Cesar and Mrša, 1996) were reported . This information is useful in determining the type of membrane filtration that will be best suited to remove valuable xylanase containing permeate while retaining fungal biomass.

There is a correlation between Daltons and microns though not exact due to the fact that Daltons are a measure of 3 – dimensional size based on atomic weights and microns are a measure of 2 – dimensional distance. The chart below provides an approximation commonly used in membrane filtration. The figure above reveals that a protein of between 25 and 35

kDa will be able to pass through a microfiltration membrane easily as this is between 0.1 and 10 microns.



**Figure 4-13: Correlation between Daltons and microns (Koch membrane systems inc)**

Zymogram analysis was performed as part of the qualitative analysis for enzyme activity. It is visualized by the hydrolysis of the substrate. Unlike with SDS PAGE analysis, the enzyme is not boiled, nor was SDS added, therefore retaining the activity of the enzyme. Lanes 3 and 4 of Figure 4-12(b) shows two halves of the gel sandwich. Lane 3 represents the translucent zones of hydrolysis on the agarose – xylan overlay gel which now contains the xylanase which has been transferred to it from the polyacrylamide gel subsequent to the sandwiching and incubation which allowed for enzymatic action on the xylan substrate. The protein migrated across the gel as the electric field was applied and here hydrolysed the substrate.

The other half of the gel was placed in Coomassie blue dye to identify the protein and compare its position to that observed in the SDS – PAGE gel. The position appears to be higher up in comparison. This can be attributed to the absence of SDS in the polyacrylamide gel during the zymogram analysis. SDS is added to bestow the proteins with uniform load density. It imparts a negative load onto the proteins. In the absence of SDS then, proteins with equal mass may migrate differently in the pores of the gel due to the load differential of their tridimensional structures (Maria de Lourdes et al., 2012).

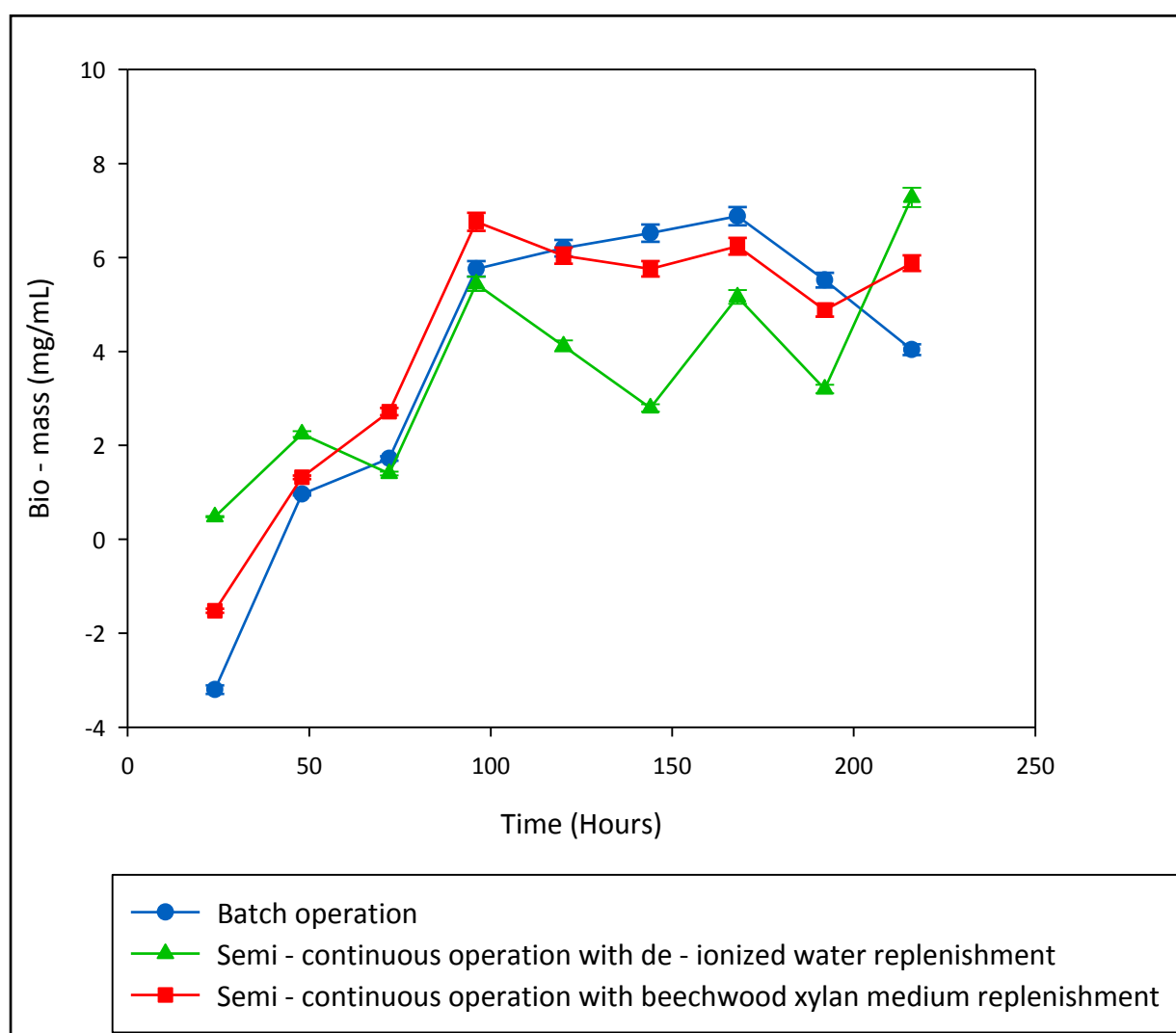
### **4.3. Experiments conducted in the membrane fermenter**

Membrane filtration provides the benefits of both concentrating the biomass and simplifying metabolite recovery. The membrane fermenter employed for these experiments consisted of

a simple vessel with a flat sheet membrane module submerged in the medium. Temperature and agitation was maintained inside an orbital shaking incubator. As such, samples were withdrawn manually via a large syringe as opposed to the use of a pump thus semi – continuous fermentation was the chosen mode of operation.

#### 4.3.1. Effect of mode of operation on dry – weight of the micro – organism

The dry – weight of the biomass measured provides an indication of the fungal growth trends during the course of the experiment.



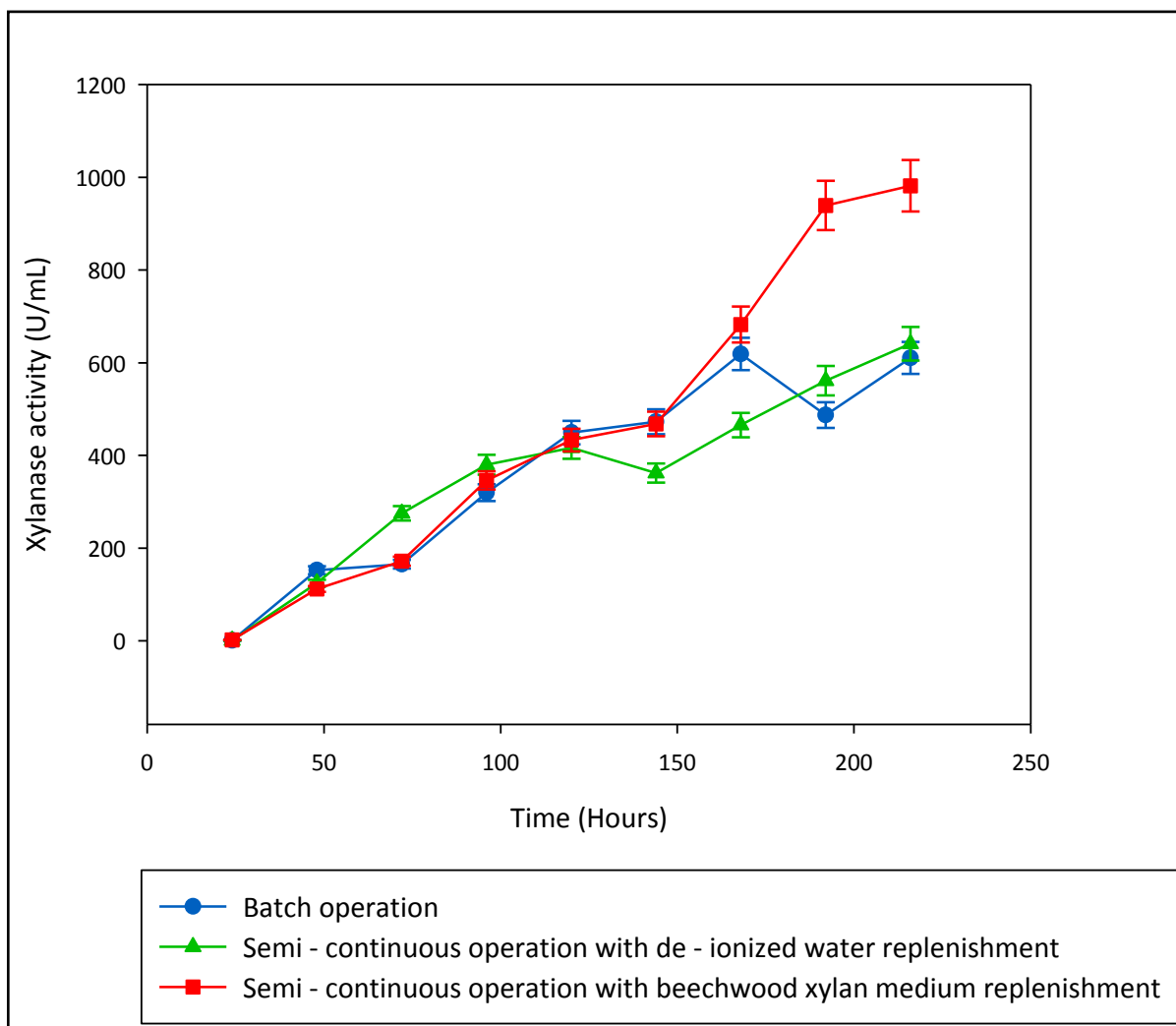
**Figure 4-14: Effect of mode of operation on dry – weight of the micro – organism**

The biomass achieved in batch mode reached a maximum of  $6.88 \pm 0.20 \text{ mg.mL}^{-1}$  after 168 hours after which it declined to  $4.04 \pm 0.11 \text{ mg.mL}^{-1}$  at the end of the fermentation period of 216 hours. The biomass recorded in semi – continuous mode with de – ionized water replenishment was erratic throughout the operation and reached a maximum of  $7.28 \pm 0.21 \text{ mg.mL}^{-1}$  after 216 hours. When replenished with beechwood xylan medium the biomass peaked at 96 hours when  $6.76 \pm 0.19 \text{ mg.mL}^{-1}$  was recorded, thereafter being maintained at  $\pm 6.00 \text{ mg.mL}^{-1}$ . In all three systems an exponential growth phase can be seen up until 96 hours. In this phase the cell metabolisms would have adjusted and all the key nutrients were available. In the batch operation, a stationary growth phase is observed between 96 and 168 hours. At this stage there may have been depletion of nutrients as well as the accumulation of toxic products. After 168 hours the death phase was observed. The number of viable cells usually follows an exponential decay curve during this period which was investigated during an extended fermentation operation, discussed in the next section.

The erratic biomass recorded for the operation with de – ionized water replenishment may be attributed to the dilution of the broth followed by a period of cell growth. It must be noted that an exponential death phase was not observed in this mode of operation but rather biomass appeared to increase after each dip. The water may have aided in the availability of the nutrients to the micro – organism. Subsequent to the exponential growth phase ending at 96 hours, biomass appeared to be in the stationary or maintenance growth phase until the end of fermentation in the operation with beechwood xylan replenishment. This trend indicates that biomass may indeed be maintained in a membrane fermenter system with sufficient nutrients being supplied to the micro – organism. The effects of toxins released by the micro – organism are not evident. They may have been removed in the permeate stream and then also been diluted upon addition of nutrient medium.

### 4.3.2. Effect of mode of operation on xylanase activity

Xylanase production achieved in each mode of operation is presented here.



**Figure 4-15: Effect of mode of operation on xylanase activity**

Xylanase activity in batch mode was  $610.58 \pm 34.54 \text{ U.mL}^{-1}$  after 216 hours. When replenished with de – ionized water in semi – continuous operation, enzyme activity reached  $641.35 \pm 36.26 \text{ U.mL}^{-1}$  while a prominent increase was observed when beechwood medium was added. A maximum xylanase activity of  $981.73 \pm 55.54 \text{ U.mL}^{-1}$  was recorded after 216 hours. The xylanase activities recorded here were much lower than that recorded in the shake flasks where fermentation proceeded in 100 mL volumes. This is not surprising as a 10 x scale – up was performed. Blakebrough (1973) stated that no matter what criteria are used for scale –

up it is not possible to reproduce exactly similar conditions on all scales of operation. It must be noted that vessels of cylindrical shape were used in the scale – up while conical flasks were used for the shake flask experiments. The larger scale fermentation vessels were also fitted with air pipes for aeration and silicon tubing for sampling. These vessels also had metal lids while the conical flasks had cotton wool stoppers covered with foil. These factors contribute to the possibility of contamination which may have contributed to the lower enzyme activities. The concentration of all components in the nutrient medium in the scale – up of experiments were reduced by 33.33 % to reduce cost. This was necessary due to the number of experiments performed as well as the large amount of commercial xylan required for 1 L of nutrient medium. This lower concentration is also a factor when comparing the xylanase activity achieved here to that in the shake flasks. This observation has been documented by Gomes et al. (1993) who reported lower xylanase activity in bioreactors than shake flasks when using xylan substrates for xylanase production by *Thermomyces lanuginosus*.

Figure 4-15 shows that enzyme activity was higher with DI water until 96 hours. In a study on chitinase production in a membrane bioreactor, Kao et al. (2007) suggested that water helps to desorb the enzyme which is coupled to the substrate in the broth. Also the cells might have experienced the depletion of enzyme which was removed in the permeate stream and hence increase enzyme secretion. Enzyme activity thereafter appeared less than in the batch operation. The percentage increase in enzyme activity between the batch operation and semi – continuous operation with beechwood xylan nutrient replenishment is 60.78 %. This can be attributed to the continuous supply of nutrients to the cell and the dilution of the growth and secretion limiting metabolites via the membrane module. These factors may have led to the high biomass and corresponding high enzyme activities (Kiy et al., 1996).

#### **4.3.3. Effect of mode of operation on volumetric productivity**

Volumetric productivity in a continuous process can be expressed by multiplying the dilution rate by the product concentration. Therefore high microbial cell concentration in the production phase results in high product concentrations and thus high productivities. A fermenter which allows for high cell density cultures is of great importance as a tool to increase process productivity.



**Table 4-1: Volumetric productivity in batch operation**

| Time (hr) | Activity (U/mL) | Productivity (U.mL <sup>-1</sup> .hr <sup>-1</sup> ) |
|-----------|-----------------|--|
| 24        | 0.9712          | 0.0405   |
| 48        | 152.5000        | 3.1771   |
| 72        | 165.1442        | 2.2937   |
| 96        | 319.7115        | 3.3303   |
| 120       | 449.5192        | 3.7460   |
| 144       | 473.0769        | 3.2853   |
| 168       | 619.2308        | 3.6859   |
| 192       | 487.5000        | 2.5391   |
| 216       | 610.5769        | 2.8267   |

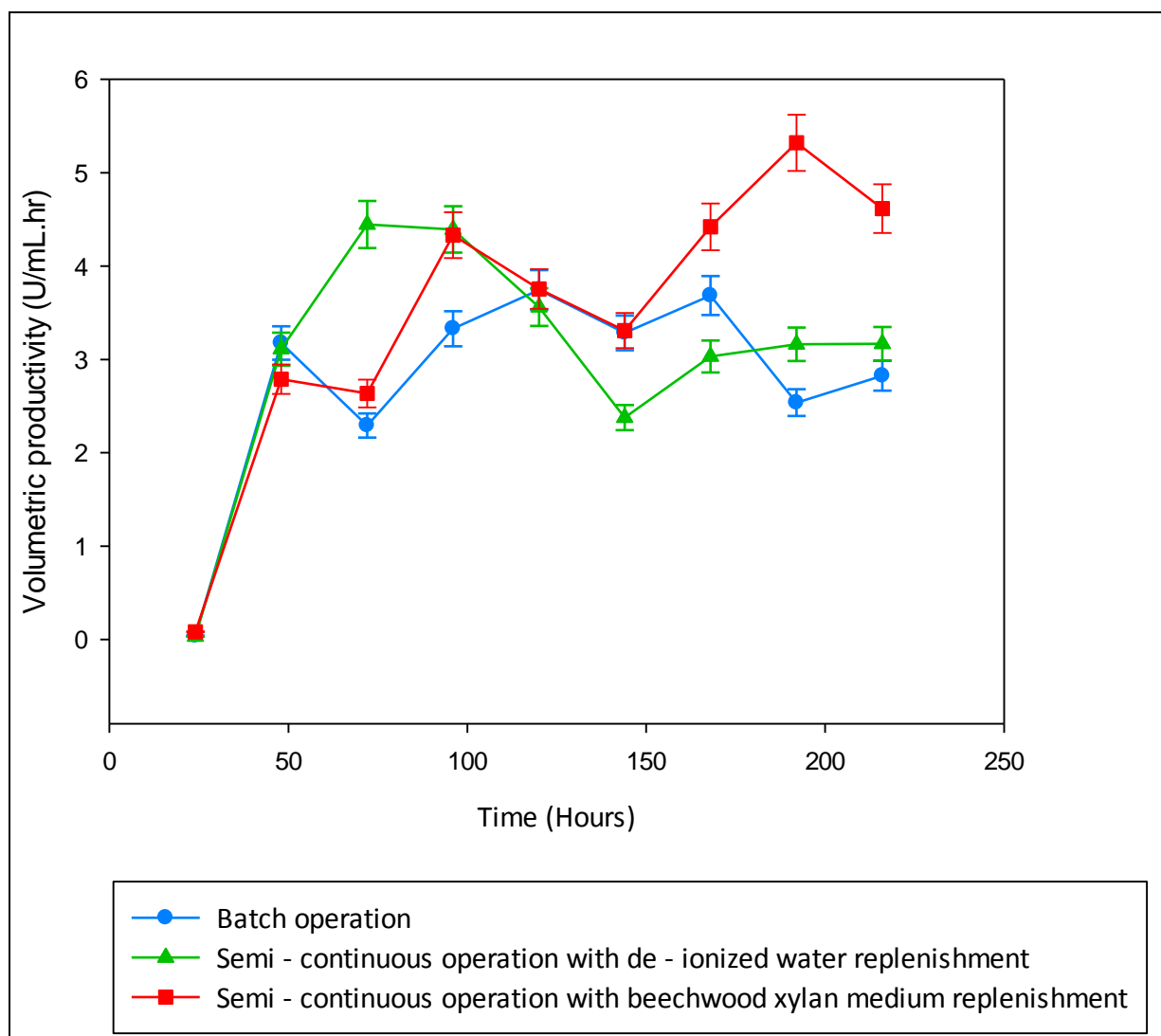
**Table 4-2: Volumetric productivity in semi – continuous operation with DI water supplementation**

| Time (hr) | Activity (U/mL) | Dilution rate (hr <sup>-1</sup> ) | Productivity (U.mL <sup>-1</sup> .hr <sup>-1</sup> ) |
|-----------|-----------------|-----------------------------------|--|
| 24        | 0.8077          | batch                             | 0.0337   |
| 48        | 124.6154        | 0.0042                            | 3.1120   |
| 72        | 275.0000        | 0.0042                            | 4.4460   |
| 96        | 379.8077        | 0.0042                            | 4.3930   |
| 120       | 416.3462        | 0.0025                            | 3.5609   |
| 144       | 362.0192        | 0.0025                            | 2.3782   |
| 168       | 465.8654        | 0.0025                            | 3.0326   |
| 192       | 561.5385        | 0.0025                            | 3.1639   |
| 216       | 641.3462        | 0.0025                            | 3.1687   |

**Table 4-3: Volumetric productivity in semi – continuous operation with beechwood xylan nutrient medium supplementation**

| Time (hr) | Activity (U/mL) | Dilution rate (hr <sup>-1</sup> ) | Productivity (U.mL <sup>-1</sup> .hr <sup>-1</sup> ) |
|-----------|-----------------|-----------------------------------|--|
| 24        | 2               | batch                             | 0.0833   |
| 48        | 111.9231        | 0.0042                            | 2.7897   |
| 72        | 171.8750        | 0.0042                            | 2.6370   |
| 96        | 346.1538        | 0.0042                            | 4.3319   |
| 120       | 433.1731        | 0.0017                            | 3.7548   |
| 144       | 468.2692        | 0.0017                            | 3.3104   |
| 168       | 682.6923        | 0.0017                            | 4.4210   |
| 192       | 939.4231        | 0.0017                            | 5.3207   |
| 216       | 981.7308        | 0.0017                            | 4.6156   |

The volumetric productivity for xylanase in batch mode is calculated by a simple measurement of the enzyme activity at the end of the fermentation process per duration of the process in hours. For continuous fermentation the volumetric productivity at each point is given by the dilution rate multiplied by the change in enzyme activity in addition to the rate of enzyme secreted in period between sampling.



**Figure 4-16: Effect of mode of operation on volumetric productivity**

The productivity achieved in batch operation was observed to be  $2.83 \pm 0.18 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 216 hours with a maximum productivity of  $3.75 \pm 0.21 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 120 hours. Volumetric productivity was maximal at  $4.45 \pm 0.25 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 72 hours in semi – continuous operation with DI water supplementation and thereafter decreased significantly to  $2.38 \pm 0.13 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 144 hours. Productivity was maintained around  $3.0 \text{ U.mL}^{-1}.\text{hr}^{-1}$  until the end of the fermentation period. In semi – continuous operation with beechwood xylan nutrient medium supplementation productivity was similar to that with DI water at  $4.33 \pm 0.25 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 72 hours. The productivity was much lower than that achieved with DI water up to this point. This is attributed to the higher xylanase activity achieved during this time with DI water supplementation. Productivity with beechwood xylan decreased to  $3.31 \pm$

0.18 U.mL<sup>-1</sup>.hr<sup>-1</sup> after 144 hours. This decrease in productivity is also observed at the same period with DI water supplementation. After 144 hours, the dilution rates in both fermenters were reduced from 0.0042 hr<sup>-1</sup> to 0.0025 hr<sup>-1</sup> with DI water and 0.0017 hr<sup>-1</sup> with beechwood xylan. This reduction was due to difficulties in sampling which may be the result of fouling on the membrane, kinks in the permeate withdrawal tubes and insufficient suction. Productivity increased to a maximum of 5.32 ± 0.30 U.mL<sup>-1</sup>.hr<sup>-1</sup> after 192 hours and decreased to 4.62 ± 0.26 U.mL<sup>-1</sup>.hr<sup>-1</sup> after 216 hours.

The productivity achieved using xylan medium and DI water is directly proportional to the xylanase activities achieved at specific periods in the fermentation process and the change in the dilution rates at the specified junctures. The maximum volumetric productivity achieved in semi – continuous operation is 2.1 times greater than that of batch operation which equates to an increase of 110.28 % in productivity measured at its peak after 192 hours.

As no other literature for continuous fermentation in a membrane fermenter for xylanase is available, it was difficult to compare the results obtained to other studies using *Thermomyces lanuginosus*. Comparisons provided here are across various micro – organisms and products for batch and continuous processing. Kariminiaae-Hamedani et al. (2005) recorded a 2.35 times increase in the productivity of baker's yeast production when operated in continuous mode compared to batch fermentation. In a study comparing the production of *Aspergillus niger* β – galactosidase by a recombinant flocculating *Saccharomyces cerevisiae*, Domingues et al. (2005) observed an 11 – fold increase in productivity in continuous mode opposed to batch mode. A study on the comparison between continuous and batch processing to produce xylanase by *Penicillium canescens* 1010c by Bakri et al. (2012) showed a maximum batch productivity of 1.31 U.mL<sup>-1</sup>.hr<sup>-1</sup> after 96 hours while productivity reached 3.46 U.mL<sup>-1</sup>.hr<sup>-1</sup> in the continuous culture which was 2.64 times greater. Mamo and Gessesse (2000) recorded volumetric productivities of 0.184 U.mL<sup>-1</sup>.hr<sup>-1</sup> for xylanase produced by immobilized alkaliphilic bacillus sp AR – 009 in batch culture and 3.20 U.mL<sup>-1</sup>.hr<sup>-1</sup> in continuous culture, which equates to a 17 – fold increase.

These studies cannot be used for direct comparison however, as the micro – organisms differ significantly from *Thermomyces lanuginosus* and the experiments conducted were not performed in membrane fermenter systems. They do however confirm the superiority of continuous fermentation to batch fermentation with regards to increased productivity.

#### 4.3.4. Effect of mode of operation on total enzyme activity

The total enzyme activity is the total number of international units of enzyme obtained in the total volume of broth for the batch operation, and in the retentate and permeate stream for the semi – continuous operations.

**Table 4-4: Total activity in batch operation**

|   |             |
|---|-------------|
| Activity in batch vessel after 216 hours (U/mL) | 610.5770    |
| Total activity in batch vessel (U)              | 610576.9231 |

**Table 4-5: Total activity of permeate during semi – continuous operation with DI water supplementation**

| Time (hr) | Activity (U/mL) | Volume of permeate (mL) | Total activity in permeate (U) |
|-----------|-----------------|-------------------------|--------------------------------|
| 24        | 0.8077          | 0                       | 0                              |
| 48        | 124.6154        | 100                     | 12461.5385                     |
| 72        | 275.0000        | 100                     | 27500.0000                     |
| 96        | 379.8077        | 100                     | 37980.7692                     |
| 120       | 416.3462        | 60                      | 24980.7692                     |
| 144       | 362.0192        | 60                      | 21721.1538                     |
| 168       | 465.8654        | 60                      | 27951.9231                     |
| 192       | 561.5385        | 60                      | 33692.3077                     |
| 216       | 641.3462        | 60                      | 38480.7692                     |

**Table 4-6: Total activity of retentate during semi – continuous operation with DI water supplementation**

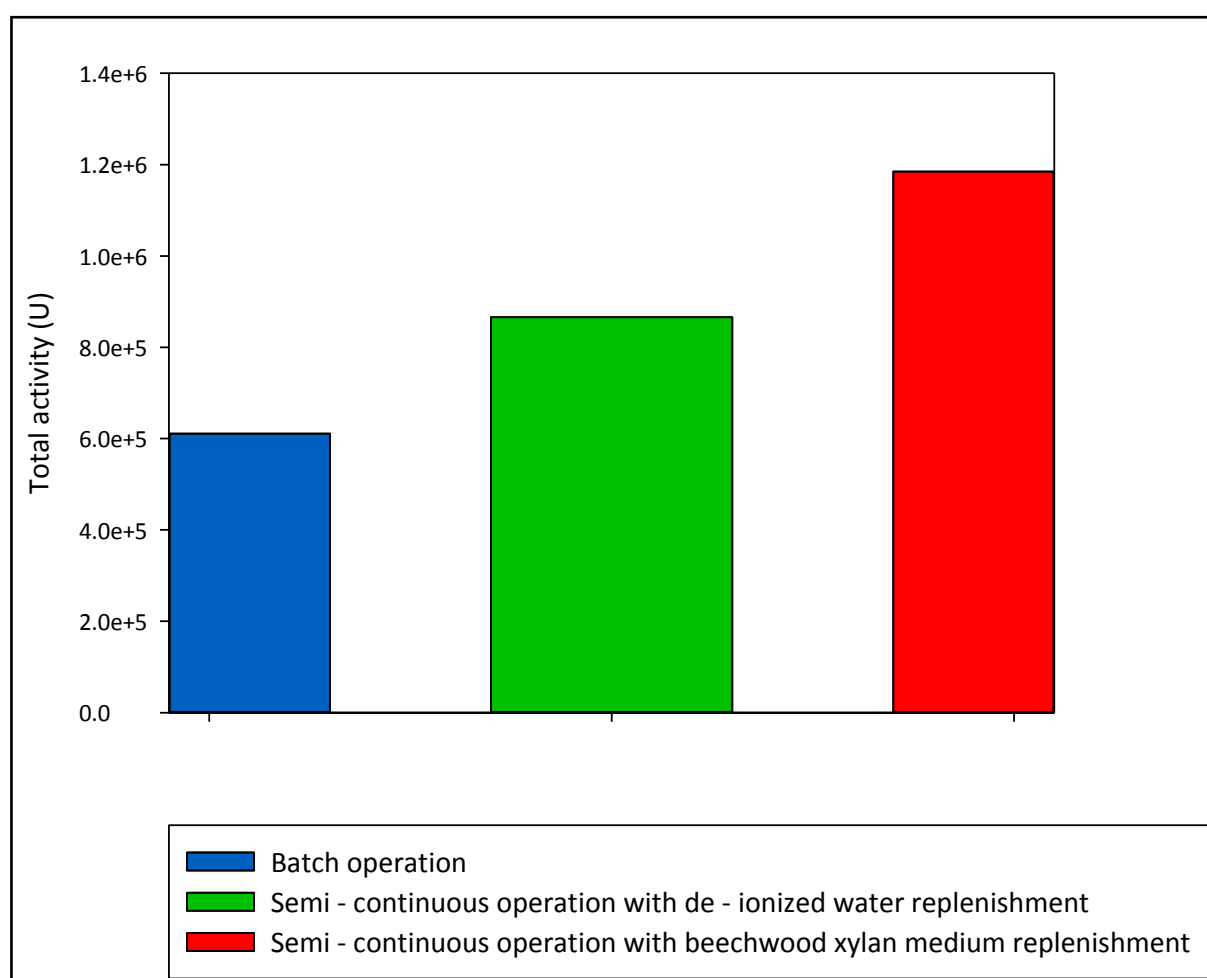
|   |             |
|---|-------------|
| Volume of retentate (mL)                                    | 1000.0000   |
| Activity in retentate after 216 hours (U.mL <sup>-1</sup> ) | 641.3462    |
| Total activity in retentate (U)                             | 641346.1538 |
| Total activity achieved (retentate + permeate) (U)          | 866115.3846 |

**Table 4-7: Total activity of permeate during semi – continuous operation with beechwood xylan nutrient medium supplementation**

| Time (hr) | Activity (U.mL <sup>-1</sup> ) | Volume of permeate (mL) | Total Activity in permeate (U) |
|-----------|--------------------------------|-------------------------|--------------------------------|
| 24        | 2.0000                         | 0                       | 0.0000                         |
| 48        | 111.9231                       | 100                     | 11192.3077                     |
| 72        | 171.8750                       | 100                     | 17187.5000                     |
| 96        | 346.1538                       | 100                     | 34615.3846                     |
| 120       | 433.1731                       | 40                      | 17326.9231                     |
| 144       | 468.2692                       | 40                      | 18730.7692                     |
| 168       | 682.6923                       | 40                      | 27307.6923                     |
| 192       | 939.4231                       | 40                      | 37576.9231                     |
| 216       | 981.7308                       | 40                      | 39269.2308                     |

**Table 4-8: Total activity of retentate during semi – continuous operation with beechwood xylan nutrient medium supplementation**

|  |              |
|--|--------------|
| Volume of retentate (mL)                           | 1000.0000    |
| Activity in retentate after 216 hours (U/mL)       | 981.7308     |
| Total activity in retentate (U)                    | 981730.7692  |
| Total activity achieved (retentate + permeate) (U) | 1184937.5000 |



**Figure 4-17: Effect of mode of operation on total xylanase activity**

Total activity represents the yield of enzyme at the end of the fermentation process. The total xylanase activity achieved was 610 576.92 U, 866 115.38 U and 1 184 937.50 U in batch mode, semi – continuous mode with DI water supplementation and semi – continuous mode with BW xylan medium supplementation. A 1.42 – fold increase is noted between batch and

DI water supplemented semi – continuous mode, equating to an increase in total activity of 41.85 %. The increase in total activity between batch and BW xylan medium supplemented semi – continuous mode is impressive at 1.94 – fold, resulting in a 94.07 % increase.

Kao et al. (2007) suggested that the increase in total activity in membrane mode with DI water supplementation compared to batch mode was because the enzyme activity level in the broth was initially in a balanced state with sufficient enzyme hydrolyzing the substrate to release the required nutrients but the addition of DI water and removal of permeate resulted in a decrease in enzymes available which then stimulated the cells to secrete more enzymes to make up the deficit. Since the enzyme activity is a measure of the ability of the xylanase to convert xylan into xylose, and enzyme activity did not continue to increase with DI water supplementation as it did with BW xylan medium supplementation, it is assumed that after a period the nutrients in the initial medium were depleted and thus biomass growth could not be maintained which in turn resulted in the lower secretion of enzymes.

The high total activity in semi – continuous mode with BW xylan medium supplementation is naturally a result of the higher enzyme activity recorded from 144 hours onwards. The reason for the small difference in total activity between the two semi – continuous operations however, was that 40 mL permeate was obtained from the xylan supplemented experiment compared to 60 mL from the DI water supplemented experiment. This may have been due to fouling of the membrane surface due to the high concentration of nutrients and kinks in the sampling tubes. The high total xylanase activities achieved in semi – continuous mode is significant as it translates into a high yield.

#### **4.4. Experiments conducted in the membrane fermenter over an extended period**

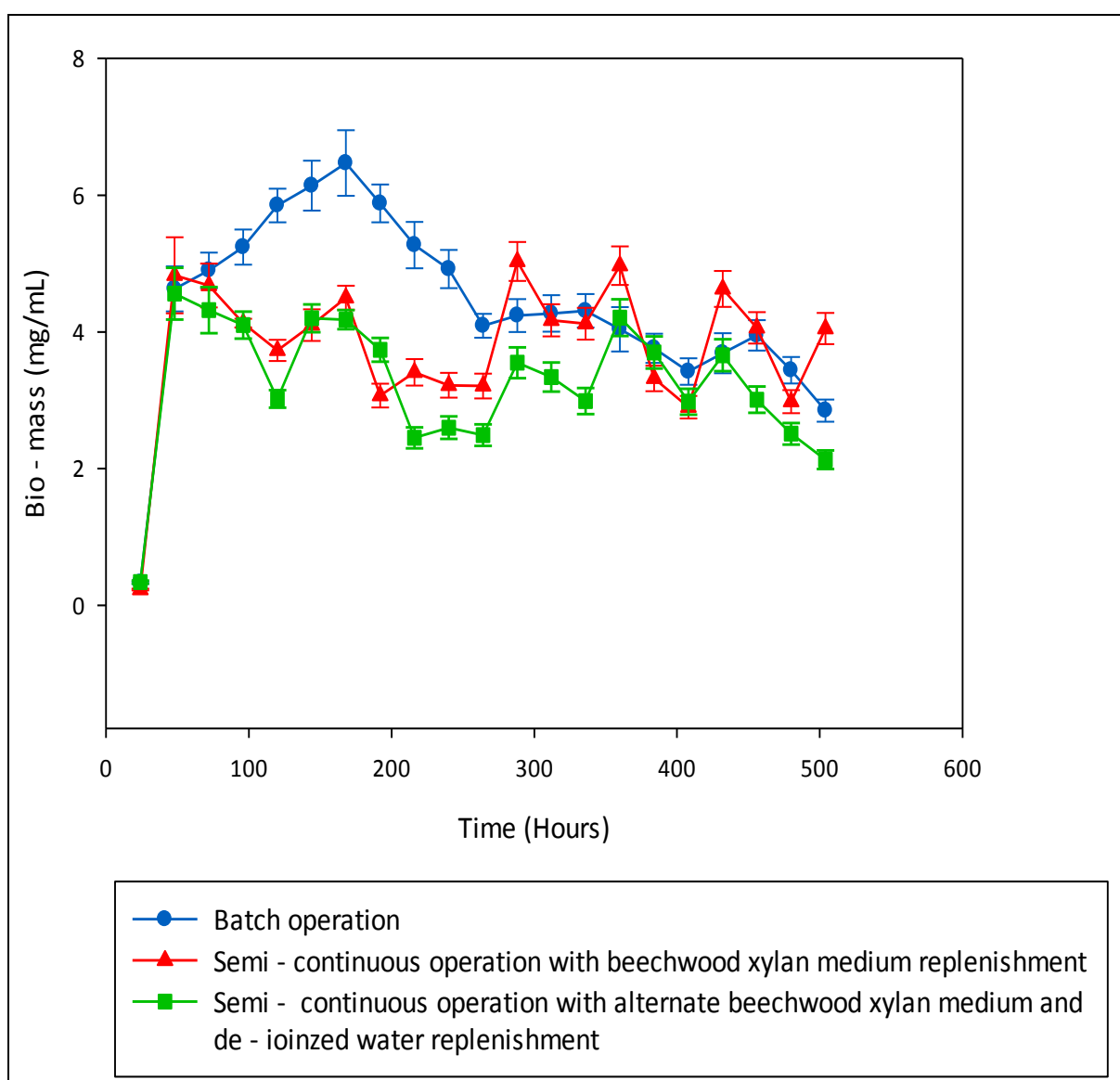
Since no persistent trends in biomass growth or enzyme activity were observed, it was necessary to perform the experiments for an increased duration. The effect of manipulating the DI water feed stream with BW xylan medium alternatively was studied while de – ionized water supplementation was not performed as the previous set of experiments provided



adequate data to draw decisive conclusions from. pH changes as well as the total protein content were measured during these experiments to better comprehend the system.

#### 4.4.1. Effect of mode of operation on dry – weight of the micro – organism over an extended period

Fungal growth trends over long runs are plotted and discussed here.



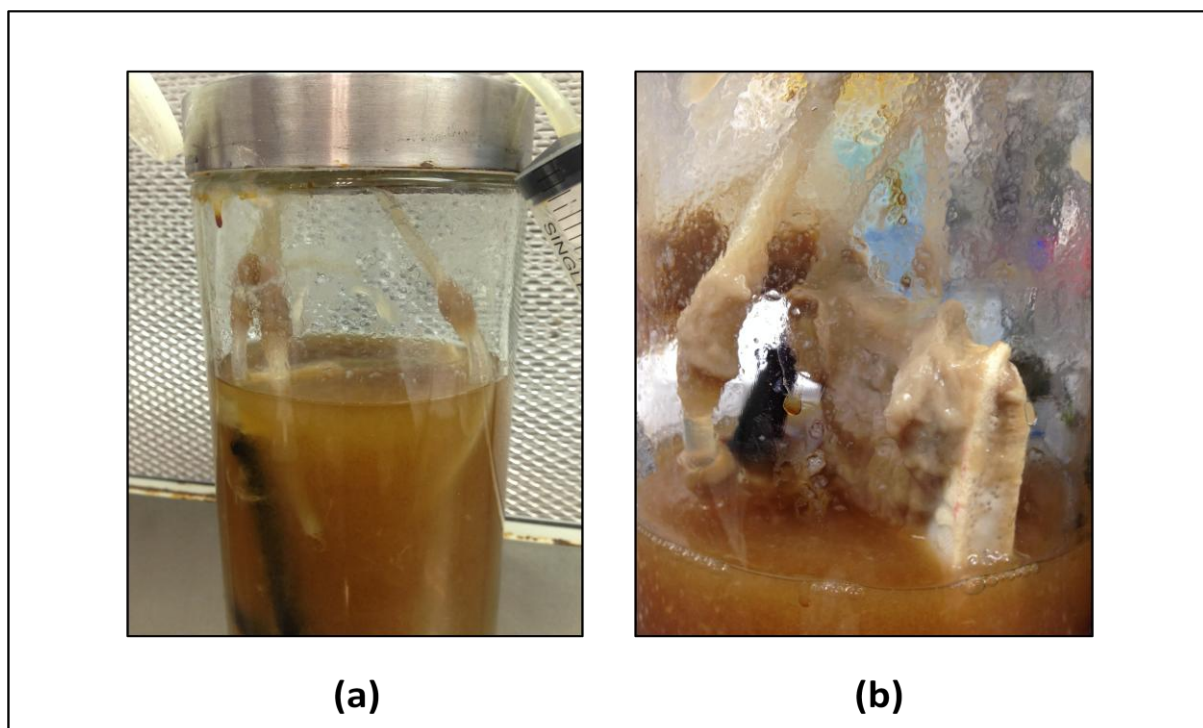
**Figure 4-18: Effect of mode of operation on dry – weight of the micro – organism over an extended period**

Figure 4-18 depicts the biomass recorded for *Thermomyces lanuginosus* DSM 5826 over a 504 hour or 21 day period. An early spike in the biomass was recorded after 48 hours in all three vessels. This is expected as all experiments were run in batch mode during this phase and the results obtained form part of the exponential phase of growth in batch systems. The biomass in the batch system continued to increase until 168 hours when a maximum of  $6.47 \pm 0.48 \text{ mg.mL}^{-1}$  was recorded. Thereafter biomass steadily decreased. The decline in biomass can be a result of depleted nutrients, toxic components secreted by the micro – organism itself and contamination. The oxygen requirements for the micro – organism could also have not been met. Denis and Boyaval (1991) stated that uninterrupted aeration is necessary. The periodic supply of sterile air to all the vessels due to the experiment being performed in a closed shaking incubator is a shortfall that should be overcome in further studies.

Biomass appears to have decreased steadily in both semi – continuous processes until 120 hours. Thereafter the recorded dry weight of biomass in both systems showed no distinctive trends in the upward or downward direction. The biomass appears to have been maintained at approximately  $4.0 \pm 1.0 \text{ mg.mL}^{-1}$  in both systems until 456 hours. The maximum biomass recorded for the semi – continuous operation with beechwood xylan nutrient medium only was  $5.03 \pm 0.28 \text{ mg.mL}^{-1}$  while  $4.56 \pm 0.38 \text{ mg.mL}^{-1}$  was recorded for the alternating nutrient and DI water system.

The method employed to determine the biomass did not take into account fungal growth on the surfaces inside the vessel. This is possibly the reason for the absence of a positive trend in the two semi – continuous plots. After a period of time the micro – organism began growing on the air tubes and on the membrane as the liquid level in the vessel decreased. It has been documented that the morphological growth of fungal organisms affect the rheology of the fermentation broth and thus the performance of the fermenter. Filamentous growth results in highly viscous broths with non – Newtonian, pseudoplastic flow behaviour and the high viscosity impacts negatively on the mass transfer properties especially the gas – liquid mass transfer rate (Papagianni, 2004). Therefore greater agitation is required for adequate agitation and oxygen transfer. It was not possible however to increase the rotational speed further due to the vessel size and volume of the liquid. The poor mass transfer rates could

have thus impacted negatively on recorded submerged fungal growth toward the latter part of the fermentation duration where the biomass decreased. Figure 4-19 below depicts clearly the growth on the pipes and membrane surface. The structure of the micro – organism below is that of a filamentous film, the natural state of the fungi. It is possible that the surfaces provided a preferred growth environment for the micro – organism and influenced positively on its metabolic behaviour.

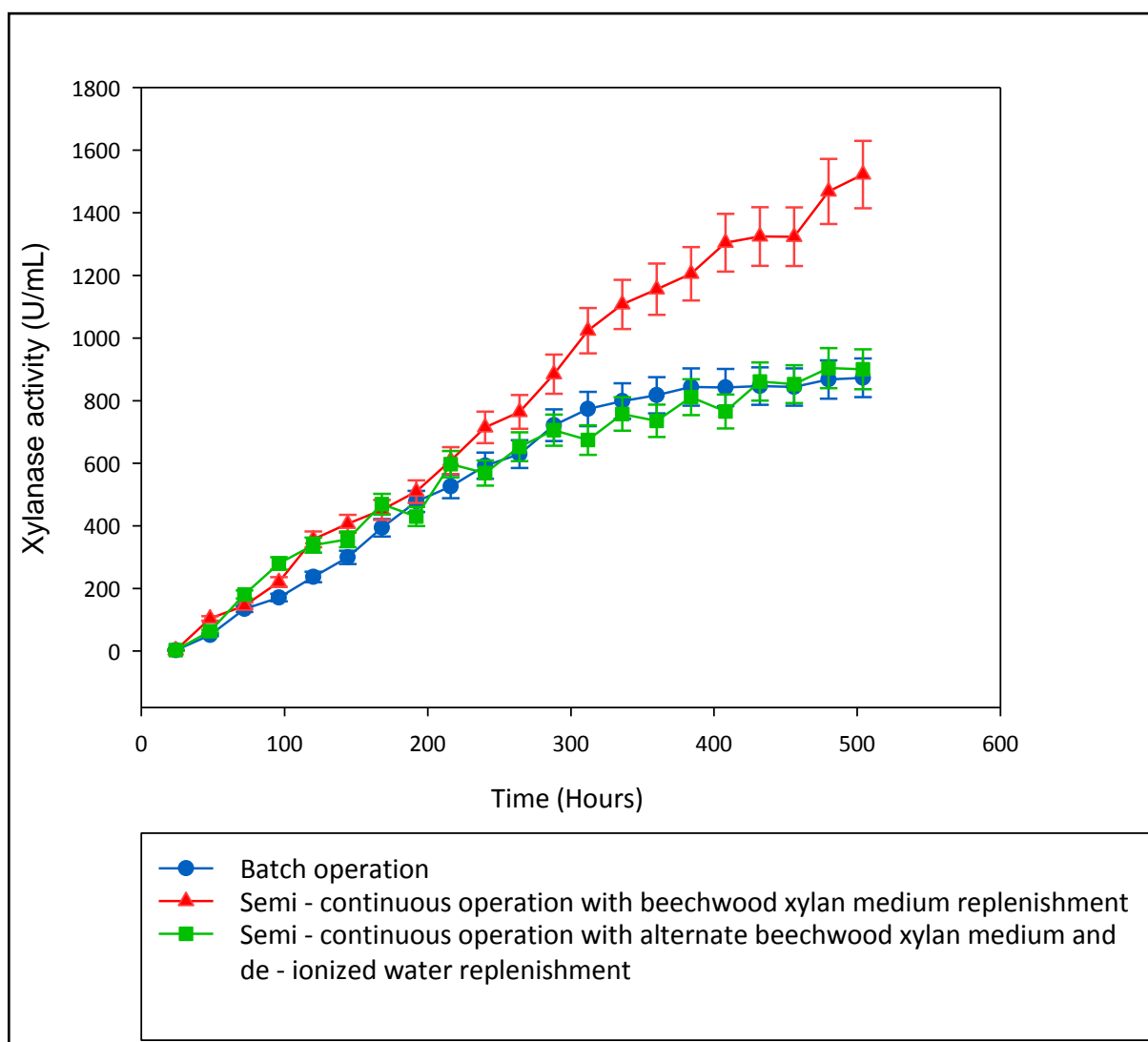


**Figure 4-19: Fungal growth in membrane vessel, (a) Growth after 216 hours, (b) Growth after 384 hours**

It is positive to note however that similar growth rates can be achieved by manipulating the feed nutrient and replacing it with DI water regularly. This technique aimed at coercing the cells into a sustained stationary or maintenance period where there was relatively little substrate used to grow the biomass and thus maximize enzyme production. Here the substrate was replaced with de – ionized water for 48 hours consecutively so that growth was restricted but the enzyme could still be secreted. Nauman (2008) stated that it is sometimes possible to maintain this state for weeks or months and to achieve high volumetric productivities.

#### 4.4.2. Effect of mode of operation on xylanase activity over an extended period

The amounts of xylanase produced over long runs are trended with possible explanations in this section.



**Figure 4-20: Effect of mode of operation on xylanase activity over an extended period**

Xylanase activity was recorded over a 504 hour period, allowing for a substantial amount of data to be collected from which to draw decisive conclusions. The plot above illustrates a marked difference in activities, with a maximum of  $1522.41 \pm 107.65 \text{ U.mL}^{-1}$  being achieved after the complete fermentation duration in the semi – continuous membrane system fed

with beechwood xylan medium only. The batch and manipulated feed membrane system produced xylanase of activities  $873.26 \pm 61.78 \text{ U.mL}^{-1}$  and  $900.70 \pm 63.69 \text{ U.mL}^{-1}$  respectively.

In these experiments the sampling techniques for the permeate stream were improved and thus a standard volume of 50 mL was removed as permeate and replaced with nutrient medium of equal volume. The results indicate that the micro – organism continued to produce enzyme in significant quantities so long as it was supplied with the substrate. Between 408 and 456 hours there was no increase in activity in the system replenished with beechwood xylan nutrient medium only. During this period the enzyme activity recorded was  $1317.62 \pm 11.38 \text{ U.mL}^{-1}$ .

There was no decline in activity in any of the systems indicating that the enzyme retained much of its activity throughout the course of the study. The removal of valuable enzyme in the permeate stream may well be the reason for continued production as well as the removal of toxins in the permeate stream and dilution of these substances when the aliquots of nutrient medium are added.

The increase in production of xylanase between the batch and xylan medium membrane systems was determined to be 74.34 %. Xylanase can thus be produced semi – continuously within a membrane fermenter system yielding increased activities over a substantial amount of time thus saving on cost, time and labour required for non – productive down – time including time for cleaning of the vessel and tubes, recharging the vessel with nutrients and sterilizing, post sterilization cooling, and the considerable lag period following inoculation.

#### 4.4.3 Effect of mode of operation on volumetric productivity over an extended period

**Table 4-9: Volumetric productivity in batch operation over an extended period**

| Time (hr) | Activity (U.mL <sup>-1</sup> ) | Productivity (U.mL <sup>-1</sup> .hr <sup>-1</sup> ) |
|-----------|--------------------------------|--|
| 24        | 2.4498                         | 0.1021   |
| 48        | 51.2365                        | 1.0674   |
| 72        | 134.4668                       | 1.8676   |
| 96        | 170.7883                       | 1.7790   |
| 120       | 236.8624                       | 1.9739   |
| 144       | 299.0726                       | 2.0769   |
| 168       | 393.7403                       | 2.3437   |
| 192       | 477.5889                       | 2.4874   |
| 216       | 525.5023                       | 2.4329   |
| 240       | 591.9629                       | 2.4665   |
| 264       | 629.0572                       | 2.3828   |
| 288       | 721.7929                       | 2.5062   |
| 312       | 773.5703                       | 2.4794   |
| 336       | 799.0726                       | 2.3782   |
| 360       | 817.6198                       | 2.2712   |
| 384       | 843.8949                       | 2.1976   |
| 408       | 842.3493                       | 2.0646   |
| 432       | 846.9861                       | 1.9606   |
| 456       | 843.8949                       | 1.8506   |
| 480       | 867.8516                       | 1.8080   |
| 504       | 873.2612                       | 1.7327   |

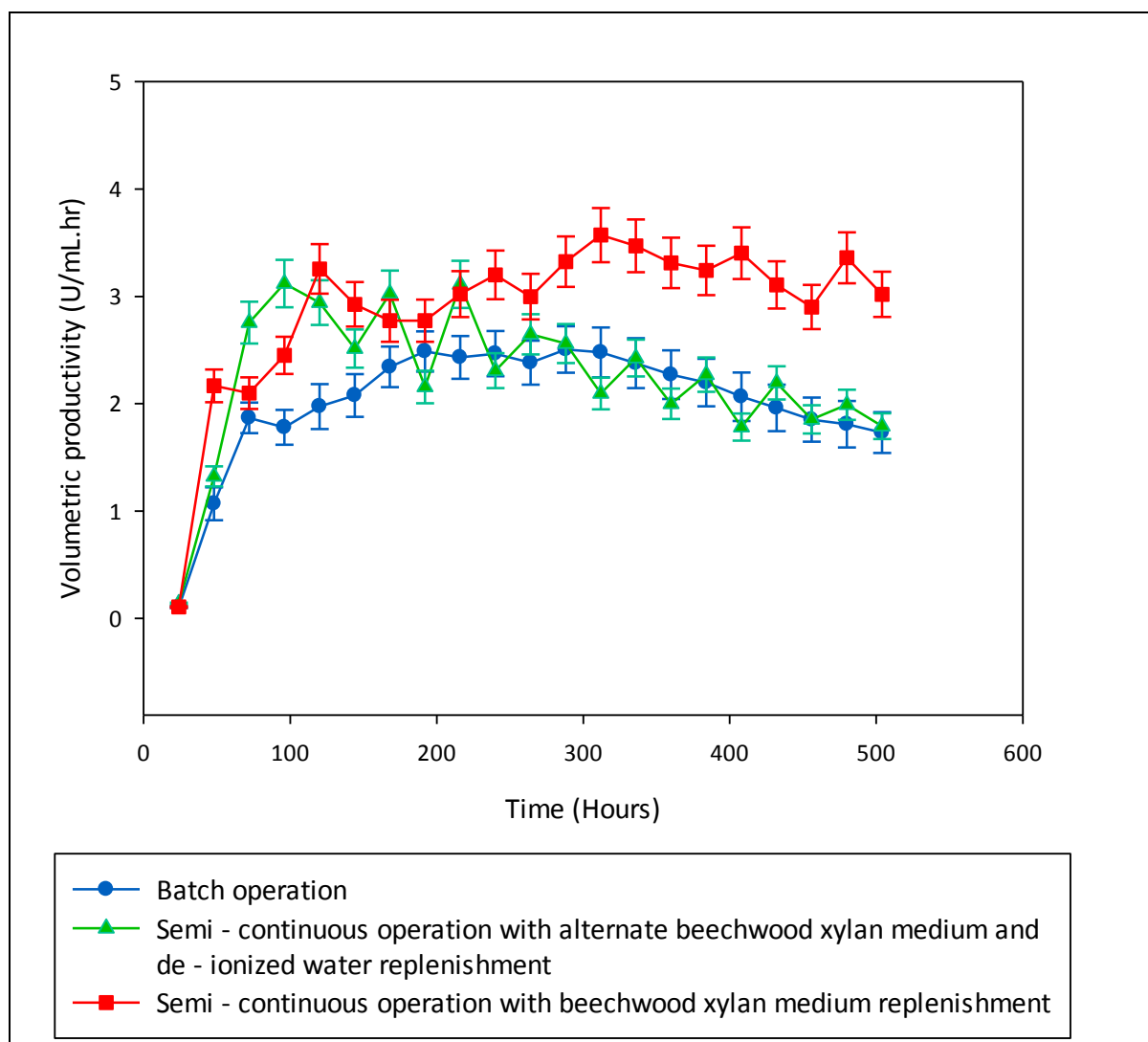
**Table 4-10: Volumetric productivity in semi – continuous operation with DI water and beechwood xylan nutrient medium supplementation over an extended period**

| Time (hr) | Activity (U.mL <sup>-1</sup> ) | Dilution rate (hr <sup>-1</sup> ) | Productivity (U.mL <sup>-1</sup> .hr <sup>-1</sup> ) |
|-----------|--------------------------------|-----------------------------------|--|
| 24        | 3.2767                         | Batch                             | 0.1365   |
| 48        | 63.4467                        | Batch                             | 1.3218   |
| 72        | 180.8346                       | 0.0021                            | 2.7562   |
| 96        | 279.7527                       | 0.0021                            | 3.1202   |
| 120       | 338.4853                       | 0.0021                            | 2.9431   |
| 144       | 356.6461                       | 0.0021                            | 2.5145   |
| 168       | 469.0881                       | 0.0021                            | 3.0264   |
| 192       | 429.6754                       | 0.0021                            | 2.1558   |
| 216       | 596.9861                       | 0.0021                            | 3.1124   |
| 240       | 568.3926                       | 0.0021                            | 2.3087   |
| 264       | 652.6275                       | 0.0021                            | 2.6476   |
| 288       | 705.7573                       | 0.0021                            | 2.5612   |
| 312       | 674.2658                       | 0.0021                            | 2.0955   |
| 336       | 757.3416                       | 0.0021                            | 2.4271   |
| 360       | 735.7032                       | 0.0021                            | 1.9985   |
| 384       | 811.4374                       | 0.0021                            | 2.2709   |
| 408       | 765.8423                       | 0.0021                            | 1.7821   |
| 432       | 861.6692                       | 0.0021                            | 2.1942   |
| 456       | 853.1685                       | 0.0021                            | 1.8533   |
| 480       | 904.1731                       | 0.0021                            | 1.9900   |
| 504       | 900.6955                       | no dilution                       | 1.7871   |

**Table 4-11: Volumetric productivity in semi – continuous operation beechwood xylan nutrient medium supplementation over an extended period**

| Time (hr) | Activity (U.mL <sup>-1</sup> ) | Dilution rate (hr <sup>-1</sup> ) | Productivity (U.mL <sup>-1</sup> .hr <sup>-1</sup> ) |
|-----------|--------------------------------|-----------------------------------|--|
| 24        | 2.5425                         | Batch                             | 0.1059   |
| 48        | 104.0185                       | Batch                             | 2.1671   |
| 72        | 144.8995                       | 0.0021                            | 2.0977   |
| 96        | 220.2473                       | 0.0021                            | 2.4512   |
| 120       | 356.6461                       | 0.0021                            | 3.2562   |
| 144       | 406.4915                       | 0.0021                            | 2.9267   |
| 168       | 450.5410                       | 0.0021                            | 2.7736   |
| 192       | 509.2736                       | 0.0021                            | 2.7748   |
| 216       | 608.1917                       | 0.0021                            | 3.0218   |
| 240       | 714.8377                       | 0.0021                            | 3.2007   |
| 264       | 764.2968                       | 0.0021                            | 2.9981   |
| 288       | 884.8532                       | 0.0021                            | 3.3236   |
| 312       | 1023.9567                      | 0.0021                            | 3.5717   |
| 336       | 1107.6507                      | 0.0021                            | 3.4709   |
| 360       | 1156.1051                      | 0.0021                            | 3.3123   |
| 384       | 1205.5641                      | 0.0021                            | 3.2425   |
| 408       | 1304.4822                      | 0.0021                            | 3.4033   |
| 432       | 1324.5750                      | 0.0021                            | 3.1080   |
| 456       | 1323.8022                      | 0.0021                            | 2.9015   |
| 480       | 1468.3153                      | 0.0021                            | 3.3601   |
| 504       | 1522.4111                      | no dilution                       | 3.0207   |





**Figure 4-21: Effect of mode of operation on volumetric productivity over an extended period**

Productivity in the batch system increased linearly during the first 196 hours consistent with the logarithmic production of xylanase enzymes during this period. A value of  $2.49 \pm 0.19$  U.mL<sup>-1</sup>.hr<sup>-1</sup> was obtained here. The productivity then remained fairly constant up to 312 hours. The constant rate of production is consistent with the linear increase in xylanase activity recorded during this period. The maximum value of  $2.51 \pm 0.22$  U.mL<sup>-1</sup>.hr<sup>-1</sup> was recorded after 288 hours. Productivity then began to decline steadily until the end of the fermentation period. The decreasing rate of production is a result of the constant xylanase activities recorded during this period which is due to the stable nature of the protein.

The first 96 hours of fermentation saw a rapid increase in the rate of production in the vessel marked for membrane fermentation with an alternate feed cycle between de – ionized water and beechwood xylan nutrient medium. The operation was run in batch mode for the first 48 hours after which the membrane was inserted and 50 mL of permeate was withdrawn and replaced with 50 mL of DI water. The addition of water would have diluted the xylanase concentration and caused the micro – organism to secrete more. This result is positive as it was the aim of the operation. The maximum recorded productivity achieved in this system was  $3.12 \pm 0.22 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 96 hours of production. Thereafter the productivity achieved was erratic, decreasing after DI water addition before reaching the maximum again. This trend was observed for 120 hours or 5 days. The decrease in productivity is due to the decrease in activity following DI water addition, while the increase to high productivities is due to the spike in xylanase secretion when substrate rich medium is added. From Figure 4-21 it is encouraging to note that the peaks achieved in productivity values were on par with those achieved when only xylan medium was added. This manipulated feed operation then could well be used as a cost saving mechanism so that expensive substrate medium can be substituted with de – ionized water intermittently during the period where xylanase activities are at its maximum.

The plot depicting the productivity achieved in the membrane fermenter operated in semi – continuous mode with beechwood xylan nutrient medium replenishment shows a sharp increase in productivity during the first 120 hours when  $3.26 \pm 0.23 \text{ U.mL}^{-1}.\text{hr}^{-1}$  was recorded, consistent with the similarly sharp increases in xylanase activity during this period. Productivity then decreased until 192 hours. During this phase the growth of the micro – organism was the lowest and the rate of enzyme activity recorded was also lower. This may have been due to many factors of which the possibilities include, insufficient mass transfer resulting in oxygen and nutrient deficiency, inconsistent sterile air input, contamination etc. After 192 hours, the productivity increased to a maximum of  $3.57 \pm 0.25 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 312 hours. Productivity then decreased to  $2.90 \pm 0.21 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 456 hours. A broader view of the plot however, reveals that the productivity was considerably well maintained between 120 and 504 hours with an average of  $3.15 \pm 0.24 \text{ U.mL}^{-1}.\text{hr}^{-1}$  being achieved during this period. An overall average increase in productivity between the batch and xylan fed membrane system was 43.25%.

#### 4.4.4 Effect of mode of operation on total activity over an extended period

The total activity is a measure of the enzyme in the entire volume of liquid involved in the experiment, a sum of permeate and retentate volumes for the semi – continuous operations and simply the batch volume in that instance.

**Table 4-12: Total activity in batch operation over an extended period**

|  |             |
|--|-------------|
| Activity in batch vessel after 504 hours (U.mL <sup>-1</sup> ) | 873.2612    |
| Total activity in batch vessel (U)                             | 873216.2056 |

**Table 4-13: Total activity of permeate during semi – continuous operation with alternate DI water and beechwood xylan nutrient medium supplementation over an extended period**

| Time (Hr) | Activity (U.mL <sup>-1</sup> ) | Volume of permeate (mL) | Total activity in permeate (U) |
|-----------|--------------------------------|-------------------------|--------------------------------|
| 24        | 3.2767                         | 0                       | -                              |
| 48        | 63.4467                        | 0                       | -                              |
| 72        | 132.5348                       | 50                      | 6626.7388                      |
| 96        | 245.3632                       | 50                      | 12268.1607                     |
| 120       | 285.5487                       | 50                      | 14277.4343                     |
| 144       | 341.1901                       | 50                      | 17059.5054                     |
| 168       | 435.0850                       | 50                      | 21754.2504                     |
| 192       | 419.6291                       | 50                      | 20981.4529                     |
| 216       | 548.6862                       | 50                      | 27434.3122                     |
| 240       | 518.5471                       | 50                      | 25927.3570                     |
| 264       | 596.5997                       | 50                      | 29829.9845                     |
| 288       | 674.2658                       | 50                      | 33713.2921                     |

|     |          |       |             |
|-----|----------|-------|-------------|
| 312 | 607.0325 | 50    | 30351.6229  |
| 336 | 733.3849 | 50    | 36669.2427  |
| 360 | 726.8161 | 50    | 36340.8037  |
| 384 | 784.3895 | 50    | 39219.4745  |
| 408 | 726.4297 | 50    | 36321.4838  |
| 432 | 842.3493 | 50    | 42117.4652  |
| 456 | 821.4838 | 50    | 41074.1886  |
| 480 | 884.0804 | 50    | 44204.0186  |
|     |          | Total | 516170.7883 |

**Table 4-14: Total activity of retentate during semi – continuous operation with alternate DI water and beechwood xylan nutrient medium supplementation**

|   |              |
|---|--------------|
| Volume of retentate (mL)                                  | 1000         |
| Activity in retentate after 21 days (U.mL <sup>-1</sup> ) | 875.9660     |
| Total activity in retentate (U)                           | 875965.9969  |
| Total activity achieved (retentate + permeate) (U)        | 1392136.7852 |

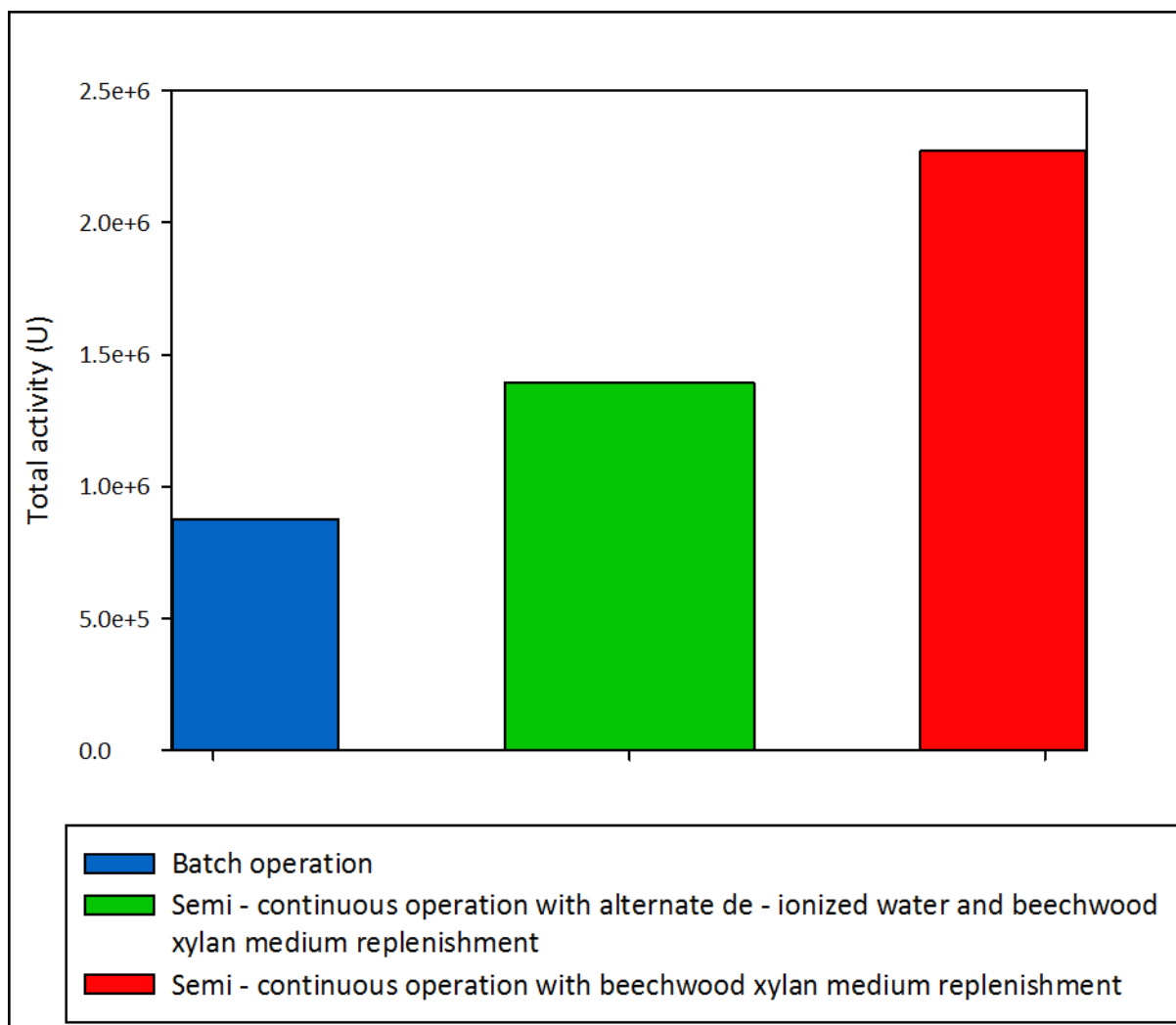
**Table 4-15: Total activity of permeate during semi – continuous operation with beechwood xylan nutrient medium supplementation**

| Time (Days) | Activity (U.mL <sup>-1</sup> ) | Vol permeate (mL) | Total Activity in permeate (U) |
|-------------|--------------------------------|-------------------|--------------------------------|
| 24          | 2.5425                         | 0                 | -                              |
| 48          | 104.0185                       | 0                 | -                              |
| 72          | 144.8995                       | 50                | 7244.9768                      |
| 96          | 220.2473                       | 50                | 11012.3648                     |
| 120         | 356.6461                       | 50                | 17832.3029                     |

|     |           |       |            |
|-----|-----------|-------|------------|
| 144 | 406.4915  | 50    | 20324.5750 |
| 168 | 450.5410  | 50    | 22527.0479 |
| 192 | 509.2736  | 50    | 25463.6785 |
| 216 | 608.1917  | 50    | 30409.5827 |
| 240 | 714.8377  | 50    | 35741.8856 |
| 264 | 764.2968  | 50    | 38214.8377 |
| 288 | 884.8532  | 50    | 44242.6584 |
| 312 | 1023.9567 | 50    | 51197.8362 |
| 336 | 1107.6507 | 50    | 55382.5348 |
| 360 | 1156.1051 | 50    | 57805.2550 |
| 384 | 1205.5641 | 50    | 60278.2071 |
| 408 | 1304.4822 | 50    | 65224.1113 |
| 432 | 1324.5750 | 50    | 66228.7481 |
| 456 | 1323.8022 | 50    | 66190.1082 |
| 480 | 1468.3153 | 50    | 73415.7651 |
|     |           | Total | 748736.476 |

**Table 4-16: Total activity of retentate during semi – continuous operation with beechwood xylan nutrient medium supplementation**

|   |              |
|---|--------------|
| Volume of retentate (mL)                                  | 1000         |
| Activity in retentate after 21 days (U.mL <sup>-1</sup> ) | 1522.4111    |
| Total activity in retentate (U)                           | 1522411.1283 |
| Total activity achieved (retentate + permeate) (U)        | 2271147.6043 |

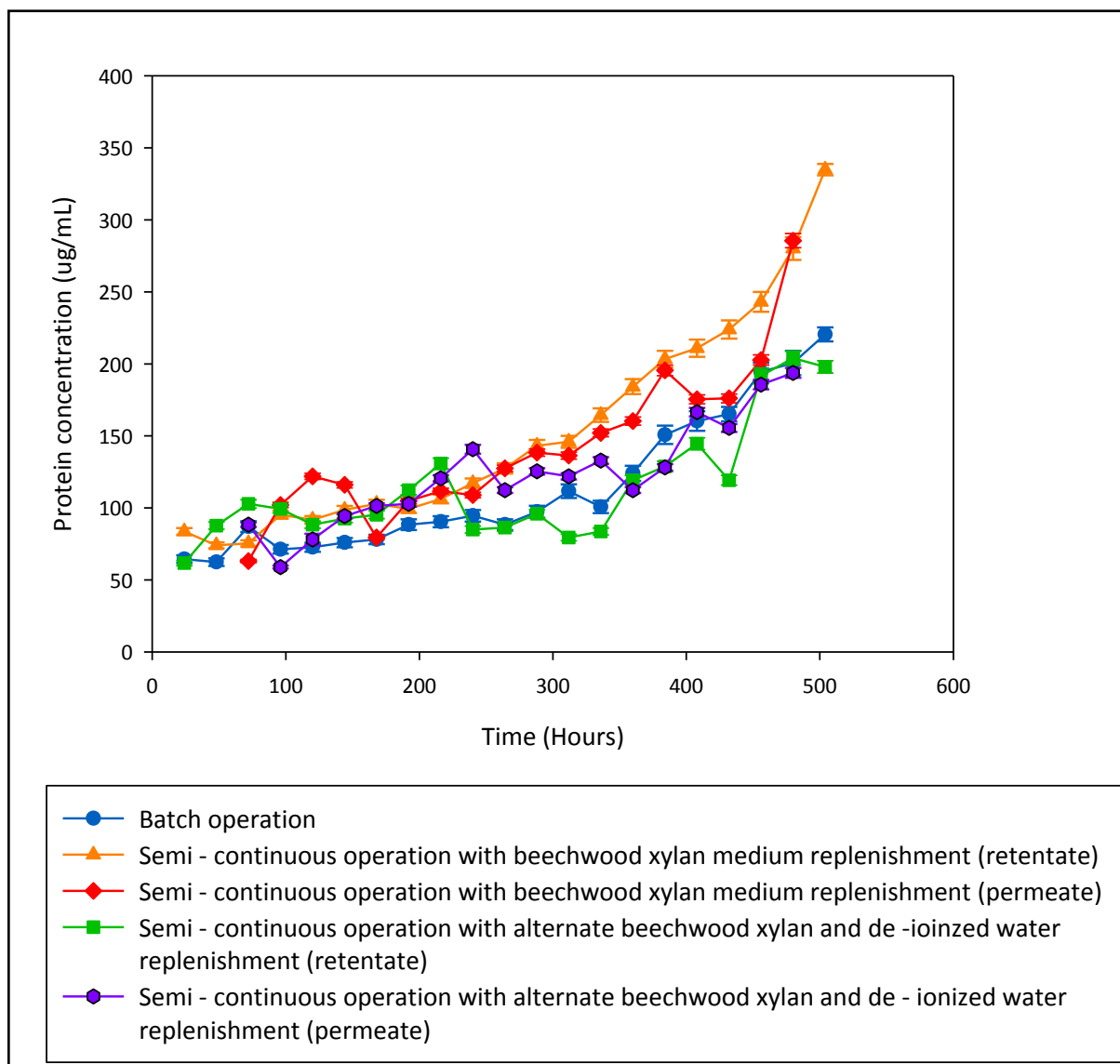


**Figure 4-22: Effect of mode of operation on total xylanase activity over an extended period**

The bar graph above provides a clearly distinguishable difference between the three experiments conducted. After 504 hours, the total activity obtained in batch process was 873 216.21 U while 1 392 136.79 U was achieved in the semi – continuous membrane operation with alternating feed, 59 % more than the batch process. 2 271 147.60 U was the total xylanase activity obtained for the semi – continuous operation with beechwood xylan nutrient medium feed which was 160 % greater than the batch process. This process offers a 2.6 – fold increase in total enzyme activity compared to conventional batch processing. The benefit of an increased enzyme yield is also coupled with the benefit of the permeate stream already being free of solids and therefore semi – processed with only the retentate having a separation stage requirement.

#### 4.4.5 Effect of mode of operation on total protein concentration over an extended period

The total protein was measured using the Bradford method of detection. Samples from both permeate and retentate streams were analyzed. This analysis was performed so as to ascertain whether the membrane had an effect on the protein concentration.



**Figure 4-23: Effect of mode of operation on total protein concentration over an extended period**

The protein concentration recorded in the batch system increased very gradually from 64.38  $\pm 2.73 \mu\text{g.mL}^{-1}$  after 24 hours to 220.55  $\pm 4.85 \mu\text{g.mL}^{-1}$  recorded after 504 hours. In contrast,

the protein concentration recorded in the retentate of the semi – continuous system with only beechwood xylan nutrient medium replenishment after 504 hours was  $334.93 \pm 3.97 \mu\text{g.mL}^{-1}$ .

The trends for the retentate and permeate streams show that for the period between 96 and 144 hour the protein concentration in the permeate stream was higher than that in the retentate stream. This could possibly be due to the membrane removing more enzyme or protein, thereby concentrating it while the retentate stream became dilute with the addition of more nutrient medium. After 288 hours however, it is evident that the protein concentration in the retentate stream was higher than that of the permeate stream. Other studies using membrane systems have encountered protein adsorption on the membranes and enzyme inactivation by shear stress due to pumping and high turbulence through the device. Since no pump was used and the manual suctioning was slow and less rigorous, it is assumed that very little if any damage to the enzymes occurred. However, the occurrence of fouling on the membrane system cannot be disregarded as a reason for lower protein concentration in the permeate stream as compared to the retentate inside the fermenter vessel. Denis and Boyaval (1991) stated that the retention of enzymes in their bioreactor during continuous fermentation was due to fouling of the membrane.

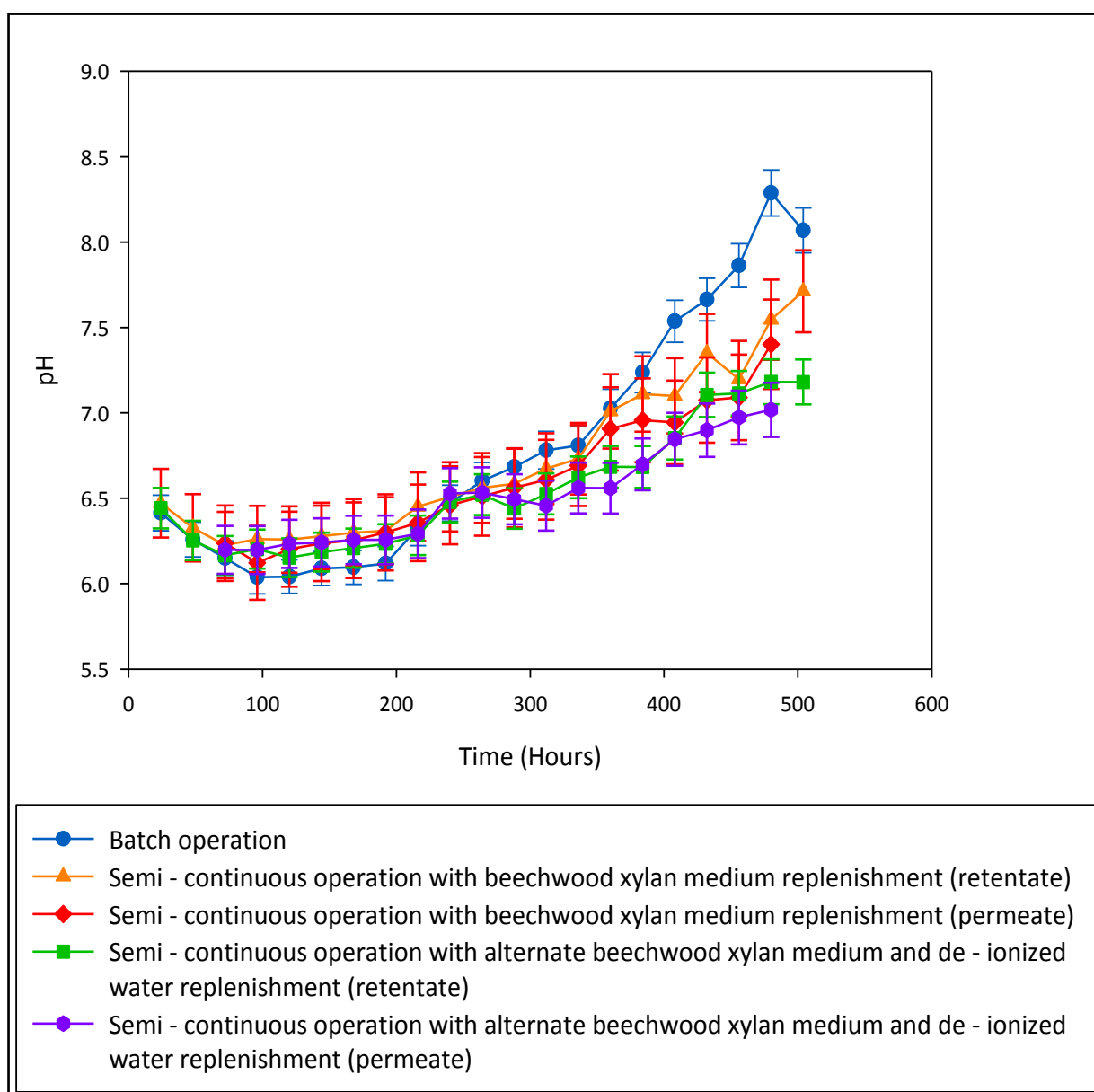
An opposing trend was observed for the alternating feed system. During the first 288 hours of fermentation the retentate contained a greater concentration of total protein than the permeate stream, after which the protein concentration in the permeate stream was higher than that recorded in the retentate stream. After 504 hours the total protein in the retentate was  $197.95 \pm 4.28 \mu\text{g.mL}^{-1}$ . This indicates that the membrane in this system may have experienced fouling to a lesser degree. A possible reason is that the de – ionized water may have dislodged particles including undissolved beechwood xylan and yeast extract powder, as well as fungal hyphae from the membrane surface into the broth thereby washing the membrane surface somewhat. Thus the passage of a larger amount of proteins into the permeate stream was allowed as compared to a severely fouled system which would have hindered filtration.



The total protein concentrations depicted in Figure 4-23 above, correspond proportionally to the xylanase activity achieved in Figure 4-20 for each of the systems. This is as expected, that is to say, the greater the protein concentration, the greater the enzyme activity.

#### 4.4.6 Effect of mode of operation on pH over an extended period

The pH of the samples were measured daily and compared to ascertain if there were any significant trends. These trends are depicted and discussed here.



**Figure 4-24: Effect of mode of operation on pH over an extended period**

Figure 4-24 illustrates a distinction in the pH measured during the course of the fermentation process for each mode. The pH was measured in both the retentate and permeate of the membrane systems. The pH recorded in the batch system remained fairly constant from the start of the process at  $6.17 \pm 0.13$  for the first 240 hours, after which the pH began to increase steadily. The maximum pH recorded was  $8.29 \pm 0.13$  after 480 hours. The retentate sample of the beechwood xylan membrane system showed a similar trend, with pH being maintained at  $6.32 \pm 0.09$  until 240 hours when it began to increase to a maximum of  $7.55 \pm 0.24$  after 480 hours. Similarly, the pH recorded for the first 240 hours in the DI water/beechwood xylan membrane system retentate was  $6.32 \pm 0.09$  and increased to  $7.18 \pm 0.13$  after 480 hours.

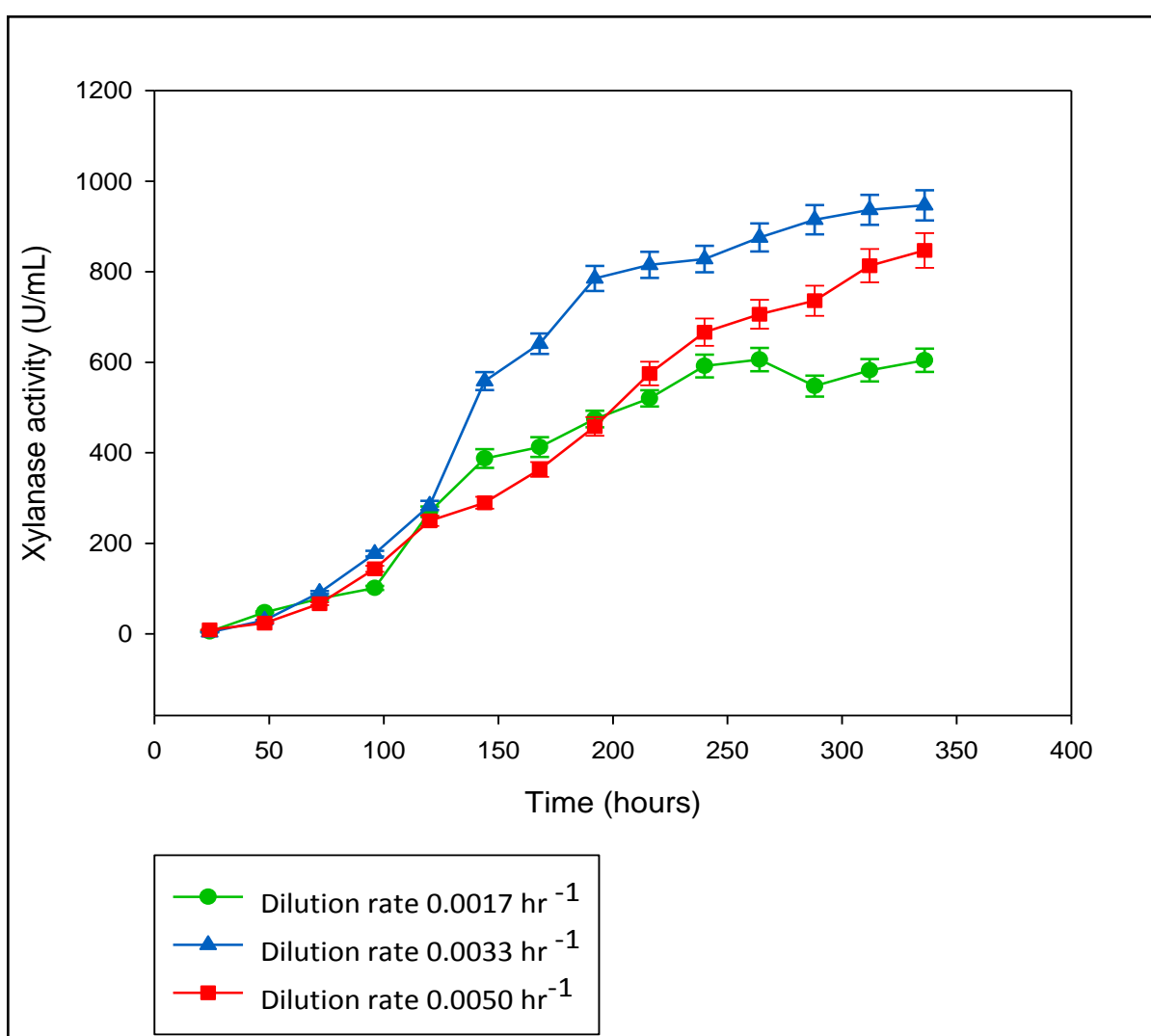
The pH of the permeate streams for both the membrane systems were slightly lower than that of the retentate but produced similar trends. It is important to maintain the pH at the desired value and to minimize its fluctuations in the fermenter liquid, however, pH is a parameter which is difficult to control. Due to the small scale of the experiments and the fact that the vessels were placed in shaking incubators for the duration of the experimentation, automated pH controllers were not installed.

The results obtained here form a platform for further work, providing the expected pH range in these systems. Earlier work presented gave the desired pH value to be 6.5 for optimal xylanase production. The pH in the alternating feed membrane system is the closest in range to this value, followed by the beechwood xylan nutrient medium membrane system, while the pH recorded in the batch system is higher. This could possibly be due to the effect of the de-ionized water on the pH of the beechwood xylan nutrient medium which, prior to adjustment is 5.5. The trend observed for all three systems is curious as the pH increases during the course of the study while it has been stated that biological metabolisms usually lead to secretion of organic acids into the medium. Therefore they are normally associated with a decline in pH (Kariminiaae-Hamedani et al., 2005). In saying as much, the alkaline tendency of the culture medium was also observed by Kumar et al. (2009) during the end of cultivation of a *Thermomyces lanuginosus* MC 134 mutant on corn cobs for the production of xylanase.

## 4.5. Optimization of dilution rate

The dilution rate was varied between 3 values. 40 mL, 80 mL and 120 mL of permeate was withdrawn from three membrane fermenter vessels and replaced equivalent volumes of beechwood xylan nutrient medium. The effect of this variation on enzyme activity and volumetric productivity are presented in this section.

### 4.5.1. Effect of different dilution rates on enzyme activity



**Figure 4-25: Effect of varying dilution rates on xylanase activity**

The effect of varying dilution rates on xylanase activity is depicted in Figure 4-25. The graph distinctly portrays this effect with the maximum xylanase being produced after 336 hours. The xylanase activity achieved after 336 hours was  $604.76 \pm 25.68 \text{ U.mL}^{-1}$ ,  $946.83 \pm 33.48 \text{ U.mL}^{-1}$  and  $847.22 \pm 38.34 \text{ U.mL}^{-1}$  for dilution rates of  $0.0017 \text{ hr}^{-1}$ ,  $0.0033 \text{ hr}^{-1}$  and  $0.0050 \text{ hr}^{-1}$  respectively.

A possible reason for the xylanase activity of the highest dilution rate being lower than at a rate of  $0.0033 \text{ hr}^{-1}$  is provided by Okafor (2007) who stated that the rate of nutrient in flow and broth out flow relates to the generation time or growth rate of the micro – organism. If the rate of nutrient addition is too high, then sufficient time is denied to the micro – organism to develop an adequate population and hence produce enzymes.

On the other hand, Okafor (2007) also states that if the rate of nutrient addition is too low, a stationary phase may set in and the population may begin to decline. This theory is validated by the trend observed for the system with the lowest dilution rate of  $0.0017 \text{ hr}^{-1}$  where the xylanase activity appears constant from 240 hours until the end of the fermentation.

It is usually unwise to operate a continuous fermentation system with a dilution rate greater than the maximum growth rate due to wash out of the culture, but this system affords the opportunity to do so as the fungal culture is retained by the membrane. The membrane fermenter system enables fungal growth at varying dilution rates without the need for cell recycle.

#### 4.5.2 Effect of different dilution rates on volumetric productivity

**Table 4-17: Volumetric productivity in semi – continuous operation at a dilution rate of  $0.0017 \text{ hr}^{-1}$**

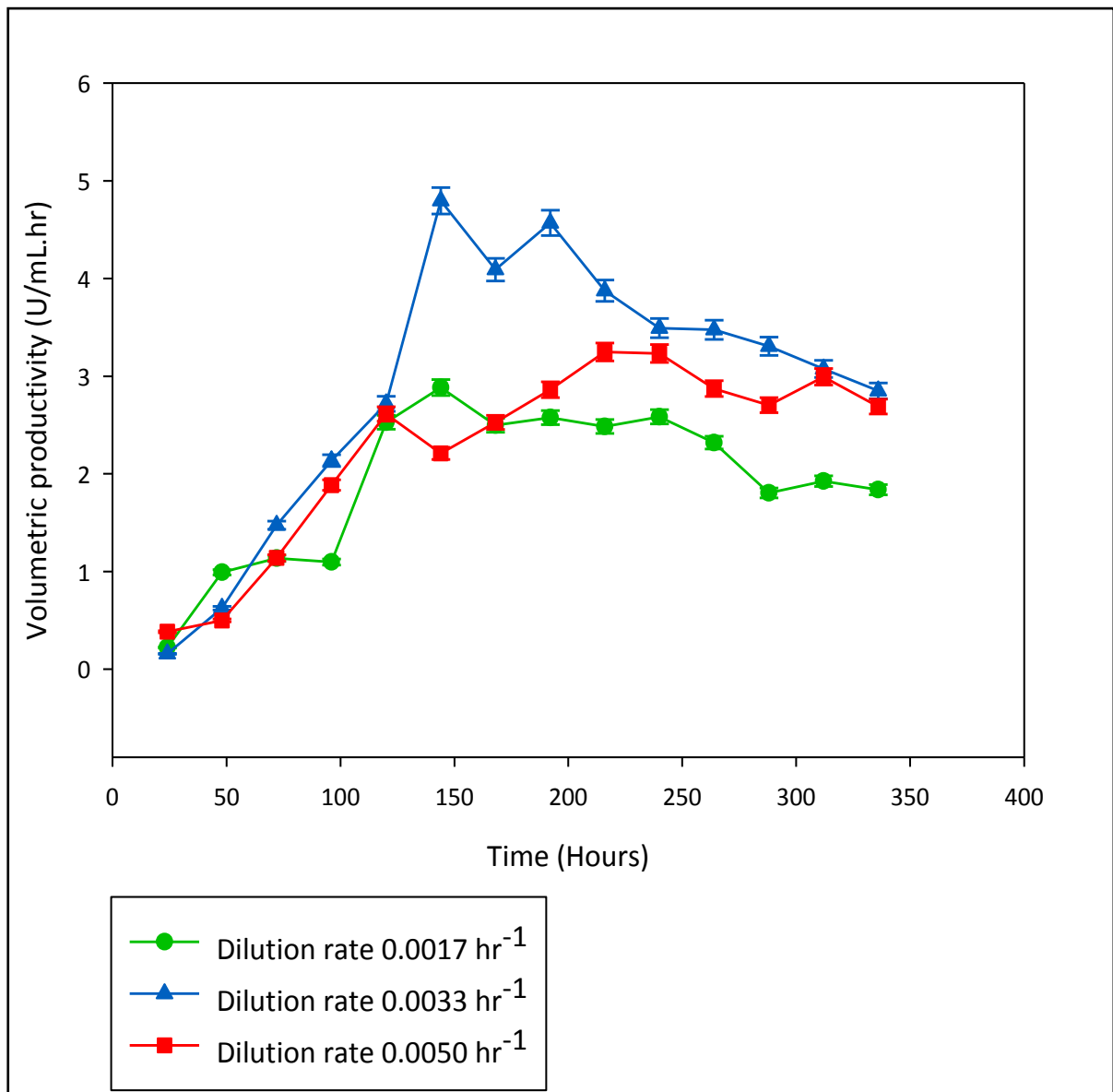
| Time (hr) | Activity ( $\text{U.mL}^{-1}$ ) | Dilution rate ( $\text{hr}^{-1}$ ) | Productivity ( $\text{U.mL}^{-1}.\text{hr}^{-1}$ ) |
|-----------|---------------------------------|------------------------------------|--|
| 24        | 5.3333                          | batch                              | 0.2222   |
| 48        | 47.6190                         | batch                              | 0.9921   |
| 72        | 78.0952                         | 0.0017                             | 1.1354   |
| 96        | 101.5873                        | 0.0017                             | 1.0974   |
| 120       | 269.8413                        | 0.0017                             | 2.5291   |
| 144       | 387.3016                        | 0.0017                             | 2.8854   |
| 168       | 412.6984                        | 0.0017                             | 2.4989   |
| 192       | 474.9206                        | 0.0017                             | 2.5772   |
| 216       | 520.6349                        | 0.0017                             | 2.4865   |
| 240       | 592.0635                        | 0.0017                             | 2.5860   |
| 264       | 606.3492                        | 0.0017                             | 2.3206   |
| 288       | 547.6190                        | 0.0017                             | 1.8036   |
| 312       | 582.5397                        | 0.0017                             | 1.9253   |
| 336       | 604.7619                        | 0.0017                             | 1.8369   |

**Table 4-18: Volumetric productivity in semi – continuous operation at a dilution rate of 0.0033 hr<sup>-1</sup>**

| Time (hr) | Activity (U.mL <sup>-1</sup> ) | Dilution rate (hr <sup>-1</sup> ) | Productivity (U.mL <sup>-1</sup> .hr <sup>-1</sup> ) |
|-----------|--------------------------------|-----------------------------------|--|
| 24        | 3.7857                         | batch                             | 0.1577   |
| 48        | 30.0000                        | batch                             | 0.6250   |
| 72        | 91.4286                        | 0.0033                            | 1.4746   |
| 96        | 177.3810                       | 0.0033                            | 2.1342   |
| 120       | 283.7302                       | 0.0033                            | 2.7189   |
| 144       | 558.7302                       | 0.0033                            | 4.7967   |
| 168       | 641.2698                       | 0.0033                            | 4.0922   |
| 192       | 785.3175                       | 0.0033                            | 4.5704   |
| 216       | 815.4762                       | 0.0033                            | 3.8759   |
| 240       | 828.1746                       | 0.0033                            | 3.4931   |
| 264       | 875.7937                       | 0.0033                            | 3.4761   |
| 288       | 915.0794                       | 0.0033                            | 3.3083   |
| 312       | 936.9048                       | 0.0033                            | 3.0757   |
| 336       | 946.8254                       | 0.0033                            | 2.8510   |

**Table 4-19: Volumetric productivity in semi – continuous operation at a dilution rate of  $0.0050 \text{ hr}^{-1}$**

| Time (hr) | Activity ( $\text{U.mL}^{-1}$ ) | Dilution rate ( $\text{hr}^{-1}$ ) | Productivity ( $\text{U.mL}^{-1}.\text{hr}^{-1}$ ) |
|-----------|---------------------------------|------------------------------------|--|
| 24        | 9.2063                          | batch                              | 0.3836   |
| 48        | 24.0079                         | batch                              | 0.5002   |
| 72        | 66.6667                         | 0.0050                             | 1.1392   |
| 96        | 143.8492                        | 0.0050                             | 1.8843   |
| 120       | 250.000                         | 0.0050                             | 2.6141   |
| 144       | 289.6825                        | 0.0050                             | 2.2101   |
| 168       | 363.0952                        | 0.0050                             | 2.5283   |
| 192       | 458.3333                        | 0.0050                             | 2.8633   |
| 216       | 575.3968                        | 0.0050                             | 3.2492   |
| 240       | 666.6667                        | 0.0050                             | 3.2341   |
| 264       | 706.3492                        | 0.0050                             | 2.8740   |
| 288       | 736.1111                        | 0.0050                             | 2.7048   |
| 312       | 813.4921                        | 0.0050                             | 2.9943   |
| 336       | 847.2222                        | 0.0050                             | 2.6901   |



**Figure 4-26: Effect of varying dilution rates on volumetric productivity**

The volumetric productivity obtained in each of the three systems is directly proportional to the rate of xylanase product in each of the systems. The irregularities in permeate and hence feed volumes as experienced in the first exercise did not factor in the productivity volumes for this experiment as all volumes were kept constant during the 14 day course of the fermentation.

As mentioned in the section above, 40 mL, 80 mL and 120 mL of permeate was removed daily from the three systems and replenished with an aliquot of beechwood xylan nutrient medium

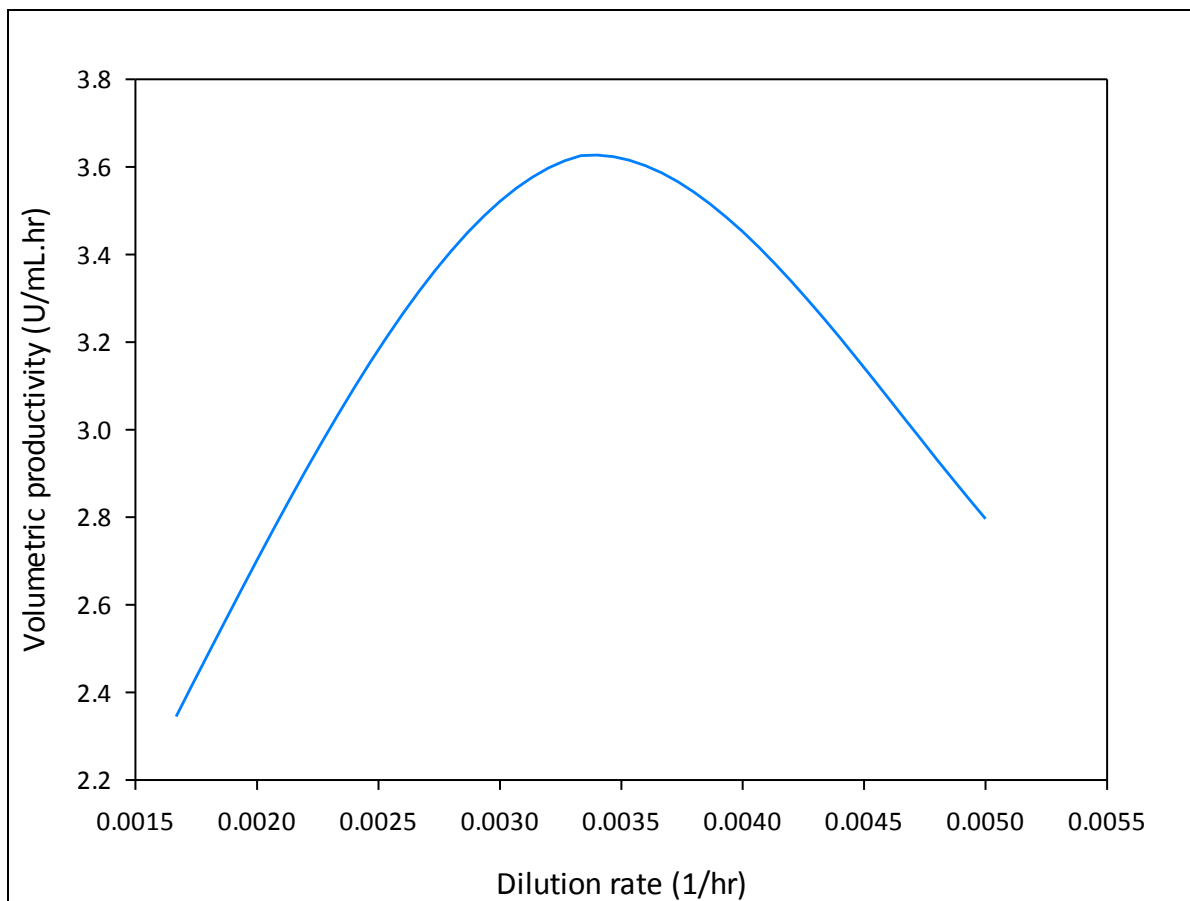


of equal volume. Figure 4-26 indicates that the maximum volumetric productivity achieved with a dilution rate of  $0.0017 \text{ hr}^{-1}$  was  $2.89 \pm 0.08 \text{ U.mL}^{-1}.\text{hr}^{-1}$  after 144 hours. Thereafter the volumetric productivity decreased before remained fairly constant at  $2.54 \pm 0.05 \text{ U.mL}^{-1}.\text{hr}^{-1}$  until 240 hours when the productivity decreased to  $1.84 \pm 0.05 \text{ U.mL}^{-1}.\text{hr}^{-1}$  on the last day of fermentation.

After 144 hours the volumetric productivity calculated for a dilution rate of  $0.0033 \text{ hr}^{-1}$  was  $4.80 \pm 0.14 \text{ U.mL}^{-1}.\text{hr}^{-1}$ . A 66.09 % increase in volumetric productivity was realized via a dilution rate of  $0.0033 \text{ hr}^{-1}$  in contrast with a dilution rate of  $0.0017 \text{ hr}^{-1}$  at 144 hours. The volumetric productivity decreased as fermentation proceeded to  $2.85 \pm 0.08 \text{ U.mL}^{-1}.\text{hr}^{-1}$  at the end of the process. Even when comparing the values obtained at the end of the process, the volumetric productivity achieved using a higher dilution rate was 55.21 % greater.

Increasing the dilution rate further to  $0.0050 \text{ hr}^{-1}$ , however, saw a decline in volumetric productivity. A maximum of  $3.25 \pm 0.09 \text{ U.mL}^{-1}.\text{hr}^{-1}$  was determined after 216 hours with a dilution rate of  $0.0050 \text{ hr}^{-1}$ , 32.26 % less than the maximum productivity achieved with a dilution rate of  $0.0033 \text{ hr}^{-1}$  while a decline to  $2.69 \pm 0.08 \text{ U.mL}^{-1}.\text{hr}^{-1}$  was observed at the end of the fermentation after 336 hours.

All three systems experienced a spike in productivity from the start of the process, corresponding to the higher rate of xylanase production observed when the micro – organism was in the exponential growth phase. Here the organism would have required a greater amount of energy and thus produced a greater amount of enzyme to release the carbon source for consumption. The plateau reached using all three dilution rates is due to the enzyme activity increasing more linearly and finally the decline in productivity is due to the gradual increase in xylanase activities achieved at the end of the fermentation process.



**Figure 4-27: Optimization of dilution rates based on volumetric productivity**

From Figure 4-26, it can be deduced that a somewhat constant volumetric productivity was achieved from 120 hours and maintained thereafter. The plot above represents the average volumetric productivity for all three dilution rates from 120 hours until 336 hours when the experiment was terminated. In order to determine optimal dilution rates, a graph of volumetric productivity versus dilution rate as in Figure 4-27 above, is typically plotted. When a large range of dilution rates is employed, this analysis is useful in describing the effect of the dilution rate on productivity and selecting the most suited. Since the dilution was only varied between three volumes in this study due to the expense and capacity of the incubator, a marked optimal is observed.

The average volumetric productivities after 120 hours were  $2.34 \pm 0.37 \text{ U.mL}^{-1}.\text{hr}^{-1}$ ,  $3.63 \pm 0.70 \text{ U.mL}^{-1}.\text{hr}^{-1}$  and  $2.80 \pm 0.32 \text{ U.mL}^{-1}.\text{hr}^{-1}$  for the dilution rates of  $0.0017 \text{ hr}^{-1}$ ,  $0.0033 \text{ hr}^{-1}$  and  $0.0050 \text{ hr}^{-1}$  respectively. The removal of 80 mL of permeate undoubtedly provided the best outcome for improving volumetric productivity. These results corroborate those

obtained from a study performed by Ahmed and Abdel-Fattah (2010) to produce protease in batch, repeated batch and continuous culture, which indicated that an increase in dilution rate leads to a gradual increase in reactor productivity, with a pronounced decrease at higher dilution rates. In that study the dilution rate was varied between 0.1 and 0.7  $\text{hr}^{-1}$  and the maximal enzyme activity was attained at a dilution rate of 0.1  $\text{hr}^{-1}$  and the maximal reactor productivity was obtained at a dilution rate of 0.4  $\text{hr}^{-1}$ .

## 5. CONCLUSION AND RECOMMENDATIONS

---

### 5.1. Conclusion

Membrane incorporated fermentation processes have received much attention in recent years, though little focus have been bestowed to enzyme production, more significantly fungal enzyme production. Numerous possibilities for this lack of inquiry include the complexity of fungal systems and the lack of information regarding their behaviour in membrane systems. Secondary to the minimal study in that respect, is the further scarcity of research on submerged membrane systems for fungal enzyme production using flat sheet woven fabric membranes. Thus the platform for this research study was provided, with the objectives of developing a laboratory scale membrane fermenter for the production of the enzyme xylanase from the thermophilic fungal species *Thermomyces lanuginosus* DSM 5826 with increased volumetric production in comparison to a batch fermenter.

Culture maintenance and enzyme production were accomplished with the optimal nutrient medium and conditions established in shake flasks. The nutrient mediums with best results were beechwood xylan (1.5 % w/v), yeast extract (1.5 % w/v) and potassium dihydrogen phosphate (0.5 % w/v), with pH being adjusted to 6.5, shaking at 150 rpm in a 50 °C incubator with 1.0 mL of inoculum. Characterization of the enzyme revealed pH optima at 6.5 and temperature optima at 70 °C and a molecular weight of 25 – 35 kDa and distinct zones of hydrolysis on a xylan infused agarose gel validating the presence of xylanase. Semi – continuous fermentation in the flasks was a basis for the membrane experiments, indicating that the micro – organism could secrete xylanase over a period of time.

Subsequent to the development of the final membrane fermentation system, xylanase was successfully produced and bio – mass free permeate was withdrawn, being replaced with nutrient medium of identical volume. Results obtained prove that fungal growth can be maintained inside a membrane fermenter system since there is no cell wash – out and by removing toxins released by the micro – organism in the permeate and replenishing the nutrient system. Increased xylanase levels were simultaneously induced by removing enzyme – containing – permeate thus decreasing the concentration of xylanase in the fermenter. De –

ionized water may be used to supplement nutrient medium to desorb the enzyme from the substrate, thus increasing production and saving on the cost of medium as well. Notable increases in volumetric productivity and total enzyme activity were also achieved in the membrane fermenter systems compared to conventional batch fermentation. These increases were maintained over an extended period thus improving productivity and minimizing time lost in cleaning, and recharging a vessel, but a shorter duration is recommended. The optimal dilution rate was also successfully established in order to achieve the best productivity in a membrane fermenter system. As the mode of operation was semi – continuous the time for filtration was shorter compared to a continuous process and so the membrane fouling was reduced, given the fact that filtration could still be conducted after 506 hours, indicating that membrane fluxes obtained are greater in semi – continuous systems. Thus a membrane fermenter for xylanase production has been developed to combine enzyme reaction with separation. It has been possible to continuously remove the enzymatic products from the reaction mix, boosting yields and enhancing fermenter productivity.

## **5.2. Recommendations**

The upscaling of biological systems from lab scale to pilot scale and subsequent upscaling to production scale poses many challenges. In particular, the hydrodynamics change significantly which greatly impact on the reaction rates, mass transfer and heat transfer processes in a biological reactor. In this study it has been shown that the concept of an integrated membrane – fermenter system greatly enhances the production of xylanase from *Thermomyces lanuginosus*. Further extension to this work will include studies in:

- A larger bioreactor with the implementation of process controls including temperature, pressure, pH, dissolved oxygen and foam control as well as a pumping system with flow rate control for permeate removal and feed input.
- Quantification of the membrane performance i.e. flux studies, fouling and cleaning mechanisms.
- The effect of multiple membranes on the volumetric productivity as well as the inclusion of an ultrafiltration unit to concentrate the enzyme.
- Development of a continuous system.

## 6. REFERENCES

---

- ABDENACER, M., KAHINA, B. I., AÏCHA, N., NABIL, N., JEAN-LOUIS, G. & JOSEPH, B. 2012. Sequential optimization approach for enhanced production of glutamic acid from *Corynebacterium glutamicum* 2262 using date juice. *Biotechnology and Bioprocess Engineering*, 17, 795-803.
- ADAM, W., LAZARUS, M., SAHA-MÖLLER, C., WEICHOLD, O., HOCH, U., HÄRING, D. & SCHREIER, P. 1999. Biotransformations with Peroxidases. *Advances in Biochemical Engineering/Biotechnology* 63, 73-108.
- ADINARAYANA, K., PRABHAKAR, T., SRINIVASULU, V., ANITHA RAO, M., JHANSI LAKSHMI, P. & ELLAIAH, P. 2003. Optimization of process parameters for cephalosporin C production under solid state fermentation from *Acremonium chrysogenum*. *Process Biochemistry*, 39, 171-177.
- AHMED, S. A. & ABDEL-FATTAH, A. F. 2010. Production of *Bacillus licheniformis* ATCC 21415 alkaline protease in batch, repeated batch and continuous culture. *Mal J Microbiol*, 6, 156-160.
- AIYER, P. V. 2005. Amylases and their applications. *African Journal of Biotechnology*, 4, 1525-1529.
- ALFENORE, S., MOLINA-JOUVE, C., GUILLOUET, S., URIBELARREA, J.-L., GOMA, G. & BENBADIS, L. 2002. Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process. *Applied Microbiology and Biotechnology*, 60, 67-72.
- ARAKI, F., NAKAMURA, H., NOJIMA, N., TSUKUMO, K. & SAKAMOTO, S. 1989. Stability of recombinant human epidermal growth factor in various solutions. *Chemical & Pharmaceutical Bulletin*, 37, 404.
- ARAUJO, M., OLIVEIRA, R., GIORDANO, R. & HOKKA, C. 1996. Comparative studies on cephalosporin C production process with free and immobilized cells of *Cephalosporium acremonium* ATCC 48272. *Chemical Engineering Science*, 51, 2835-2840.

- ASENJO, J. A. 1990. *Separation processes in biotechnology*, CRC Press, 209-263.
- AZAD, K., HALIM, M. A. & HOSSAIN, F. 2013. Optimization of culture conditions for the production of xylanase by two thermophilic fungi under solid state fermentation. *Journal of the Asiatic Society of Bangladesh, Science*, 39, 43-51.
- BABU, K., SWAMINATHAN, S., MARTEN, S., KHANNA, N. & RINAS, U. 2000. Production of interferon- $\alpha$  in high cell density cultures of recombinant *Escherichia coli* and its single step purification from refolded inclusion body proteins. *Applied Microbiology and Biotechnology*, 53, 655-660.
- BAILEY, M. J., BIELY, P. & POUTANEN, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*, 23, 257-270.
- BAKER, R. 2012. *Membrane Technology and Applications (3rd Edition)*, Somerset, NJ, USA, Wiley.
- BAKRI, Y., AKEED, Y. & THONART, P. 2012. Comparison between continuous and batch processing to produce xylanase by *Penicillium canescens* 10-10c. *Brazilian Journal of Chemical Engineering*, 29, 441-448.
- BAMFORTH, C. W. 2008. *Food, fermentation and micro-organisms*, Wiley-Blackwell.
- BARAKAT, M. & SCHMIDT, E. 2010. Polymer-enhanced ultrafiltration process for heavy metals removal from industrial wastewater. *Desalination*, 256, 90-93.
- BARTELS, C., FRANKS, R., RYBAR, S., SCHIERACH, M. & WILF, M. 2005. The effect of feed ionic strength on salt passage through reverse osmosis membranes. *Desalination*, 184, 185-195.
- BELKACEMI, K. & HAMOUDI, S. 2003. Enzymatic hydrolysis of dissolved corn stalk hemicelluloses: reaction kinetics and modeling. *Journal of Chemical Technology and Biotechnology*, 78, 802-808.
- BERRY, D. R. & PATERSON, A. 1990. Enzymes in the food industry. *Enzyme Chemistry*. Springer, 306-351.

- BHAT, M. 2000. Cellulases and related enzymes in biotechnology. *Biotechnology Advances*, 18, 355-383.
- BHATIA, M. S. 2009. *Principles of Microbiology*, Delhi, IND, Global Media.
- BISHOP, D. F. & BRENNER, R. C. Fixed Film Bioreactors. Seminars on bioremediation of hazardous waste sites practical approaches to implementation., 1993. DIANE Publishing.
- BLAKEBROUGH, N. 1973. Fundamentals of fermenter design. *Pure and Applied Chemistry*, 36, 305-316.
- BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.
- BRAUTASET, T. & ELLINGSEN, T. E. 2011. 3.47 - Lysine: Industrial Uses and Production. In: EDITOR-IN-CHIEF: MURRAY, M.-Y. (ed.) *Comprehensive Biotechnology (Second Edition)*. Burlington: Academic Press.
- CARRIER, D. J., RAMASWAMY, S. & BERGERON, C. 2012. *Wiley Series in Renewable Resource : Biorefinery Co-Products : Phytochemicals, Primary Metabolites and Value-Added Biomass Processing*, Hoboken, NJ, USA, Wiley.
- CARSTENSEN, F., APEL, A. & WESSLING, M. 2012. *In situ* product recovery: Submerged membranes vs. external loop membranes. *Journal of Membrane Science*, 394, 1-36.
- CASEY, E., GLENNON, B. & HAMER, G. 1999. Review of membrane aerated biofilm reactors. *Resources, Conservation and Recycling*, 27, 203-215.
- CESAR, T. & MRŠA, V. 1996. Purification and properties of the xylanase produced by *Thermomyces lanuginosus*. *Enzyme and Microbial Technology*, 19, 289-296.
- CHAIN, E., PALADINO, S., CALLOW, D., UGOLINI, F. & VAN DER SLUIS, J. 1952. Studies on aeration-I. *Bulletin of the World Health Organization*, 6, 73.



- CHAKRABARTY, B., GHOSHAL, A. & PURKAIT, M. 2008. Ultrafiltration of stable oil-in-water emulsion by polysulfone membrane. *Journal of Membrane Science*, 325, 427-437.
- CHANG, R. 2006. *Chemistry*, New York, McGraw-Hill.
- CHATTERJEE, A., DAS, N. C., RAHA, S., BABBIT, R., HUANG, Q., ZAITLIN, D. & MAITI, I. B. 2010. Production of xylanase in transgenic tobacco for industrial use in bioenergy and biofuel applications. *In Vitro Cellular & Developmental Biology-Plant*, 46, 198-209.
- CHEDRESE, P. J. 2009. Recombinant DNA Technology. *Reproductive Endocrinology*. Springer.
- CHERRY, J. R. & FIDANTSEF, A. L. 2003. Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology*, 14, 438-443.
- CLARK, D. & LENTZ, C. 1961. Submerged citric acid fermentation of sugar beet molasses: effect of pressure and recirculation of oxygen. *Canadian Journal of Microbiology*, 7, 447-453.
- COLLINS, T., GERDAY, C. & FELLER, G. 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews*, 29, 3-23.
- CONGDON, R. W., MUTH, G. W. & SPLITTGERBER, A. G. 1993. The binding interaction of Coomassie blue with proteins. *Analytical Biochemistry*, 213, 407-413.
- CORAL, G., ARIKAN, B., UNALDI, M. & GUVENMEZ, H. 2003. Thermostable alkaline protease produced by an *Aspergillus niger* strain. *Annals of Microbiology*, 53, 491-498.
- CULLEN, D., GRAY, G. L., WILSON, L. J., HAYENGA, K. J., LAMSA, M. H., REY, M. W., NORTON, S. & BERKA, R. M. 1987. Controlled expression and secretion of bovine chymosin in *Aspergillus nidulans*. *Biotechnology*, 5, 369-376.
- CUYVERS, S., DORNEZ, E., MOERS, K., POLLET, A., DELCOUR, J. A. & COURTIN, C. M. 2011. Evaluation of the xylan breakdown potential of eight mesophilic endoxylanases. *Enzyme and Microbial Technology*, 49, 305-311.
- DEMAIN, A. L. 1974. HOW DO ANTIBIOTIC-PRODUCING MICROORGANISMS AVOID SUICIDE?\*. *Annals of the New York Academy of Sciences*, 235, 601-612.

- DENIS, S. & BOYAVAL, P. 1991. Microbial enzyme production in a membrane bioreactor. *Applied Microbiology and Biotechnology*, 34, 608-612.
- DHIMAN, S. S., SHARMA, J. & BATTAN, B. 2008. Industrial applications and future prospects of microbial xylanases: A review. *BioResources*, 3, 1377-1402.
- DIEBOLD, G., MOSENTHIN, R., PIEPHO, H.-P. & SAUER, W. 2004. Effect of supplementation of xylanase and phospholipase to a wheat-based diet for weanling pigs on nutrient digestibility and concentrations of microbial metabolites in ileal digesta and feces. *Journal of Animal Science*, 82, 2647-2656.
- DION, P., RUMMEL, J. & NAUTIYAL, C. S. 2007. *Microbiology of extreme soils*, Springer.
- DODSWORTH, N., HARRIS, R., DENTON, K., WOODROW, J., PC, W. & QUIRK, A. 1996. Comparative studies of recombinant human albumin and human serum albumin derived by blood fractionation. *Biotechnology and Applied Biochemistry*, 24, 171-176.
- DOMINGUES, L., LIMA, N. & TEIXEIRA, J. A. 2005. *Aspergillus niger*  $\beta$ -galactosidase production by yeast in a continuous high cell density reactor. *Process Biochemistry*, 40, 1151-1154.
- DORIDÍ, M. Y. 2013. Pastel pencils drawing of: Two-empire system tree. Six Kingdoms of living things tree. Phylogenetic tree of life. Symbiogenetic evolution of eukaryotes.
- DUNFORD, N. T. 2012. *Food and industrial bioproducts and bioprocessing*, John Wiley & Sons.
- EL-SHAMI, A. & HAMED, E. 2007. Production of lovastatin by *Pleurotus ostreatus*. *Egyptian Journal of Biotechnology*, 25, 102-110.
- ENNIS, B. & MADDOX, I. 1989. Production of solvents (ABE fermentation) from whey permeate by continuous fermentation in a membrane bioreactor. *Bioprocess Engineering*, 4, 27-34.
- ENZMINGER, J. & ASENJO, J. 1986. Use of cell recycle in the aerobic fermentative production of citric acid by yeast. *Biotechnology Letters*, 8, 7-12.
- FABER, K. 2011. *Biotransformations in organic chemistry*, Springer-Verlag Berlin Heidelberg.

- FLEMING, A. 1932. On the specific antibacterial properties of penicillin and potassium tellurite. Incorporating a method of demonstrating some bacterial antagonisms. *The Journal of Pathology and Bacteriology*, 35, 831-842.
- FODA, M. I. & LOPEZ-LEIVA, M. 2000. Continuous production of oligosaccharides from whey using a membrane reactor. *Process Biochemistry*, 35, 581-587.
- FRITZMANN, C., LÖWENBERG, J., WINTGENS, T. & MELIN, T. 2007. State-of-the-art of reverse osmosis desalination. *Desalination*, 216, 1-76.
- FURU, L., KAZMER, G., STRAUSBAUGH, L. & ZINN, S. 1999. Cloning and characterization of the bovine somatostatin gene. *Journal of Animal Science*, 77, 492-493.
- GAFFNEY, M., DOYLE, S. & MURPHY, R. 2009. Optimization of xylanase production by *Thermomyces lanuginosus* in solid state fermentation. *Bioscience, Biotechnology, and Biochemistry*, 73, 2640-2644.
- GEORGE-NASCIMENTO, C., GYENES, A., HALLORAN, S. M., MERRYWEATHER, J., VALENZUELA, P., STEIMER, K. S., MASIARZ, F. R. & RANDOLPH, A. 1988. Characterization of recombinant human epidermal growth factor produced in yeast. *Biochemistry*, 27, 797-802.
- GERDAY, C. G., NICOLAS 2007. *Physiology and Biochemistry of Extremophiles*, Washington, DC, USA, ASM Press.
- GHILDYAL, N. P., LONSANE, B. K. & KARANTH, N. G. 1988. Foam control in submerged fermentation: state of the art. *Advances in Applied Microbiology*, 33, 173.
- GHOSH, R. 2006. *Principles of bioseparations engineering*, World Scientific.
- GINOVART, M., PRATS, C., PORTELL, X. & SILBERT, M. 2011. Exploring the lag phase and growth initiation of a yeast culture by means of an individual-based model. *Food Microbiology*, 28, 810-817.
- GOEL, R. 2007. *Laboratory techniques in sericulture*, APH Publishing.
- GOLDBERG, E. 1997. *Handbook of downstream processing*, Chapman & Hall.

- GOMES, J., PURKARTHOFFER, H., HAYN, M., KAPPLMÜLLER, J., SINNER, M. & STEINER, W. 1993. Production of a high level of cellulase-free xylanase by the thermophilic fungus *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. *Applied Microbiology and Biotechnology*, 39, 700-707.
- GONCHARUK, V. V., KAVITSKAYA, A. A. & SKIL'SKAYA, M. D. 2011. Nanofiltration in drinking water supply. *Journal of Water Chemistry and Technology*, 33, 37-54.
- GOTTSCHALK, U. 2009. *Process Scale Purification of Antibodies*, Hoboken, NJ, USA, Wiley.
- GOVENDER, S., JACOBS, E., LEUKES, W. & PILLAY, V. 2003. A scalable membrane gradostat reactor for enzyme production using *Phanerochaete chrysosporium*. *Biotechnology Letters*, 25, 127-131.
- GREENLEE, L. F., LAWLER, D. F., FREEMAN, B. D., MARROT, B. & MOULIN, P. 2009. Reverse osmosis desalination: Water sources, technology, and today's challenges. *Water Research*, 43, 2317-2348.
- GUTIÉRREZ-CORREA, M., LUDEÑA, Y., RAMAGE, G. & VILLENA, G. K. 2012. Recent advances on filamentous fungal biofilms for industrial uses. *Applied Biochemistry and Biotechnology*, 167, 1235-1253.
- HALTRICH, D., NIDETZKY, B., KULBE, K. D., STEINER, W. & ŽUPANČIČ, S. 1996. Production of fungal xylanases. *Bioresource Technology*, 58, 137-161.
- HEDDEN, P. & PHILLIPS, A. L. 2000. Gibberellin metabolism: new insights revealed by the genes. *Trends in Plant Science*, 5, 523-530.
- HELD, P. 2012. Enzymatic Digestion of Polysaccharides  
Part II: Optimization of Polymer Digestion and Glucose Production in Microplates. .  
*Application Note*. Vermont, USA: BioTek Instruments, Inc.
- HENZE, M. 2008. *Biological Wastewater Treatment: Principles, Modeling, and Design*, International Water Assn, 335-370.

- HEW, L., RAVINDRAN, V., MOLLAH, Y. & BRYDEN, W. 1998. Influence of exogenous xylanase supplementation on apparent metabolisable energy and amino acid digestibility in wheat for broiler chickens. *Animal Feed Science and Technology*, 75, 83-92.
- HOUP, R. C. 2009. Ultrafiltration and Diafiltration. *Journal of Validation Technology*, 15, 40-45.
- HUDSON, H. J. 1991. *Fungal biology*, Cambridge University Press.
- ITO, K., KIZU, M. & KASUGAI, T. 1972. The effect of pepstatin, a specific acid protease inhibitor on gastric secretion and peptic activity (V). *Journal of Gastroenterology*, 7, 199-200.
- JENKINS, R. 1992. *Product recovery in bioprocess technology*, Butterworth Heinemann Limited, Oxford.
- JOHNSON, I. S. 1983. Human insulin from recombinant DNA technology. *Science*, 219, 632-637.
- JUDD, S. 2010. *The MBR book: principles and applications of membrane bioreactors for water and wastewater treatment*, Butterworth-Heinemann.
- JUNG OH, S., MOON, S.-H. & DAVIS, T. 2000. Effects of metal ions on diffusion dialysis of inorganic acids. *Journal of Membrane Science*, 169, 95-105.
- JUWON, A. D. & EMMANUEL, O. F. 2012. Experimental investigations on the effects of carbon and nitrogen sources on concomitant amylase and polygalacturonase production by *Trichoderma viride* BITRS-1001 in submerged fermentation. *Biotechnology Research International*, 2012.
- KALE, V. V. & BHUSARI, K. P. 2007. *Applied Microbiology*, Mumbai, IND, Global Media.
- KAMPEN, W. H. 1996. *Nutritional Requirements in Fermentation Processes*, Oxford, Elsevier.
- KANAI, M., FERRE, V., WAKAHARA, S., YAMAMOTO, T. & MORO, M. 2010. A novel combination of methane fermentation and MBR—Kubota Submerged Anaerobic Membrane Bioreactor process. *Desalination*, 250, 964-967.

- KANG, S., PARK, Y., LEE, J., HONG, S. & KIM, S. 2004. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. *Bioresource Technology*, 91, 153-156.
- KAO, P.-M., HUANG, S.-C., CHANG, Y.-C. & LIU, Y.-C. 2007. Development of continuous chitinase production process in a membrane bioreactor by *Paenibacillus* sp. CHE-N1. *Process Biochemistry*, 42, 606-611.
- KAPOOR, K. 2010. *Illustrated Dictionary of Microbiology*, Jaipur, IND, Global Media.
- KARGUPTA, K., DATTA, S. & SANYAL, S. K. 1998. Analysis of the performance of a continuous membrane bioreactor with cell recycling during ethanol fermentation. *Biochemical Engineering Journal*, 1, 31-37.
- KARIMINIAAE-HAMEDAANI, H.-R., SHAHROKHI, M. & ROOSTAAZAD, R. 2005. Experimental Evaluation of Batch and Continuous Production of Baker's Yeast under Computer Controlled pH. *Iran. J. Chem. & Chem. Eng*, 24.
- KATRAHALLI, U., KALANUR, S. S. & SEETHARAMAPPA, J. 2010. Interaction of bioactive coomassie brilliant blue g with protein: insights from spectroscopic methods. *Scientia Pharmaceutica*, 78, 869-880.
- KHACHATOURIANS, G. G. & ARORA, D. K. 2001. *Applied Mycology and Biotechnology, Volume 1 : Agriculture and Food Production*, New York, NY, USA, Elsevier Science & Technology.
- KHAJURE, P. V., METI, R. S. & AMBARISH, S. 2011. Enzymes of Ammonia Assimilation in Fungi: An Overview. *Recent Research in Science and Technology*, 3.
- KHALIKOVA, E., SUSI, P. & KORPELA, T. 2005. Microbial dextran-hydrolyzing enzymes: fundamentals and applications. *Microbiology and Molecular Biology Reviews*, 69, 306-325.
- KHUCHAROENPHAISAN, K. & SINMA, K. 2010. Beta-xylanase from *Thermomyces lanuginosus* and its biobleaching application. *Pakistan Journal of Biological Sciences: PJBS*, 13, 513.

- KILKOWSKI, W. J. & GROSS, G. G. 1999. Color reaction of hydrolyzable tannins with Bradford reagent, Coomassie brilliant blue. *Phytochemistry*, 51, 363-366.
- KIM, J. Y., KAVAS, M., FOUAD, W. M., NONG, G., PRESTON, J. F. & ALTPETER, F. 2011. Production of hyperthermostable GH10 xylanase Xyl10B from *Thermotoga maritima* in transplastomic plants enables complete hydrolysis of methylglucuronoxylan to fermentable sugars for biofuel production. *Plant Molecular Biology*, 76, 357-369.
- KIRK, O., BORCHERT, T. V. & FUGLSANG, C. C. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology*, 13, 345-351.
- KIY, T., SCHEIDGEN-KLEYBOLDT, G. & TIEDTKE, A. 1996. Production of lysosomal enzymes by continuous high-cell-density fermentation of the ciliated protozoon *Tetrahymena thermophila* in a perfused bioreactor. *Enzyme and Microbial Technology*, 18, 268-274.
- KOBAYASHI, K. 2006. Summary of recombinant human serum albumin development. *Biologicals*, 34, 55-59.
- KRISHNA, C. & CHANDRASEKARAN, M. 1996. Banana waste as substrate for  $\alpha$ -amylase production by *Bacillus subtilis* (CBTK 106) under solid-state fermentation. *Applied Microbiology and Biotechnology*, 46, 106-111.
- KULKARNI, N. S. & DESHPANDE, M. S. 2007. *General Enzymology*, Mumbai, IND, Global Media.
- KUMAR, C. G. & TAKAGI, H. 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnology Advances*, 17, 561-594.
- KUMAR, D., SAINI, N., PANDIT, V. & ALI, S. 2012. An Insight To Pullulan: A Biopolymer in Pharmaceutical Approaches. *International Journal of Basic and Applied Sciences*, 1, 202-219.
- KUMAR, D., VERMA, R. & BHALLA, T. 2010. Citric acid production by *Aspergillus niger* van. Tieghem MTCC 281 using waste apple pomace as a substrate. *Journal of Food Science and Technology*, 47, 458-460.

- KUMAR, K. S., MANIMARAN, A., PERMAUL, K. & SINGH, S. 2009. Production of  $\beta$ -xylanase by a *Thermomyces lanuginosus* MC 134 mutant on corn cobs and its application in biobleaching of bagasse pulp. *Journal of Bioscience and Bioengineering*, 107, 494-498.
- KUROIWA, T., IZUTA, H., NABETANI, H., NAKAJIMA, M., SATO, S., MUKATAKA, S. & ICHIKAWA, S. 2009. Selective and stable production of physiologically active chitosan oligosaccharides using an enzymatic membrane bioreactor. *Process Biochemistry*, 44, 283-287.
- KWON, S.-G., PARK, S.-W. & OH, D.-K. 2006. Increase of xylitol productivity by cell-recycle fermentation of *Candida tropicalis* using submerged membrane bioreactor. *Journal of Bioscience and Bioengineering*, 101, 13-18.
- LADISCH, M. R. & KOHLMANN, K. L. 1992. Recombinant human insulin. *Biotechnology Progress*, 8, 469-478.
- LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.
- LARROCHE, C., SOCCOL, C. R. & PANDEY, A. *Fermentation Processes Engineering in the Food Industry (Contemporary Food Engineering)*, Boca Raton, Florida, CRC Press Taylor & Francis Group.
- LEE, Y. K. 2003. *Microbial biotechnology: principles and applications*, World Scientific Publishing Company.
- LEUCHTENBERGER, W., HUTHMACHER, K. & DRAUZ, K. 2005. Biotechnological production of amino acids and derivatives: current status and prospects. *Applied Microbiology and Biotechnology*, 69, 1-8.
- LEVY, R. V. & JORNITZ, M. W. 2006. Types of filtration. *Sterile Filtration*. Springer.
- LI, N. N., FANE, A. G., HO, W. W. & MATSUURA, T. 2011. *Advanced membrane technology and applications*, John Wiley & Sons.



- LI, X.-Y. & CHU, H. P. 2003. Membrane bioreactor for the drinking water treatment of polluted surface water supplies. *Water Research*, 37, 4781-4791.
- LIN, S. H. & LO, M. C. 1998. Recovery of sulfuric acid from waste aluminum surface processing solution by diffusion dialysis. *Journal of Hazardous Materials*, 60, 247-257.
- LITCHFIELD, J. H. 1983. Single-cell proteins. *Science*, 219, 740-746.
- LUO, J., WU, C., XU, T. & WU, Y. 2011. Diffusion dialysis-concept, principle and applications. *Journal of Membrane Science*, 366, 1-16.
- MACHVE, K. K. 2009. *Fermentation technology*, Global Media, 24-40.
- MADIGAN, M. T., MARTINKO, J. M., PARKER, J. & BROCK, T. D. 1997. Biology of Microorganisms.
- MAHARAJ, M., KUMAR, A., PERMAUL, K. & SINGH, S. 2011. Purification and Fluorescence Spectroscopy Analysis of A Xylanase from *Thermomyces lanuginosus* DSM 5826 and its Thermostability Under Denaturation Conditions. *Atlas Journal of Chemistry & Biochemistry*, 1, 1-9.
- MAHONEY, R. & WHITAKER, J. 1978. PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF  $\beta$ -GALACTOSIDASE FROM *Kluyveromyces fragilis*. *Journal of Food Science*, 43, 584-591.
- MAMO, G. & GESSESSE, A. 2000. Immobilization of alkaliphilic *Bacillus sp.* cells for xylanase production using batch and continuous culture. *Applied Biochemistry and Biotechnology*, 87, 95-101.
- MANDELS, M. & WEBER, J. 1969. The production of cellulases. *Adv. Chem. Ser.*, 95, 391-414.
- MANN, U. 2009. *Principles of Chemical Reactor Analysis and Design : New Tools for Industrial Chemical Reactor Operations*, Hoboken, NJ, USA, Wiley.
- MARIA DE LOURDES, T., PEIXOTO-NOGUEIRA, S. C., DA SILVA, T. M., MALLER, A. & CABRAL, H. 2012. *Gel Electrophoresis for Investigating Enzymes with Biotechnological Application*.

- MIYANO, K.-I., YE, K. & SHIMIZU, K. 2000. Improvement of vitamin B<sub>12</sub> fermentation by reducing the inhibitory metabolites by cell recycle system and a mixed culture. *Biochemical Engineering Journal*, 6, 207-214.
- MOHANTY, A., MUKHOPADHYAY, U., GROVER, S. & BATISH, V. 1999. Bovine chymosin: Production by rDNA technology and application in cheese manufacture. *Biotechnology Advances*, 17, 205-217.
- MONOD, J. 1949. The growth of bacterial cultures. *Annual Reviews in Microbiology*, 3, 371-394.
- MOORE, D. & FRAZER, L. A. N. 2002. *Essential Fungal Genetics*, Secaucus, NJ, USA, Springer.
- MOSTAFA, N. 2001. Production of acetic acid and glycerol from salted and dried whey in a membrane cell recycle bioreactor. *Energy Conversion and Management*, 42, 1133-1142.
- MUNRO, A. 1970. Measurement and control of pH values. *Methods in Microbiology*, 2, 39-89.
- NAIR, S. & PANDA, T. 1997. Statistical optimization of medium components for improved synthesis of pectinase by *Aspergillus niger*. *Bioprocess Engineering*, 16, 169-173.
- NASR, S., SOUDI, M. R. & HAGHIGHI, M. 2007. Xanthan production by a native strain of *X. campestris* and evaluation of application in EOR. *Pakista Journal of Biological Sciences: PJBS*, 10, 3010.
- NATH, K. 2008. *Membrane Separation Process: Basic Concepts*, Phi Learning.
- NAUMAN, E. B. 2008. *Chemical Reactor Design, Optimization, and Scaleup (2nd Edition)*, Hoboken, NJ, USA, Wiley.
- NAYLOR, L. H. 1999. Reporter gene technology: the future looks bright. *Biochemical pharmacology*, 58, 749-757.
- NEUSHUL, P. 1993. Science, government and the mass production of penicillin. *Journal of the history of medicine and allied sciences*, 48, 371-395.

- OKAFOR, N. 2007. *Modern industrial microbiology and biotechnology*, Science Publishers Enfield.
- OLEMPСКА-BEER, Z. S., MERKER, R. I., DITTO, M. D. & DINOVI, M. J. 2006. Food-processing enzymes from recombinant microorganisms—a review. *Regulatory toxicology and Pharmacology*, 45, 144-158.
- PANIKOV, N. S. 1995. *Microbial growth kinetics*, Chapman & Hall.
- PAPAGIANNI, M. 2004. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances*, 22, 189-259.
- PARIHAR, P. 2008. *Applied Microbiology*, Delhi, IND, Global Media.
- PARK, Y., SANTI, M. & PASTORE, G. 1979. PRODUCTION AND CHARACTERIZATION OF  $\beta$ -GALACTOSIDASE FROM ASPERGILLUS ORYZAE. *Journal of Food Science*, 44, 100-103.
- PASHA, S. Y., ALI, M. N., TABASSUM, H. & MOHD, M. K. 2011. Comparative studies on production of Glutamic acid using wild type, mutants, immobilized cells and immobilized mutants of *Corynebacterium glutamicum*. *International Journal of Engineering Science*, 3.
- PATIL, S. R. & DAYANAND, A. 2006. Production of pectinase from deseeded sunflower head by *Aspergillus niger* in submerged and solid-state conditions. *Bioresource Technology*, 97, 2054-2058.
- PENG, Y., HUANG, Y., CAI, Y. & CAO, R. 2008. Screening for *Streptomyces hygroscopicus* strains with high production of agricultural antibiotics by streptomycin resistance. *Agricultural Science and Technology*, 9.
- PERRY, R., GREEN, D. & MALONEY, J. 1997. *Perry's Chemical Engineers' Handbook*, New York, McGraw-Hill
- PERSSON, I., TJERNELD, F. & HAHN-HÄGERDAL, B. 1991. Fungal cellulolytic enzyme production: a review. *Process biochemistry*, 26, 65-74.

- PINELO, M., ZEUNER, B. & MEYER, A. S. 2010. Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. *Food and Bioproducts Processing*, 88, 259-265.
- PIRT, S. J. 1975. *Principles of microbe and cell cultivation*, Blackwell Scientific Publications.
- PITT, J. I. & HOCKING, A. A. D. 2009. *Fungi and food spoilage*, Springer.
- POLIZELI, M., RIZZATTI, A., MONTI, R., TERENCE, H., JORGE, J. & AMORIM, D. 2005. Xylanases from fungi: properties and industrial applications. *Applied Microbiology and Biotechnology*, 67, 577-591.
- POPE, D. H. & BERGER, L. R. 1973. Inhibition of metabolism by hydrostatic pressure: what limits microbial growth? *Archiv für Mikrobiologie*, 93, 367-370.
- PRADE, R. A. 1996. Xylanases: from biology to biotechnology. *Biotechnology & Genetic Engineering Reviews*, 13, 101.
- PRASHAD, M. & TARRACH, K. 2006. Depth filtration: Cell clarification of bioreactor offloads. *Filtration & Separation*, 43, 28-30.
- PUMPHREY, B. & JULIEN, C. 1996. An Introduction to Fermentation. *Fermentation Basics, New Brunswick Scientific (UK and The Netherlands) May*, 1-24.
- PURKARTHOFFER, H. & STEINER, W. 1995. Induction of endo- $\beta$ -xylanase in the fungus *Thermomyces lanuginosus*. *Enzyme and Microbial Technology*, 17, 114-118.
- PURMONEN, M., VALJAKKA, J., TAKKINEN, K., LAITINEN, T. & ROUVINEN, J. 2007. Molecular dynamics studies on the thermostability of family 11 xylanases. *Protein Engineering Design and Selection*, 20, 551-559.
- RAI, M., TIDKE, G. & WASSER, S. P. 2005. Therapeutic potential of mushrooms. *Natural Product Radiance*, 4, 246-257.
- RAMACHANDRAN, S., SINGH, S. K., LARROCHE, C., SOCCOL, C. R. & PANDEY, A. 2007. Oil cakes and their biotechnological applications—A review. *Bioresource Technology*, 98, 2000-2009.

- RAMAKRISHNA, S., MA, Z. & MATSUURA, T. 2011. *Polymer membranes in biotechnology: preparation, functionalization and application*, World Scientific, 1-35.
- RAMCHANDRAN, L., SANCIOLO, P., VASILJEVIC, T., BROOME, M., POWELL, I. & DUKE, M. 2012. Improving cell yield and lactic acid production of *Lactococcus lactis* ssp. *cremoris* by a novel submerged membrane fermentation process. *Journal of Membrane Science*, 403-404, 179-187.
- RATHORE, A. & SHIRKE, A. 2011. Recent developments in membrane-based separations in biotechnology processes: review. *Preparative Biochemistry and Biotechnology*, 41, 398-421.
- REDDY, S. M., REDDY, R. S. & BABU, G. N. 2012. *Basic Industrial Biotechnology*, Daryaganj, IND, New Age International.
- RICHMOND, A. 2008. *Handbook of microalgal culture: biotechnology and applied phycology*, Wiley-Blackwell.
- RIESENBERG, D., MENZEL, K., SCHULZ, V., SCHUMANN, K., VEITH, G., ZUBER, G. & KNORRE, W. A. 1990. High cell density fermentation of recombinant *Escherichia coli* expressing human interferon alpha 1. *Applied Microbiology and Biotechnology*, 34, 77-82.
- RIFFAT, R. 2012. *Fundamentals of wastewater treatment and engineering*, CRC Press.
- ROBINSON, R. K., BATT, C. A. & PATEL, P. 1999. *Encyclopedia of Food Microbiology, Three-Volume Set*, Academic press.
- ROMANOWSKA, I., POLAK, J. & BIELECKI, S. 2006. Isolation and properties of *Aspergillus niger* IBT-90 xylanase for bakery. *Applied Microbiology and Biotechnology*, 69, 665-671.
- ROYER, J. C. & NAKAS, J. P. 1990. Simple, sensitive zymogram technique for detection of xylanase activity in polyacrylamide gels. *Applied and Environmental Microbiology*, 56, 1516-1517.
- SALE, G. S., DARR, H. J. & HOOVER, W. L. 1988. *Water purification process*. US Patent 4724079 A.

- SANDHYA, C., SUMANTHA, A., SZAKACS, G. & PANDEY, A. 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. *Process Biochemistry*, 40, 2689-2694.
- SAVVIDES, A. L., KATSIFAS, E. A., HATZINIKOLAOU, D. G. & KARAGOUNI, A. D. 2012. Xanthan production by *Xanthomonas campestris* using whey permeate medium. *World Journal of Microbiology and Biotechnology*, 28, 2759-2764.
- SCHWARTZ, L. 2003. Diafiltration: A Fast, Efficient Method for Desalting or Buffer Exchange of Biological Samples. *Pall Scientific & Technical Report*.
- SCHWINGE, J., NEAL, P., WILEY, D., FLETCHER, D. & FANE, A. 2004. Spiral wound modules and spacers: Review and analysis. *Journal of Membrane Science*, 242, 129-153.
- SHAH, A. R., SHAH, R. & MADAMWAR, D. 2006. Improvement of the quality of whole wheat bread by supplementation of xylanase from *Aspergillus foetidus*. *Bioresource Technology*, 97, 2047-2053.
- SHARMA, P. D. 2007. *Microbiology*, Global Media.
- SILVERSIDES, F. & BEDFORD, M. 1999. Effect of pelleting temperature on the recovery and efficacy of a xylanase enzyme in wheat-based diets. *Poultry Science*, 78, 1184-1190.
- SINGH, S., MADLALA, A. M. & PRIOR, B. A. 2003. *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *FEMS Microbiology Reviews*, 27, 3-16.
- SINGH, S., REDDY, P., HAARHOFF, J., BIELY, P., JANSE, B., PILLAY, B., PILLAY, D. & PRIOR, B. A. 2000. Relatedness of *Thermomyces lanuginosus* strains producing a thermostable xylanase. *Journal of Biotechnology*, 81, 119-128.
- SINGH, U. S. & KAPOOR, K. 2010. *Microbial Biotechnology*, Jaipur, IND, Global Media.
- SIRKAR, K. K. 1997. Membrane separation technologies: current developments. *Chemical Engineering Communications*, 157, 145-184.
- SIVALINGAM, M. & FARRINGTON, K. 2007. Haemodialysis. *Medicine*, 35, 461-465.

- SMITH, A., DATTA, S. P., SMITH, G. H., CAMPBELL, P. N., BENTLEY, R. & MCKENZIE, H. 2000. *Oxford dictionary of biochemistry and molecular biology*, Oxford University Press (OUP).
- SOLOMON, M. & PETERSEN, F. 2002. Membrane bioreactor production of lignin and manganese peroxidase. *Membrane Technology*, 2002, 6-8.
- STANBURY, P. F., WHITAKER, A. & HALL, S. J. 1995. *Principles of fermentation technology* Oxford, Pergamon.
- STOKES, R. & ROBINSON, R. 1949. Standard Solutions for Humidity Control at 25° C. *Industrial & Engineering Chemistry*, 41, 2013-2013.
- SUGIURA, M., ITO, A., OGISO, T., KATO, K. & ASANO, H. 1973. Studies on dextranase: Purification of dextranase from *Penicillium funiculosum* and its enzymatic properties. *Biochimica et Biophysica Acta (BBA)-Enzymology*, 309, 357-362.
- SUMBALI, G. & MEHROTRA, R. 2009. *Principles of microbiology*, Tata McGraw-Hill.
- SURVASE, S. A., KAGLIWAL, L. D., ANNAPURE, U. S. & SINGHAL, R. S. 2011. Cyclosporin A—A review on fermentative production, downstream processing and pharmacological applications. *Biotechnology Advances*, 29, 418-435.
- SUTHERLAND, K. 2008. Filtration overview: A closer look at depth filtration. *Filtration & Separation*, 45, 25-28.
- SUTHERLAND, K. 2010. The rise of membrane bioreactors. *Filtration & Separation*, 47, 14-16.
- TAKAYA, M., MATSUMOTO, N. & YANASE, H. 2002. Characterization of membrane bioreactor for dry wine production. *Journal of Bioscience and Bioengineering*, 93, 240-244.
- TAKEUCHI, T. 1973. *Biologically active substrate, pepstatin and production processes thereof*. US Patent 3740319 A.
- TAN, T., CHEN, B.-Q. & YE, H. 2006. Enzymatic synthesis of 2-ethylhexyl palmitate by lipase immobilized on fabric membranes in the batch reactor. *Biochemical Engineering Journal*, 29, 41-45.

- TASKIN, M., TASAR, G. E. & INCEKARA, U. 2012. Citric acid production from *Aspergillus niger* MT-4 using hydrolysate extract of the insect *Locusta migratoria*. *Toxicology and Industrial Health*.
- THALI, M. 1995. Cyclosporins: immunosuppressive drugs with anti-HIV-1 activity. *Molecular Medicine Today*, 1, 287-291.
- TILL, S. & MALLIA, H. Membrane bioreactors: Wastewater treatment applications to achieve high quality effluent. 64th Annual Water Industry Engineers and Operators' Conference, 2001, 57-65.
- TODAR, K. 2006. *Todar's online textbook of bacteriology*, University of Wisconsin-Madison Department of Bacteriology.
- TODARO, C. M. & VOGEL, H. C. 2014. *Fermentation and biochemical engineering handbook*, William Andrew.
- TÖRRÖNEN, A., HARKKI, A. & ROUVINEN, J. 1994. Three-dimensional structure of endo-1, 4-beta-xylanase II from *Trichoderma reesei*: two conformational states in the active site. *The EMBO Journal*, 13, 2493.
- TRIPATHI, R. C. 2006. *Biotechnological processing steps for enzyme manufacturing*, Global Media.
- TROLLER, J. A. 1980. Influence of water activity on microorganisms in foods. *Food Technology*, 34.
- TUDZYNSKI, B. 1999. Biosynthesis of gibberellins in *Gibberella fujikuroi*: biomolecular aspects. *Applied Microbiology and Biotechnology*, 52, 298-310.
- UFFEN, R. 1997. Xylan degradation: a glimpse at microbial diversity. *Journal of Industrial Microbiology and Biotechnology*, 19, 1-6.
- VANDOOREN, J., GEURTS, N., MARTENS, E., VAN DEN STEEN, P. E. & OPDENAKKER, G. 2013. Zymography methods for visualizing hydrolytic enzymes. *Nature Methods*, 10, 211-220.



- VARNAM, A. H. & EVANS, M. G. 2000. *Environmental Microbiology*, London, GBR, Manson Publishing Ltd.
- VERBELEN, P. J., DE SCHUTTER, D. P., DELVAUX, F., VERSTREPEN, K. J. & DELVAUX, F. R. 2006. Immobilized yeast cell systems for continuous fermentation applications. *Biotechnology Letters*, 28, 1515-25.
- VISVANATHAN, C., AIM, R. B. & PARAMESHWARAN, K. 2000. Membrane separation bioreactors for wastewater treatment. *Critical Reviews in Environmental Science and Technology*, 30, 1-48.
- WAITES, M. J., MORGAN, N. L., ROCKEY, J. S. & HIGTON, G. 2009. *Industrial microbiology: an introduction*, Wiley-Blackwell.
- WAKSMAN, S. A., REILLY, H. C. & JOHNSTONE, D. B. 1946. Isolation of Streptomycin-producing Strains of *Streptomyces griseus*. *Journal of Bacteriology*, 52, 393.
- WALKER, J. M. 1996. *The protein protocols handbook*, Springer.
- WANG, H. & ZHOU, H. 2013. Understand the Basics of Membrane Filtration. *Chemical Engineering Progress*, 109, 33-40.
- WHARTON, D. A. 2002. *Life at the Limits: Organisms in Extreme Environments*, Port Chester, NY, USA, Cambridge University Press.
- WHITTAKER, R. H. 1969. New concepts of kingdoms of organisms. *Science*, 163, 150-160.
- WILLAERT, R. & DE VUYST, L. 2006. Continuous production of l (+)-tartaric acid from cis-epoxysuccinate using a membrane recycle reactor. *Applied Microbiology and Biotechnology*, 71, 155-163.
- WILLIAMS, D. H., STONE, M. J., HAUCK, P. R. & RAHMAN, S. K. 1989. Why are secondary metabolites (natural products) biosynthesized? *Journal of Natural Products*, 52, 1189-1208.
- WILSON, M. 2009. *Bacteriology of Humans : An Ecological Perspective*, Hoboken, NJ, USA, Wiley-Blackwell.

- WIZANI, W., ESTERBAUER, H., STEINER, W. & GOMES, J. 1993. Preparation of xylanase by cultivating *Thermomyces lanuginosus* DSM 5826 in a medium containing corn cobs. US Patent 5183753 A.
- XIONG, H., NYSSÖLÄ, A., JÄNIS, J., PASTINEN, O., WEYMARN, N. V., LEISOLA, M. & TURUNEN, O. 2004. Characterization of the xylanase produced by submerged cultivation of *Thermomyces lanuginosus* DSM 10635. *Enzyme and Microbial Technology*, 35, 93-99.
- YACUBOWICZ, H. & YACUBOWICZ, J. 2005. Nanofiltration: properties and uses. *Filtration & Separation*, 42, 16-21.
- YADAV, B., RANI, U., DHAMIJA, S., NIGAM, P. & SINGH, D. 1996. Process optimization for continuous ethanol fermentation by alginate-immobilized cells of *Saccharomyces cerevisiae* HAU-1. *Journal of Basic Microbiology*, 36, 205-210.
- ZHAO, Z. & CHEN, L. 2009. Dynamic analysis of lactic acid fermentation in membrane bioreactor. *Journal of Theoretical Biology*, 257, 270-278.
- ZIAIE-SHIRKOLAEI, Y., TALEBIZADEH, A. & SOLTANALI, S. 2008. Comparative study on application of *T. lanuginosus* SSBP xylanase and commercial xylanase on biobleaching of non wood pulps. *Bioresource Technology*, 99, 7433-7437.