DEVELOPMENT OF A FLAT SHEET WOVEN FABRIC MEMBRANE FERMENTER FOR XYLANASE PRODUCTION BY THERMOMYCES LANUGINOSUS

[1]V THORULSLEY, [2]S RATHILAL, [3]S RAMSUROOP, [4]VL PILLAY

of Chemical Engineering, Institute of Water and Wastewater Treatment, Durban University of Technology, PO Box 1334, Durban 4000, South Africa
[2]Stellenbosch University, Department of Process Engineering, Private Bag X1, Matieland 7602, South Africa
E-mail: vthorulsley@yahoo.com, rathilals@dut.ac.za, ramsuros@dut.ac.za, pillayvl@sun.ac.z

Abstract- A submerged membrane fermenter system using a flat sheet woven fabric membrane to increase the rate of xylanase production, from the thermophilic fungal species Thermomyces lanuginous was developed and evaluated. During short run membrane fermenter experiments the percentage increase in enzyme activity between the batch operation and semi-continuous operation with beechwood xylan nutrient replenishment was 60.8%. The maximum volumetric productivity achieved with beechwood supplementation after 192 hours in semi-continuous operation was 2.1 times greater than that of batch operation which equates to an increase of 110% in productivity measured at its peak. During long run experimental periods, the increase in production of xylanase between the batch and the xylan medium membrane system was determined to be 74.3% whereas an overall average increase in productivity between the batch and xylan fed membrane system was 43.3%. Experiments where deionized water was alternated with beechwood xylan nutrient medium had no significant impact on the productivity or enzyme activity. 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.

Index Terms- Membrane fermenter, Thermomyces lanuginosus, woven fabric membrane, xylanase.

I. INTRODUCTION

Fermentation is developing significantly in 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 [1]. 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 [2]. Since fermentation is affected by the mass culture of micro-organisms in the appropriate apparatus and 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 [3]. 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 [4] 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.

The product of interest is the enzyme xylanase which catalyses the hydrolysis of xylan. Xylan is a polymer chain found in plant tissue, which can be hydrolysed by xylanase to its simple monomer sugar xylose. Xylose, 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 biofuel 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 [5]. 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. The micro-organism used was Thermomyces lanuginosus, 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.

II. MATERIALS AND METHODS

A. Maintenance of culture

The fungal culture Thermomyces lanuginosus was part of the German Type Collection (DSM 5826), which was grown on potato dextrose agar (PDA) and periodically sub-cultured. Fungal plugs, approximately 1 cm x 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. For use as an inoculum, the fungal spores were scraped in an aqueous solution of 0.9% NaCl and 0.05% Tween 80.

B. Temperature and pH 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. 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.

C. Membrane fermenter description

The final membrane fermenter apparatus illustrated in Fig. 1, consisted of a 1.5 L glass vessel into which a framed 75 mm x 100 mm engineering PVC membrane module was inserted. The membrane module consisted of two sheets of woven fabric, separated by a plastic grid as in Fig. 2. 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.

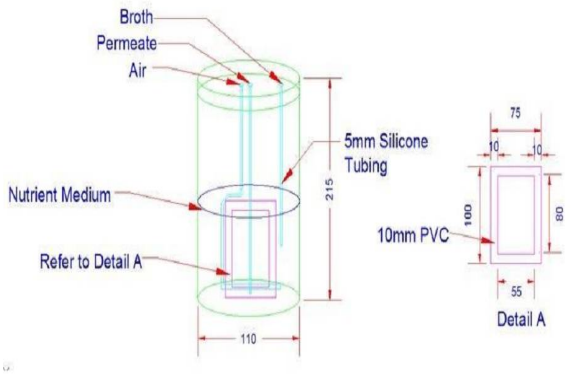


Figure 1: Schematic of membrane fermenter system

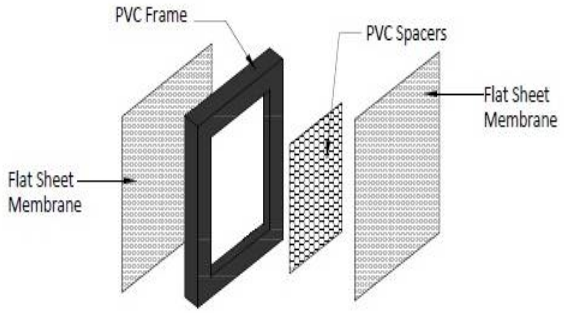


Figure 2: Membrane module fabrication

A 60 mL syringe was used to withdraw permeate from the membrane within the vessel, whereas 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.

D. Effect of mode of operation on xylanase production

Initial trials were conducted over a 9 day period. For this set of experiments the three modes of operation were batch mode, semi-continuous mode with beechwood xylan nutrient replenishment, and semi-continuous mode with deionized (DI) 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 (KH_2PO_4) (0.33% w/v) was prepared in 1.5 L fermenter vessels.

E. Effect of mode of operation on xylanase production over an extended period

Further trials were conducted over a longer period of time 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 was also 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, and semi-continuous mode with alternating beechwood xylan and deionized water replenishment.

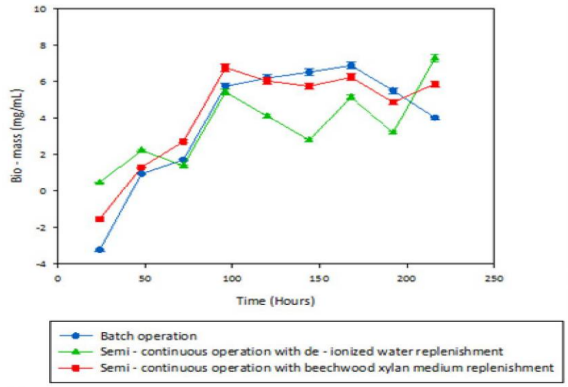
F. Optimization of nutrient medium dilution rate

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 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 analysis.

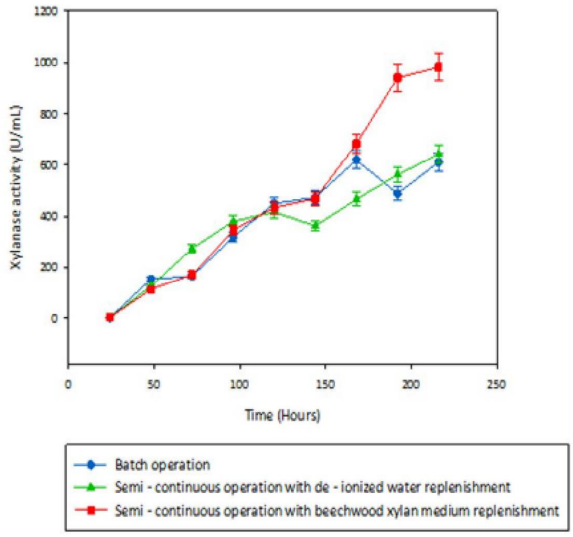
III. RESULTS AND DISCUSSION

G. Effect of mode of operation on xylanase production

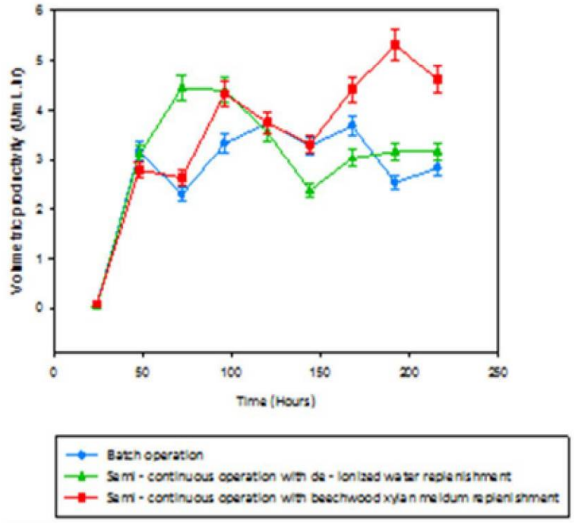
The biomass concentration achieved in batch mode reached a maximum of 6.88 ± 0.20 mg.mL^{-1} after 168 hours after which it declined to 4.04 ± 0.11 mg.mL^{-1} at the end of the fermentation period of 216 hours (Fig. 3a). The biomass recorded in semi-continuous mode with deionized water replenishment was erratic throughout the operation and reached a maximum of 7.28 ± 0.21 mg.mL^{-1} after 216 hours. When replenished with beechwood xylan medium the biomass peaked at 96 hours when 6.76 ± 0.19 mg.mL^{-1} was recorded, thereafter being maintained at ± 6.00 mg.mL^{-1}. The erratic biomass recorded for the operation with deionized water replenishment may be attributed to the dilution of the broth followed by a period of cell growth. 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. Xylanase activity in batch mode was 610 ± 35 U.mL^{-1} after 216 hours (Fig. 3b). When replenished with deionized water in semi-continuous operation, enzyme activity increased slightly whereas a prominent increase was observed when beechwood medium was added. The percentage increase in enzyme activity between the batch operation and semi-continuous operation with beechwood xylan nutrient replenishment is 60.8%. 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 [6].



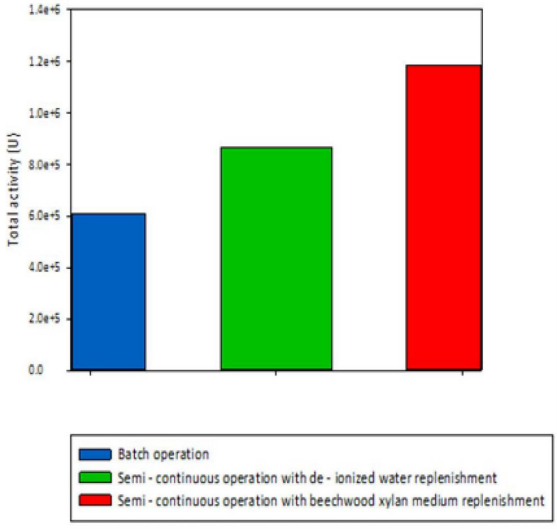
(a)



(b)



(c)



(d)

Figure 3 Effect of mode of operation on xylanase production. (a) Effect of mode of operation on biomass concentration; (b) Effect of mode of operation on xylanase activity; (c) Effect of mode of operation on volumetric productivity; (d) Effect of mode of operation on total activity.

The productivity achieved in batch operation was observed to be 2.83 ± 0.18 $U.mL^{-1}.hr^{-1}$ after 216 hours with a maximum productivity of 3.75 ± 0.21 $U.mL^{-1}.hr^{-1}$ after 120 hours (Fig. 3c). Volumetric productivity was maximal at 4.45 ± 0.25 $U.mL^{-1}.hr^{-1}$ after 72 hours in semi-continuous operation with DI water supplementation and thereafter decreased significantly to 2.38 ± 0.13 $U.mL^{-1}.hr^{-1}$ after 144 hours. Productivity was maintained around 3.0 $U.mL^{-1}.hr^{-1}$ 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 ± 0.25 $U.mL^{-1}.hr^{-1}$ 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 ± 0.18 $U.mL^{-1}.hr^{-1}$ 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^{-1} to 0.0025 hr^{-1} with DI water and 0.0017 hr^{-1} 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.

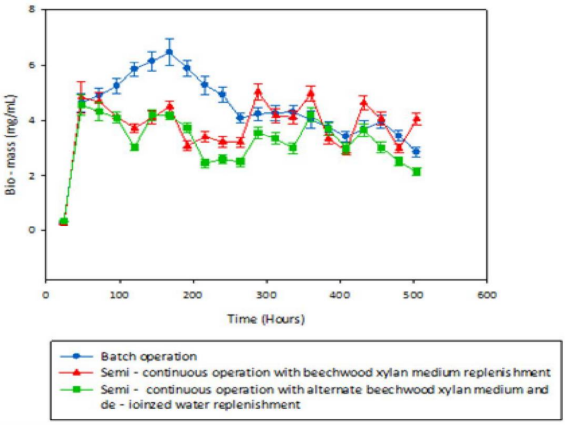
Productivity increased to a maximum of 5.32 ± 0.30 $U.mL^{-1}.hr^{-1}$ after 192 hours and decreased to 4.62 ± 0.26 $U.mL^{-1}.hr^{-1}$ after 216 hours. The productivity achieved using xylan medium and DI water is directly proportional to the xylanase activities achieved at specific periods in the fermentation process and the change in the dilution rates at the specified junctures. The maximum volumetric productivity achieved in semi-continuous operation was 2.1 times greater than that of batch operation which equates to an increase of 110 % in productivity measured at its peak after 192 hours.

Total activity represents the yield of enzyme at the end of the fermentation process. The total xylanase activity achieved was 610 577 U, 866 115 U and 1 184 938 U in batch mode, semi-continuous mode with DI water supplementation and semi-continuous mode with beechwood xylan medium supplementation (Fig. 3d). 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 is in total activity between batch and beechwood xylan medium supplemented semi-continuous mode is impressive at 1.94-fold, resulting in a 94.07% increase. Reference [7] 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.

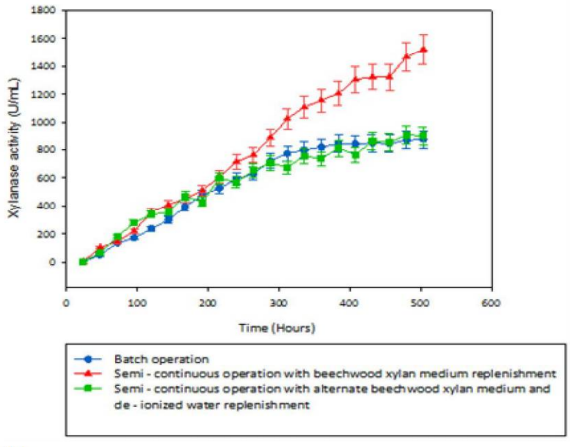
H. Effect of mode of operation on xylanase production over an extended period

Fig. 4a depicts the biomass concentration recorded over a 504 hour or 21 day period. The biomass in the batch system continued to increase until 168 hours when a maximum 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. 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.

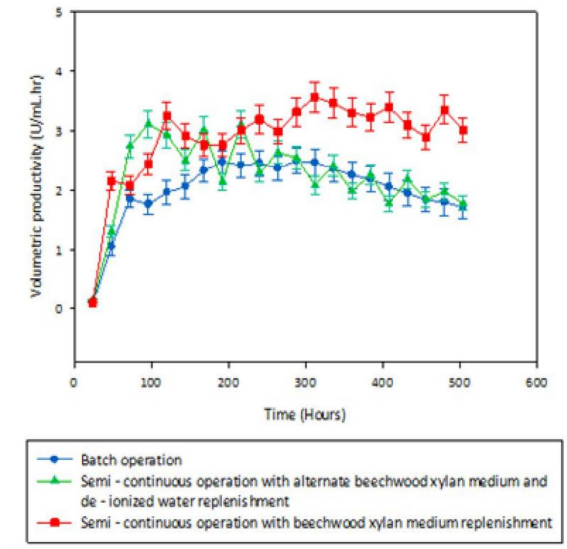
The maximum biomass recorded for the semi-continuous operation with beechwood xylan nutrient medium only was 5.03 ± 0.28 $mg.mL^{-1}$ whereas 4.56 ± 0.38 $mg.mL^{-1}$ was recorded for the alternating nutrient and DI water system. The method employed to determine the biomass did not take into account 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.



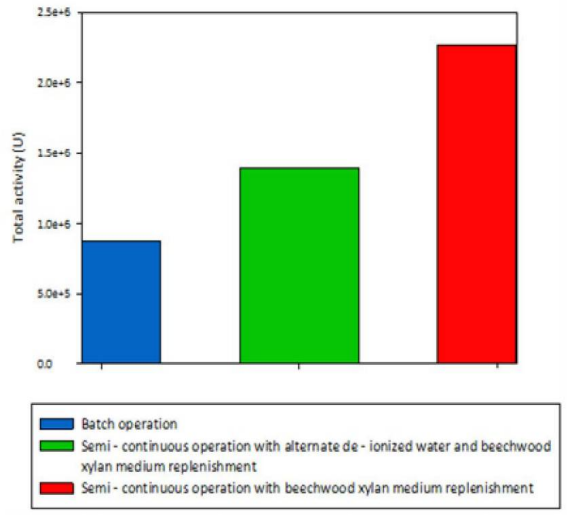
(a)



(b)



(c)



(d)

Figure 4: Effect of mode of operation on xylanase production over an extended period. (a) Effect of mode of operation on biomass concentration over an extended period; (b) Effect of mode of operation on xylanase activity over an extended period; (c) Effect of mode of operation on volumetric productivity over an extended period; (d) Effect of mode of operation on total activity over an extended period.

It has been documented that the morphological growth of fungal organisms affects 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 behaviours and the high viscosity impacts negatively on the mass transfer properties especially the gas – liquid mass transfer rate [8]. Therefore greater agitation is required for adequate mixing 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. 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 deionized water for 48 hours consecutively so that growth was restricted but the enzyme could still be secreted. Reference [9] stated that it is sometimes possible to maintain this state for weeks or months and to achieve high volumetric productivities.Fig. 4b illustrates a marked difference in activities, with a maximum of 1522.41 ± 107.65 $U.mL^{-1}$ 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 with activities to a maximum of 900.70 ± 63.69 $U.mL^{-1}$. 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. 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.

Productivity in the batch system increased linearly during the first 196 hours (Fig. 4c). The productivity then remained fairly constant up to 312 hours, consistent with the linear increase in xylanase activity recorded during this period. The maximum value of 2.51 ± 0.22 $U.mL^{-1}.hr^{-1}$ was recorded after 288 hours. Productivity then began to decline steadily until the end of the fermentation period, resulting from the constant xylanase activities recorded during this period, due to the stable nature of the protein. In the vessel marked for membrane fermentation with an alternate feed cycle between deionized water and beechwood xylan nutrient medium, there was a rapid increase in the rate of production during the first 96 hours. 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 ± 0.22 $U.mL^{-1}.hr^{-1}$ after 96 hours of production. Thereafter the productivity achieved was erratic, with a decrease in productivity due to the decrease in activity following DI water addition, whereas the increase to high productivities is due to the spike in xylanase secretion when substrate rich 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 deionized water intermittently during the period where xylanase activities are at its maximum. Productivity achieved in the membrane fermenter operated in semi-continuous mode with beechwood xylan nutrient medium replenishment only, increased sharply during the first 120 hours. Productivity then decreased. This may have been due to many factors including insufficient mass transfer resulting in oxygen and nutrient deficiency, inconsistent sterile air input, contamination etc. Productivity increased to a maximum after 312 hours and then decreased. 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 ± 0.24 $U.mL^{-1}.hr^{-1}$ being achieved during this period. An overall average increase in productivity between the batch and xylan fed membrane system was 43%.
Fig. 4d provides a clearly distinguishable difference between the three experiments conducted. After 504 hours, the total activity obtained in the semi-continuous membrane operation with alternating feed was 59% more than the batch process whereas the total xylanase activity obtained for the semi-continuous operation with beechwood xylan nutrient medium feed 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.

CONCLUSIONS

Results obtained prove that fungal growth can be maintained inside a membrane fermenter system since there is no cell washout 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. Deionized 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. 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.

REFERENCES

[1] N. T. Dunford, "Food and industrial bioproducts and bioprocessing," John Wiley & Sons, 2012.
[2] P. J. Verbelen, D. P. de Schutter, F. Delvaux, K. J. Verstrepen, and F. R. Delvaux, "Immobilized yeast cell systems for continuous fermentation applications," Biotechnology Letters, vol. 28, pp. 1515-25, 2006.
[3] J. A. Asenjo, "Separation processes in biotechnology," CRC Press, pp. 209-263, 1990.
[4] F. Carstensen, A. Apel, and M. Wessling, "In situ product recovery: Submerged membranes vs. external loop membranes," Journal of Membrane Science, vol. 394, pp. 1-36, 2012.
[5] S. S. Dhiman, J. Sharma, and B. Battan, "Industrial applications and future prospects of microbial xylanases: A review," BioResources, vol. 3, pp. 1377-1402, 2008.
[6] T. Kiy, G. Scheidgen-Kleyboldt, and A. Tiedtke, "Production of lysosomal enzymes by continuous high-cell-density fermentation of the ciliated protozoon Tetrahymena thermophila in a perfused bioreactor," Enzyme and Microbial Technology, vol. 18, pp. 268-274, 1996.
[7] P. M. Kao, S. C. Huang, Y. C. Chang, and Y. C. liu, "Development of continuous chitinase production process in a membrane bioreactor by Paenibacillus sp. CHE-N1," Process Biochemistry, vol. 42, pp. 606-611, 2007.
[8] M. Papagianni, "Fungal morphology and metabolite production in submerged mycelial processes," Biotechnology Advances, vol. 22, pp. 189-259, 2004.
[9] E. B. Nauman, "Chemical Reactor Design, Optimization, and Scaleup," 2nd Edition, Hoboken, NJ, USA, Wiley, 2008.

★★★