ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MEMBRANE BIOREACTOR (MBR) ASSISTED BACTERIAL CONTAMINATION REMOVAL FROM BIOETHANOL FERMENTATION SYSTEM

M.Sc. THESIS

Beray ÇAYLI

Department of Civil Engineering

Environmental Engineering & Sciences Programme

DECEMBER 2017

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MEMBRANE BIOREACTOR (MBR) ASSISTED BACTERIAL CONTAMINATION REMOVAL FROM BIOETHANOL FERMENTATION SYSTEM

M.Sc. THESIS

Beray ÇAYLI
501131739

Department of Civil Engineering

Environmental Engineering & Sciences Programme

Thesis Advisor: Assoc. Prof. Dr. Tuğba ÖLMEZ HANCI
Thesis Co-Advisor Prof. Dr. Mohammad J. TAHERZADEH

DECEMBER 2017

ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MEMBRAN BİYO-REAKTOR YARDIMIYLA BİYOETANOL FERMANTASYON SİSTEMİNDE BAKTERİ KONTAMİNASYONU GİDERİMİ

YÜKSEK LİSANS TEZİ

Beray ÇAYLI
501131739

İnşaat Mühendisliği Anabilim Dalı

Çevre Bilimleri ve Mühendisliği Programı

Tez Danışmanı: Doç. Dr. Tuğba ÖLMEZ HANCI
Eş Danışman: Prof. Dr. Mohammad J. TAHERZADEH

ARALIK 2017

Beray Çaylı, a M.Sc. student of İTU Graduate School of Science Engineering and Technology student 501131739 successfully defended the thesis entitled "Membrane Bioreactor (MBR) Assisted Bacterial Contamination Removal From Bioethanol Fermentation System", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Assoc. Prof. Dr. Tuğba ÖLMEZ HANCI**
İstanbul Technical University

Co-advisor : **Prof. Dr. Mohammad J. TAHERZADEH** ......
University of Boras

Jury Members : **Prof. Dr. Hayrettin Güçlü İNSEL**
İstanbul Technical University

Prof. Dr. Kadir ALP
İstanbul Technical University

Prof. Dr. Bülent KESKİNLER
Gebze Technical University

Date of Submission : 16.11.2017
Date of Defense : 22.12.2017

To my beloved family and friends,

FOREWORD

I would first like to thank my thesis advisor Assoc. Prof. Dr. Tuğba Ölmez Hancı for all good counsel and kindness she has given me over the long years since I have stepped into ITU. I could not thank her enough for setting into this path I took and shaping me as an engineer and scholar.

I thank from the bottom of my heart my co-advisor Professor Mohammed Taherzadeh for accepting me to Hogskolan Boras and give me the chance to be a part of this project. Professor Taherzadeh had always been supportive and encouraging and had steered me to right path whenever I needed help without being reproaching.

I would also like to thank my supervisor Amir Mahboubi Soufiani for the hours of hard work he had put into this project along with me. Few supervisors would freely offer the amount of support given to me by Mr. Mahboubi and I am ever grateful for it.

Last, I give my thanks to my family and friends especially to my partner Gülru Bulkan who helped much more than she ought to; in and out of laboratory.

November 2017

Beray Çaylı
Environmental Engineer

TABLE OF CONTENTS

Page

ABBREVIATIONS

DR	: Dilution Rate
HPLC	: High Performance Liquid Chromatography
IPC	: Integrated Permeate Channel
iMBR	: Immersed Membrane Bio Reactor
MBR	: Membrane Bio Reactor
PBS	: Phosphate Buffered Saline
PES	: Polyethersulfone
SS	: Suspended Solids
TMP	: Trans Membrane Pressure

LIST OF TABLES

Page

LIST OF FIGURES

Page

MEMBRANE BIOREACTOR (MBR) ASSISTED BACTERIAL CONTAMINATION REMOVAL FROM BIOETHANOL FERMENTATION SYSTEM

SUMMARY

Bioethanol fermentation is an anaerobic process in which the monomeric sugars are converted into ethanol and carbon dioxide by yeast fermentation. As a sustainable source of energy that can be used as an alternative for fossil-fuel consumption, the production volumes have been shown to increase over the years.

A major issue hindering efficiency in1st generation industrial ethanol fermentation from sugar based feed stock is unwanted bacterial contamination. The presence of bacterial contamination in a fermentation process leads to the drop in final ethanol yields, process profitability, yeast metabolic activity and the quality of final product, and increasing downstream processing costs.

In industrial scale fermentation, a contamination free process is hardly achieved as complete sterilization and maintaining sterility of the feedstock and instruments involved is very costly and laborious. As the contamination may occur from various pathways, the use of conventional anti-microbial compounds are not fully effective for long term bacterial control and system disinfection.

In this study, applying a physical bacterial decontamination of the fermentation to replace acid or base treatments and antibiotic addition was investigated. A submerged membrane bioreactor (MBR) using Integrated Permeate Chanel (IPC) flat sheet membrane panels were used for this purpose. Ethanol producing microorganism was selected as Saccharomyces cerevisiae as it is the most widely used strain for bioethanol production. The contaminant - Enterobacter cloacae- had been previously isolated from a lab-scale bioethanol fermentor located in Biotechnology Department of University of Boras.

Both strains were cultivated separately and concomitantly to determine growth characteristics, metabolites and cell morphology in synthetic medium and analyzedthroughspectrophometry, HPLC analysis, microscopy, plate inoculationand cell dry weight measurements.

A selective separation of a co-culture of Saccharomyces cerevisiae and complex of Enterobacter cloacae were first investigated by placing the cultures within a phosphate buffered saline solution in MBR to determine the interaction of both strains with the membrane surface in the absence of nutrients and therefore metabolites. Dilution rates of 0.25 to 0.75 h^{-1}were practiced using poly ethersulfune (PES) membrane with pore sizes of 1-2.4 µm.

The effect of initial bacterial inoculum size (up to 0.25 g/l in the bioreactor) on its washout regime was studied and it was observed that in optimum conditions up to 93% could be effectively removed through the permeate line by this MBR set up.

Upon establishing the optimum filtration conditions for bacteria strain in the absence of nutrients, the concomitant presence of both species in continuous fermentation using sugar based nutrient medium was studied. As expected in fermentation conditions the process was hindered by the growth of bacteria as well as yeast; never the less continuous bacteria washout was achieved with a smaller bacteria inoculum (0.001 g/l) and slower dilution rate (0.11 h^{-1}).

MEMBRAN BİYO-REAKTOR YARDIMIYLA BİYOETHANOL FERMANTASYON SİSTEMİNDE BAKTERİ KONTAMİNASYONU GİDERİMİ

ÖZET

.

Biyoetanol fermantasyonu monomerik şeker gruplarının maya fermantasyonu sonucunda etanol ve karbon dioksite dönüşmesini sağlayan anaerobik bir prosestir. Başlıca kullanılan hammaddeler mısır veya şeker kamışı gibi tarım ürünleri olmakla birlikte lignoselüloz içerikli tarımsal atıklardan üretimi de mevcuttur.

Fosil yakıtların gideren artarak kullanımı çevre kirlenmesinin yanı sıra küresel iklim değişikliği gibi çok yönlü sorunlara yol açtığı göz önünde bulundurulduğunda, yerel olarak üretilebilen bir likit enerji kaynağı olan bioethanol, fosil yakıt yerine geçebilecek sürdürülebilir bir alternatiftir.Kendi başına kullanılabileceği gibi, benzinle karıştırılarak (%30' a kadar) da taşıt yakıtı olarak kullanılabilir. Oksitlenmiş bir yakıt olduğundan benzin yerine taşıt yakıtı olarak kullanılması hava kalitesinde de pozitif bir etki yaratır.

Dünya çapında devlet birimlerinin ve sanayinin biyoetanole artan ilgisi sayesinde 2007-2015 arasında biyoetanol üretimi iki katına çıkmıştır. Üretimde başı çeken ülkeler Brezilya ve ABD'dir ve 2015 RFA (Yenilenebilir Yakıtlar Birliği) verilerine göre toplam küresel biyoetanol üretiminin %85'i bu iki ülke tarafından gerçekleştirilmiştir.

Bununla birlikte biyoethanol sanayisinde verimi etkileyen başlıca sorunlardan biri proses sırasında ve öncesi çeşitli yollardan sisteme girebilen bakteri kontaminasyonudur. Fermantasyon prosesinde ortaya çıkan bakteri kontaminasyonu nihai etanol üretimi verimliliği, maya metabolik aktivitesi ve nihai ürünün kalitesini olumsuz etkilemekte ve sonraki işlem kademelerinin bedelini artırmaktadır.

Sanayi ölçekte yapılan fermantasyonda tamamen kontaminasyondan uzak bir sistem elde edilmesi oldukça zor, besleme çözeltisi ve ekipmanların steril tutulması ise masraflı ve zahmetli bir iştir. Kontaminasyonun çok çeşitli yollardan sisteme dahil olabileceği göz önünde tutulduğunda konvansiyonel anti-mikrobiyal bileşiklerin kullanımı uzun dönemde bakteri kontrolü ve sistem dezenfeksiyonu için yeterli olmamaktadır.

Bu çalışmada fermantasyon sürecinde oluşan bakteri kontaminasyonunun asit-baz işlemi veya antibiyotik katımı yerine fiziksel yollarla giderilmesi araştırılmıştır. Bu amaçla batık membran biyoreaktör ve IPC (Integrated Permeate Chanel) düz levha membran panellerinin birlikte yer aldığı bir konfigürasyon kullanılmıştır. Bu konfigürasyon ile maya hücreleri biyoreaktör içinde tutulurken kontaminasyona neden

olan bakteri hücrelerinin reaktör dışına atılabileceği sürekli beslemeli bir bioethanol fermantasyon sistemi elde edilmesi amaçlanmıştır.

Etanol üretici mikroorganizma olarak en yaygın kullanılan maya türü olan Saccharomyces cerevisiae seçilmiştir. Kontamine bakteri türü olarak daha önce Boras Üniversitesi dahilindelaboratuar ölçekli bir etanol fermantöründe kontaminasyona neden olduğu tespit edilerek izole edilmiş olan Enterobacter cloacae seçilmiştir.

Her iki tür ayrı ve beraber olarak fermantasyon koşullarında (30 C° ve pH 5) ve sentetik besleme çözeltisi içerisinde,sallanan su banyosunda24 saat büyütülmüştür. Kültürler olgunlaştığında steril koşullarda örnekler alınmış ve gram boyama yöntemiyle ışık mikroskobu altında hücre morfolojileri incelenerek hücre boyutları maya için hücre çapı 3-4 µm, bakteri için 1-1.5µm olarakbelirlenmiştir.

Ayrıca mikroorganizmaların metabolik hareketlerinin ve büyüme hızlarının belirlenmesi amacıyla spektrofotometri, HPLC, plaka aşılama ve kuru ağırlık ölçümleri yapılmıştır.

Saccharomyces cerevisiae veEnterobacter cloacae bakteri kompleksinin selektif olarak ayrılmasını incelemek amacıyla öncelikle kültürler MBR’a PBS çözeltisi içinde konulmuştur. Nütrient yokluğunda bakteri ve maya kültürlerinin metabolik aktiviteleri yavaşlayarak hücredışı bileşik üretimleri durma noktasında geldiğinden sadece boyutsal olarak membran tarafından reaktör içinde tutulup tutulmadıklarını gözlemlemek mümkün olmuş, mikroorganizmaların ayrı ve beraber olarak konulduklarında membran yüzeyiyle etkileşimleri incelenmiştir. Bu sayede kullanılacak membran plakalarının por büyüklüğü, sistemin optimum seyreltme oranı ve reaktöre konacak kültürlerin miktarının belirlenmesi amaçlanmıştır. 1.5 ve 2 litrelik hacimleri olan laboratuar ölçekli iki ayrı reaktör kullanılmış ve bu reaktörlerde 0.25 ve 0.75 1/saat aralığında seyreltme oranları, 1 ve 2.4 µm por çapına sahip iki ayrı polieter sülfan (PES) membran panel tipi denenmiştir.

İlk aşamada maya ve bakteri kültürleri ayrı olarak reaktöre konularak membran özelliklerinin mayanın reaktör içinde tutulması ve bakterinin atılması için uygun olup olmadığı araştırılmıştır. Bu amaçla sadece maya bulunan kültürle 1 ve 2.4 µm por çaplı iki ayrı membran panelinin kullanıldığı iki ayrı deney yapılmıştır.Sistem akısının 110 L/m^2/saat ve reaktör içindeki kültür konsantrasyonunun 0.6 g/L olduğu deneylerde membran yüzeyindeki basıncın ve reaktör içindeki türbiditenin sabit olduğu gözlemlenmiş ve membranın mayayı reaktör içinde tutmak için uygun olduğu sonucu çıkarılmıştır.

Sadece bakteri bulunun kültürle PBS çözeltisi içinde yapılan deneyler sonucunda optimum konfigürasyonda (akı; 150 L/m^2/saat, 2,4 µmpor çaplı membran, reaktör içindeki bakteri kültür konsantrasyonu; 0.04 g/L)membran yüzeyinde basınç artışı olmadığı halde % 93 oranında kontaminasyon gideriminin sağlanabildiği spektrofotometrik yöntemlerle belirlenmiştir.

Sonraki aşamada bakteri ve maya kültürleri ayrı ve beraber olarak kesikli beslemeli reaktör sisteminde, membran yokluğunda, sentetik besleme çözeltisinde büyütülmüş ve bu koşullar altındaki metabolik aktiviteleri ve büyüme hızları gözlemlenmiştir. Yapılan deneyler sonucunda mayanın varlığının bakterinin büyümesini glikoz tüketiminde rekabete yol açması ve etanol üretimi sayesinde olumsuz etkilediği sonucuna varılmıştır.

PBS çözeltide yapılan MBR ve kesikli beslemeli reaktör sisteminde yapılan deneyler sonucunda elde edilen bulgular doğrultusunda MBR sisteminde sentetik nütrient için bakteri büyütülmüş ve sistemden atılması denenmiştir. Ancak akının 37 L/m^2/saat, reaktör içindeki başlangıç bakteri konsantrasyonunun ise 0.001 g/L'ye düşürülmesine rağmen 6. saatte bakterinin eksponansiyel büyüme fazına geçmesini takiben membran yüzeyindeki basınç artışı kontrol edilememiş ve sürekli bakteri giderimi yapılamıştır.

Bunun üzerine bakteri ve maya MBR sistemine sentetik besleme çözeltisinde birlikte konulmuş ve fermantasyon koşullarında büyütülmüştür. Bu aşamada akının 72-16 L/m^2/saat, reaktör içindeki başlangıç bakteri konsantrasyonunun 0.001-0.0001 g/L, mayanın ise 0.14-0.36 g/L olduğu koşullarda denemeler yapılmıştır. Reaktör içindeki pH'ınmanüel olarak 10. ve 24. saaate konsantre NaOH eklenerek 5'te kontrol edildiği deneylerde akının 16 L/m^2/saat, reaktör içindeki başlangıç bakteri konsantrasyonunun 0.001g/L, mayanın ise 0.36 g/L olduğu koşullarda sürekli bakteri giderimi sağlanabildiği membran yüzeyindeki basıncın sabit gittiğinin gözlenmesiyle ispatlanmıştır.

Daha sonra manüel olarak yapılan pH kontrolü yerine otomatik olarak konsantre NaOH eklenmesiyle pH'ın 5'te tutulması denenmiş, ancak reaktör içindeki başlangıç bakteri konsantrasyonunun 0,00001 g/L'ye kadar düşürülmesine rağmen membran yüzeyindeki basıncın kontrol edilememesi nedeniyle sürdürebilir bir bakteri giderimi sağlanamamıştır.

Sonuç olarak optimum sistem konfigürasyonunda (ideal por büyüklüğü, akı ve başlangıç maya ve bakteri kültürlerinin büyüklüğü) pH'ın 4-5 arasında tutulması halinde beslemeli membran biyoreaktör sisteminde sürekli bakteri kontaminasyonu gideriminin sağlanabileceği sonucuna varılmıştır.

1. INTRODUCTION

1.1 Purpose of Thesis

A major issue hindering efficient industrial ethanol fermentation from sugar based feed stock is unwanted bacterial contamination. The presence of bacterial contamination in a fermentation process leads to the drop in final ethanol yields, process profitability, yeast metabolic activity and the quality of final product, and increasing downstream processing costs. In industrial scale fermentation a contamination free process is hardly achieved as complete sterilization and maintaining sterility of the feedstock and instruments involved is very costly and laborious and the use of conventional anti-microbial compounds are not fully effective for long term bacterial control and system disinfection.

On the other hand a physical method for decontamination shall lead to a high quality product free of endocrine disruptors that are harmful to human health and environment as well as a result in a fully biodegradable waste stream that should not disturb the aquatic environment and would not force the producer to apply harsher treatment methods for the environment. Since the process could be continuous it can be also modified to be more energy efficient when overall energy consumption is considered.

Membrane separation also has the advantage of eliminating the need for constant addition of chemicals or antibiotics once the system is installed; based on the lifespan of the bioethanol production plant it could save the production, transportation and usage+ treatment of a significant amount of chemicals and /or antibiotics.

In conclusion, through membrane separation the product could be more eco-friendly, healthy and economical.

1.2 Scope of Thesis

In this study physical bacterial decontamination of the fermentation was successfully practiced by means of a submerged membrane bioreactor (MBR) using Integrated Permeate Chanel (IPC) flat sheet membrane panels. In this regard, selective separation of a co-culture of Saccharomyces cerevisiae and complex of Enterobacter cloacae form a continuous fermentation medium at dilution rates of 0.25 to 0.75 h-1 was practiced using poly ethersulfune (PES) with pore sized of 0.3-2.4 µm at different filtration conditions. The interaction between the bacterial complex, yeast and membrane was separately analyzed and the concomitant presence of both species in continuous fermentation using nutrient medium was studied. The effect of initial bacterial inoculum size (up to 0.25 g/l in the bioreactor) on its washout regime was studied and it was observed that in optimum conditions up to 93% could be effectively removed through the permeate line by this MBR set up. However in fermentation conditions the process was hindering by the growth of bacteria as well as yeast; never the less continuous bacteria washout was achieved with a smaller bacteria inoculum (0.001 g/l) and slower dilution rate (0.11 h^{-1}).

2. LITERATURE REVIEW

2.1 Bioethanol Industry; an Overview

Current practice of industries around the world strongly depends on fossil fuel for production of energy as well as manufacturing of goods; however the pollution created during the extraction, transportation and consumption of fossil fuels had a negative effect on the environment while contributing to global climate change (Ballesteros et al. 2006). Though increase in population worldwide results in a higher demand for production of fossil fuel, global production is predicted to decrease over the next years due to diminishing resources (Campbell et al. 1998).

Biofuels may be part of a solution to all these problems. These fuels are derived through the conversion of sugars by biomass; hence they are sustainable and can be supplied locally. Major biofuels are biodiesel, bio-hydrogen and bioethanol. Among these, bioethanol has the highest share of total biofuel production, with USA and Brazil being the top producers in the world and covering 85% of total production (Renewable Fuels Association, 2016). According to Lopes et al., Brazil is the most successful in substituting gasoline with ethanol, however there are also several other countries who have initiated similar programs for the sake of environmental and economic concerns (2016).As seen in Figure 2-1 production volume has doubled between 2007 and 2015.

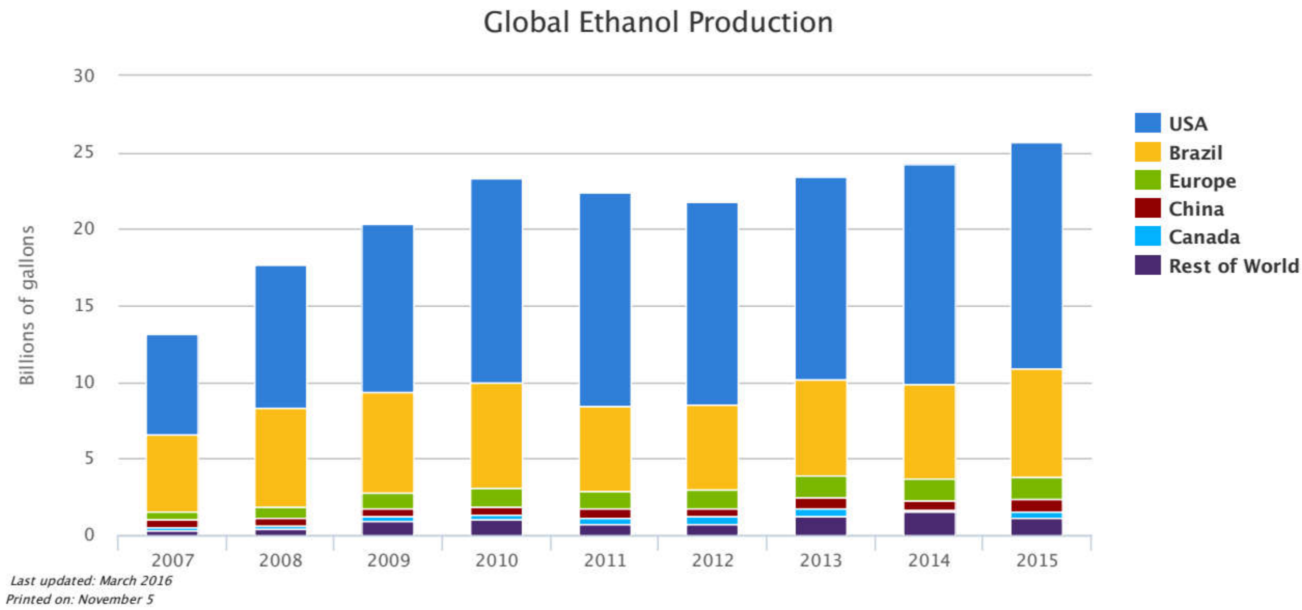

Figure 2-1 : Global ethanol production by country or region, from 2007 to 2015, Renewable Fuels Association, March 2016

Bioethanol may directly be used in modified engines for transportation or mixed with gasoline (Staniszewski et al. 2007) either as fuel or octane enhancer. As an octane enhancer, ethanol is capable of replacing toxic chemicals such as lead which is banned from several countries due to environmental and health concerns (Olympio et al. 2009).

Bioethanol also provides other advantages over gasoline such as “broader flammability limits, higher flame speeds and increased heats of vaporization” (Azhar et al. 2017).

In comparison to fossil fuels; bioethanol is more environmentally friendly, biodegradable and less polluting to air as well as water (John et al. 2011). In certain city centers using ethanol blends in place of gasoline has been reported to reduce carbon monoxide emission from 50 to 5.8 g per kilometer driven (Goldemberg, 2008).

There has been also two important factors contributing to the rising interest in ethanol consumption; invention of flex-fuel cars that may either run on pure ethanol or an ethanol blend and the fluctuating oil prices since conversion to ethanol have provided a stability to fuel suppliers (Amorim & Lopes 2005).

2.2 Bioethanol Production

Bioethanol, also known as ethyl alcohol, is produced from a variety of sugar sources as demonstrated on Table 2-1; sugar sources that do not require pretreatment are utilized to produce 1st generation bioethanol whereas sugar sources that require pretreatment before utilization by biomass such as lignocellulose material fall under 2nd generation bioethanol production; 3rd generation bioethanol production is achieved through the ethanol production by algae (Nigam et al. 2011).Main agricultural products used for ethanol production are corn in USA and sugarcane in Brazil respectively (Wang et al. 2016).

Table 2-1 : Bioethanol pathways from different raw materials (Demirbas 2007)

Raw Material	Processing
Wood	Acid hydrolysis + fermentation
Wood	Enzymatic hydrolysis + fermentation
Straw	Acid hydrolysis + fermentation
Straw	Enzymatic hydrolysis + fermentation
Wheat	Malting + fermentation
Sugar cane	Fermentation
Sugar beet	Fermentation
Corn grain	Fermentation
Corn stover	Acid hydrolysis + fermentation
Sweet sorghum	Fermentation

As 1st generation ethanol is produced from agricultural crops that are consumable by humans there are certain concerns that producing fuels from these crops may cause global supply problems of food, causing many researches to focus on ethanol production from lignocelluloses as well as algae (Reddy et al. 2008 & Amigun et al. 2008). Another problem is the cost; raw material yields to 40–70% of the total production cost in 1st general ethanol (Galbe and Zacchi, 2002). Further concerns are raised about the production stages of these crops; as significant amounts of water, fertilizers and fuel is used (Sims et al. 2008). However as 2nd generation ethanol production requires pretreatment of lignocellulosic materials for the utilization of microorganisms, the process is not yet economically feasible and requires further optimization (Ishola et al. 2013).

The production stages for 1st generation ethanol include preparation of raw material, fermentation and distillation steps (Naik et. al. 2010). Main steps involved in producing ethanol by wet milling and dry milling from corn are represented in Figure 2-2, in each case the sugar source is prepared for easy glucose intake by yeast, yeast cells are cultivated to achieve high cell density and ethanol is removed from the broth through distillation.

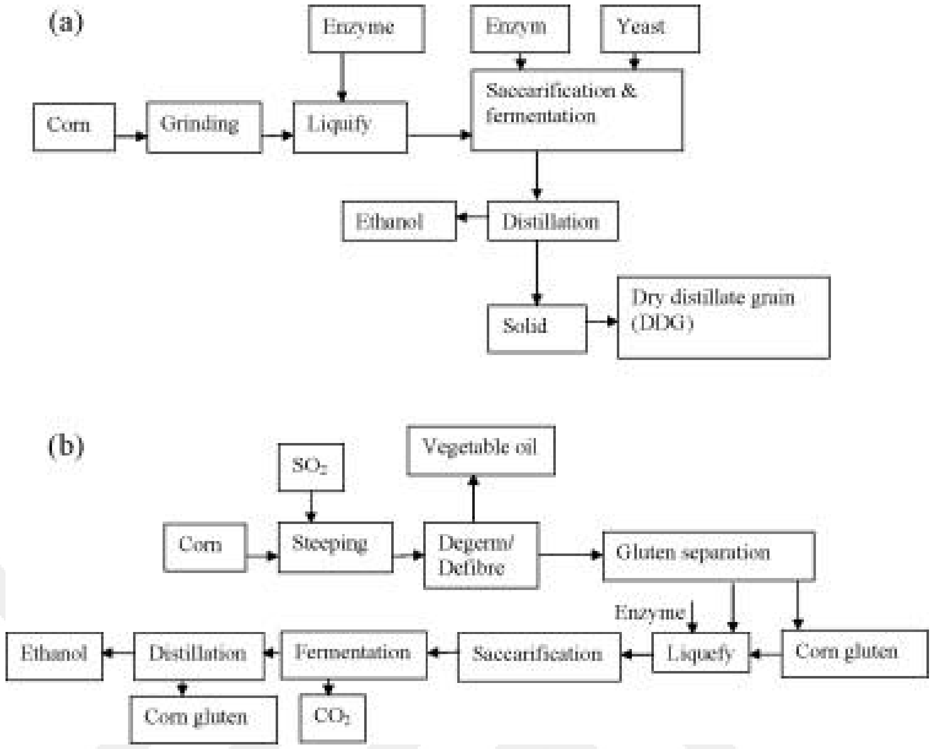

Figure 2-2 : 1st generation ethanol production by (a) Dry mill process and (b) Wet mill process (Naik et al. 2010)

2.2.1 Bioethanol fermentation by Saccharomyces cerevisiae

Though there are several organisms capable of converting sugars into ethanol such as *Zymomonas mobilis, Escherichia coli, Scheffersomyces stipitis, Candida pseudotropicalis, Candida utilis*, S. *cerevisiae* remains the most popular and widely used (Ishola et al. 2014). There are various reasons for this; the major reasons being the safety of usage, high ethanol yield as well as process tolerance and the extensive sources of previously conducted research on this organism (Attfield 1997, Zaldibar et al. 2001). Desirable qualities for an ethanol producing microorganism are summarized in Table 2-1.

Table 2-2 : Desirable characteristics of bioethanol production microorganisms (Díaz-Montaño, 2013)

Fermentation Properties	Technological Properties
Rapid initiation of fermentation	High genetic stability
High fermentation efficiency	Low foam formation
High ethanol tolerance	Flocculation properties
High osmo tolerance	Compacts sediment
Low temperature optimum	Low nitrogen demand
Moderate biomass production	

S. *cerevisiae* is capable of directly utilizing monomeric sugars such as sucrose, glucose and fructose, more complex sugars such as starch on the other hand would need to be hydrolyzed prior to fermentation stage of ethanol (Wheals et al. 1999, Amorim et al. 2011). However S. *cerevisiae* cannot utilize pentose sugars such as xylose, which is one of the challenges that is investigated by several researches (Diao et al. 2013).

Ethanol conversion takes place through glycolysis (Figure 2-3) under anaerobic conditions, in which one glucose molecule is broken down by the cell to two pyruvate (or pyruvic acid) and two ATP molecules. Under anaerobic conditions the pyruvate is then fermented into lactic acid or ethanol. The theoretical yield based on mass balance is 0.511g/ g for ethanol and 0.489 g/g for CO_2 (Bai et al. 2008).

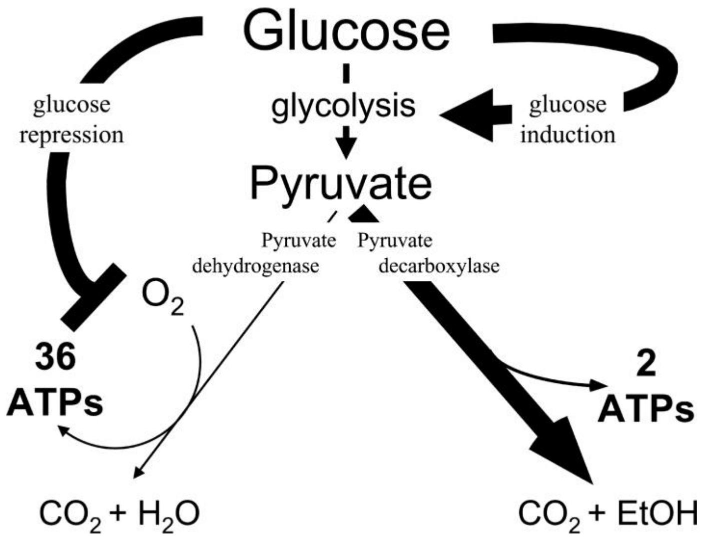

Figure 2-3 : Simplified diagram of glucose metabolism in yeast (Johnston & Kim, 2005)

When overall energy conversion is concerned S. *cerevisiae* again proves rather effective; 93% of the energy from sugar source is contained in ethanol and only 7% is used within the process (Bhatia, 2014). Macedo et al. (2008) reports that 9.3 units of renewable energy were generated for every unit of fossil energy required in Brazilian distilleriesin 2005 and it is estimated to rise to 11.6 units by 2020.

Glycerol is also produced during alcoholic fermentation -approximately 2.5–3.6 wt % of the produced ethanol- as a by-product by various yeast strains (Petrovska et al. 1999) which is required to re-oxidise NADH in anaerobic conditions (Albers et al. 1998).

2.2.2 Main processes for bioethanol production

Ethanol fermentation may take place in 3 different operation modes; continuous, batch and fed-batch (Figure 2-4). For batch fermentation, yeast culture is placed into the reactor along with substrate and nutrients; as such this system is easy to operate and sterilize and does not require skilled labor (Jain &Chaurasia, 2014). As there is no addition or removal of any type of medium, biomass, product and substrate concentrations vary with time. Once fermentation is complete, the reactor is completely emptied and cleaned before a new batch can be started (Dunn et al. 2003)

Since fermentation takes place in a closed system this mode results in a high ethanol concentration; however due to inhibitory effect of the product as well as high sugar concentration on yeast cells the productivity is low (Cheng et al. 2009).Yeast cells are usually reused for the next batch by separating the cells from the fermentation medium through centrifugation or sedimentation in order to increase overall productivity (Gutiérrez-Correa & Villena, 2010).

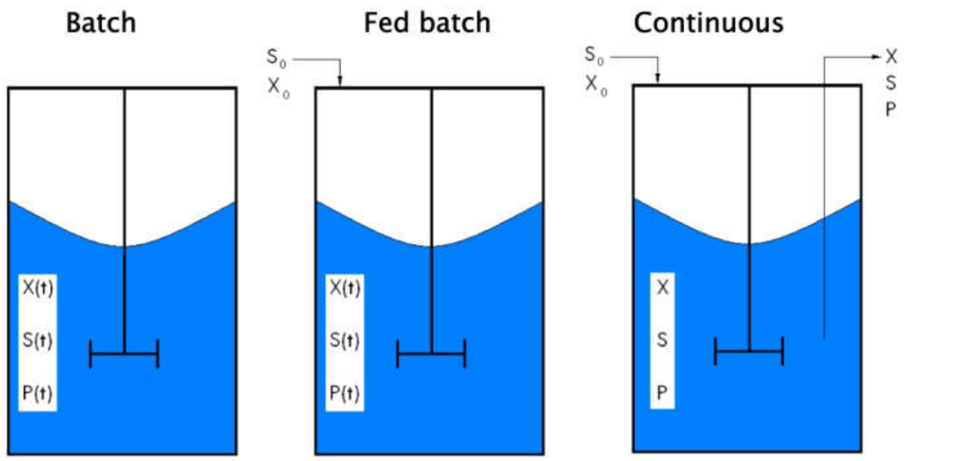

Figure 2-4 : Different modes of fermentation processes; x: biomass, s: sustrate, p: product, t: time (Díaz-Montaño, 2013)

In the fed-batch mode, also known as Melle-Boinot process, the yeast cells are fed the substrate in portions and the product is not removed since from the beginning of the fermentation, hence the volume of the fermentation medium increases during the operation (Sanchez & Cardona, 2008). This mode decreases the inhibition effect and catabolic repression allowing greater volumetric productivity. As such fed-batch mode is the most common mode used in Brazil, accounting 75% of all distilleries. Problems common in continuous applications such contamination and cell escape are also avoided through fed-batch processing (Basso et al, 2011).

Continuous mode, combined with cell immobilization produces the highest cell concentrations inside the reactor, resulting in higher productivity rates compared to conventional batch fermentation mode. As ethanol is constantly removed during the fermentation stage, inhibition factor caused by ethanol on yeast cells are eliminated

(Ishola, 2014). Even though, continuous mode is not popular as reported by Andrietta et. al. (2007) due to ineffective transformation of batch plants to continuous plants.

2.2.3 Factors affecting bioethanol production

Major factors that affect bioethanol production are;

- Temperature (as it would affect the growth speed of the biomass)
- sugar concentration (high concentrations of sugar could cause cell stress),
- pH,
- fermentation time,
- agitation rate,
- inoculum size (Zabed et al. 2014 &Charoenchaiet al.1998 &MarelneCot et al. 2007)

The ideal conditions would depend on the strain used for producing the bioethanol. For the purposes of this study, further information on the ideal conditions for growth and productivity of S. *cerevisiae*are given in Section 2.4.1 .

2.2.4 Contamination issue

A major issue hindering efficient industrial fermentation of sugar based feed stock to ethanol is unwanted bacterial contamination (Khullar et al., 2013; Zia et al., 2011). The bacterial contamination can be introduced to the fermentation system from different sources such as instruments, reactors and pipe lines to feed stream and added chemical and nutrients(Hines et al., 2010; Zia et al., 2011).There have been 500 or more different strains of bacteria isolated from different stages of a fermentation process. These bacterial contaminations can also be introduced to the system by recycled/reused yeast. However the majority of the bacteria contaminating the fermentation stage of bioethanol production are lactic acid producing bacteria as they have a relatively better tolerance for low pH environment as well as higher ethanol concentration (Kandler, 1983;Skinner and Leathers, 2004).

The presence of bacterial contamination in a fermentation process can lead to the drop in final ethanol yields and process profitability. In fermentation processes running in continuous, semi-batch and batch modes, the substrate and nutrient sources are provided for Saccharomyces cerevisiae to thrive in and produce ethanol, therefore, the competition between yeast and bacteria in utilizing nutrients, survival, growth and reproduction leaves inadequate levels of food for the yeast resulting in considerably lower ethanol yield. Moreover, the contaminating microorganisms produce metabolites such as lactic and acetic acids that disturb and inhibit the metabolic activity of yeast as well as reducing the quality of the final product(Zia et al., 2011). When the undissociated acetic acid enters yeast cell plasma it would cause a decrease in intracellular resulting in decreased ethanol yield or stock fermentation (Ylitervo et al. 2014).

In addition, in processes where yeast cells are being recycled and used for consecutive fermentation batches high level of bacterial contamination may lead to yeast flocculation which is an unfavorable phenomenon leading to poor mass transfer, lower cell viability and reduction in ethanol production. Moreover it would lead to excessive foam formation and in order to prevent this, antifoam agents are usually added to the process which results in increase in overall costs (Basso et. al., 2011). In certain cases the bioethanol yield may decrease as far 30%, causing a major loss for the producers. (Neto and Yokoya, 1997)

In industrial large scale fermentation a totally contamination free process is hardly achieved as complete sterilization and maintaining sterility of the feedstock and instruments involved is very costly and laborious. In this regard, in order to remediate the contamination issue and control bacterial growth in fermentation systems, conventional anti-microbial compounds such as virginiamycin, sodium fluoride, hydrogen peroxide etc. are applied(Chang et al., 1997; Limayem et al., 2011; Liu et al., 2015; Viegas et al., 2002). However, none of the anti-bacterial agents have proven to be fully effective for long term bacterial control and system disinfection. More over the residual levels of antibiotics make certain by-products such as dried yeast unavailable for commercialization for human consumption or animal feed due to adverse health effects (Basso et. al., 2011).

Other means of decontamination involves the use of strong acids; for example in Brazil, sulphuric acid (pH 1.8-2.5) is used for the treatment of diluted yeast cream for a duration of 2 hours (Basso et. al., 2011). This treatment has been reported to decrease the bacteria population by 44.55%(Lopes et el. 2016). However this method has been reported to decrease yeast viability (Ferreira et al. 1999) in terms of a drop in yeast cellular trehalose.

2.3 Immersed Membrane Bio Reactors

It is possible to resolve the issues addressed in the previous section through Membrane bioreactor (MBR) systems. By principle, membrane applications are selectively permeable hence are able to retain certain substances based on size or chemical affinity. A selective washout of bacterial contamination while retaining the larger yeast cells in the MBR can be achieved through properly adjusting the membrane pore size, hydrophobicity and affinity to different cells and compounds (Mahboubi *et al.*, 2016). Membrane bioreactors would also prevent cell escape by immobilizing the cells between layers. In this regard, not only bacterial contamination in fermentation process is mechanically removed but also higher ethanol yields are achieved as high yeast cell concentrations are present in the medium. According to Azhar et al. (2017) the benefits of immobilized cells over free cells include "higher cell density per volume of reactor, easier separation from the reaction medium, higher substrate conversion, less inhibition by products, shorter reaction time and control of cell replication."

However, the viability of this physical decontamination method is challenged by the fact that suspended bacteria can cause biofouling by internal and external membrane pore blockage and/or biofilm formation, causing increased pressure on membrane surface and eventual system failure (Lim and Bai, 2003; Chae *et al.*, 2006; Orantes *et al.*, 2004). Proposed remedies to this issue are addressed within the following sections.

2.4 Microorganisms Studied in This Thesis

Two types of microorganisms were required for the execution of this research; the bioethanol fermenting organism and the contaminant bacteria. *Saccharomyces cerevisiae* was selected for the first and *Enterobacter cloacae* for the latter.

2.4.1 Saccharomyces cerevisiae

Saccharomyces cerevisiae is a species of yeast, commonly used for fermentation. Cells produce by budding and appear as ovoid or elipsoidal under microscope and in average 5-10 µm for the large diameter and 1-7 µm for the small diameter. *Saccharomyces cerevisiae* is one of the few yeasts with the capacity to grow rapidly under anaerobic conditions and is classified as facultative anaerobic. (Feldman, 2010; Snoek and Steensma, 2006)

Baker's yeast prefers mesophilic conditions, optimum temperature being 30.0°C-35.0°C. Woo et al. (2014) have performed cultivation between 30 to 42 °C and have reported that even though carbon utilization was not affected significantly when cultivation temperature was increased to 38 °C, cell structures had changed at cultivation temperatures of 40 and 42 °C and growth rate decreased by half. Kasemets et al. (2007) have also found that growth rate started to decrease in case the cultivation temperature is increased to 38 °C and observed optimum temperature for growth as 34–35 °C.

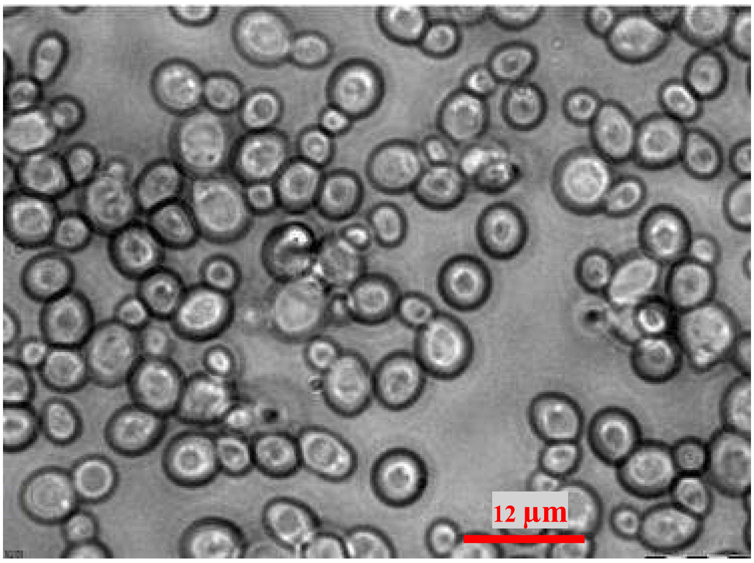

Figure 2-5 : Microscope image of mature yeast culture at high magnification; Volkov (2015)

S. *cerevisiae* is able to utilize Saccharose, maltose, melibiose, glucose and ethanol for carbon source; however if present, prefers glucose (Bekatorou et al. 2006). One of the challenges of using baker's yeast for ethanol production is that though it is able to utilize hexoses for fermentation, it cannot process pentoses (Azhara, Abdullaa, Jamboa, & Marbawia, 2017). When grown in YPD broth at a regulated pH of 5.5 for 64 hours, it has shown to enter the stationary phase after approximately 48 h. (Bassi et al. 2012) Main metabolites produced by baker's yeast are; ethanol, glycerol, and lactic acid (Wurst et al. 1980; Kasemets et al. 2007)

Though preferring mildly acidic conditions, S. *cerevisiae* can tolerate a range of pH.; Wasungu and Simard (1982) have observed optimum cell growth when the pH is between 3.5 and 5.0, preferably at pH 4.0-4.5. Eroshin et al. (1976) reported optimum pH for Saccharomyces cerevisiae growth on ethanol as 4.2. Buzas et al. (1988) have observed that cell viability decreases significantly below 3, however remains stable between pH 3 to 6. Buzas et al. (1988) have reported pH between 3.5-5 optimum for the ethanol production (based on the ethanol concentration at 100th min of fermentation) by free or immobilized S. *cerevisiae* cells, however for long term fermentation pH 3.9 was found as optimum. (Azhara, Abdullaa, Jamboa, & Marbawia, 2017)have determined ideal range as 4-5 for ethanol production and observed a decrease once the pH exceeded 5.

For growth Baker's yeast is able to utilize a variety of nitrogen sources including; ammonia, glutamine, asparagines, proline, urea, glutamic acid and amino acids (Albers et al. 1996; Zahed et al. 2016; terSchure et al. 2000). Zahed et al. (2016) have tested both organic (malt extract, yeast extract, bacto-tryptone, casein) and mineral nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulfate, ammonium molybdate) for fermentation (performed at 30 °C, pH 5) and have reported that maximum ethanol and biomass production is achieved when yeast extract is used as the sole nitrogen.

2.4.2 Enterobacter cloacae

Enterobacter cloacae is a facultative anaerobic bacterium that can grow well in mesophilic condition; Khaled et al. (2015) has reported the optimum temperature for growth is 37°C and that after 40°C the biomass growth significantly decreases. Kumar et al (2000) has also observed 36 °C as the temperature with highest metabolic activity and 45°C as critical temperature for *E. cloacae*. Microscopic observation of *Enterobacter cloacae* reveal the bacterial cells as; gram-negative, non-spore forming, short, straight, rod shaped (with a length of 0.7–1.2 µm and thickness of 0.3–0.5 µm) with round ends (Kumar et al. 1999&n.d.Mosby's Medical Dictionary 2009). Cells occur singly and in pairs and exhibit gram-negative staining.

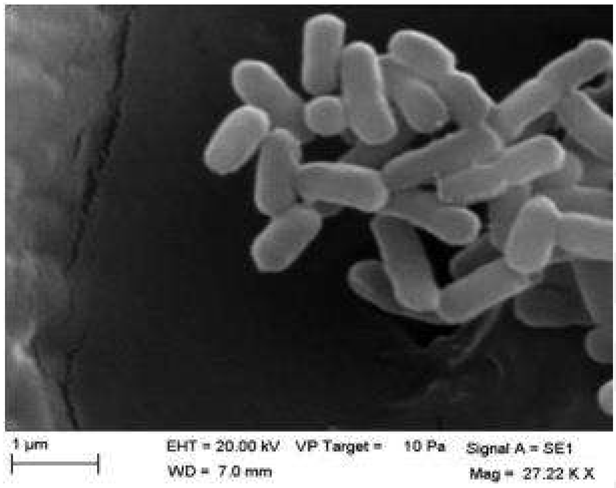

Figure 2-6 : Scanning electron microscopy (SEM) image Enterobacter cloacae DT-1. (Subuhdi et al., 2012)

E. *cloacae* can utilize various carbon sources; in their study on hydrogen production by *Enterobacter cloacae,* Subudhi et al (2012) reported that E. *cloacae* can utilize glycerol, CMCellulose, glucose and xylose at wide range of pH (5–10), optimum pH being 8 at 37 °C. The biomass was observed through optical density of the cultures in which glucose gave the highest OD_{600} as 1.6 followed by xylose, CMCellulose and glycerol OD_{600}1.4, 0.4 and 0.5 respectively.

Khaled et al. (2015) has also tried different carbons sources for the growth of *E. cloacae* such as glucose, maltose, sucrose and so on. When the optical density was observed after 80 hours of cultivation when the pH is 7.5 and temperature is 37 C° it was observed that mannitol and maltose had provided the highest biomass concentration (OD_{600} 0.84 and 0.82), where glucose showed $OD_{600}$of 0.73.

Subuhdi et al., (2012) has reported that during H_2 fermentation with glucose and xylose as carbon source initial pH 7, at 37 °C; acetate and ethanol were the major metabolites and small amounts of butyric acid were detected. Khanna et al.,(2010)has also observed acetate, butyrate, and ethanol as the major end metabolites of fermentation by E. cloacae under pH conditions between (5.5-7.5) and that production of metabolites were very pH dependent. 6.5 was reported as the optimum pH for acetate and butyrate, 7.5 for ethanol. Kumar et al (2000) has also reported that initial pH of the medium is very important for the growth of E. cloacae; it was observed that while at pH 4 cell growth and hydrogen production were limited, cell density increased with rise in initial pH from 4 to 8 the optimum pH for cell growth being 6.5.Khanna et al (2010) has also observed that E. cloacae produces highest biomass when pH is kept between 5.5- 7.5 with the optimum pH being 6.5. Khaled et al. (2015) has tried different nitrogen sources for the growth of E. cloacae such as Urea, Casein, and yeast extract and so on. When the optical density was observed after 80 hours of cultivation when the pH is 7.5 and temperature is 37 C° it was observed that urea and casein had provided the highest biomass concentration (OD_{600} 0.85 for both), where yeast extract, potassium nitrate and ammonium nitrate showed OD_{600} 0.65, 0.8 and 0.68 respectively.

2.5 Previous Studies

Though there have been several studies concerning bioethanol production in an MBR system, a study combining MBR technology for the purpose of bacteria decontamination during fermentation have not yet been conducted.

Mahboubi et al. (2017) studied a combination of MBR system with IPC (integrated permeate channel) membrane modules to develop a method in which pentose and hexose sugars were utilized by a xylose-utilizing strain of Saccharomyces cerevisiae to produce ethanol. The developed system could operate under high SS concentrations of 20% in which wheat straw hydrolysate was used as feed. In optimized conditions permeate flux and transmembrane pressure remained acceptable, facilitating a continuous process in which 83% of the theoretical ethanol yield was achieved.

Mahboubi et al. (2016) have conducted research on yeast cell retention for improvement of ethanol yield through reverse membrane bioreactors (rMBR) by combining MBR and cell encapsulation techniques. The study also included a review on the comparable characteristics of rMBR and conventional MBRs and cell encapsulation methods in terms of biofuel production. The study concludes that the use of rMBR may result in a possibility to convert substrates with significant amount of inhibitorycompounds.

Zahed et al. (2016) researched the utilization of lignocellulosic waste material (rice straw) for the production of ethanol and xylitol in an MBR system. The optimum parameters for sugar yield of 81% from rice straw were found as diluted sulfuric acidpretreatment (3.5 %)at 100 C^0 for 30 minutes to release glucose and xylose compounds and an enzymaticconversion by a commercial cellulase enzyme. Active carbon and resin were used for the removal of inhibitory compounds such as furfural, hydroxyl methyl furfural, and acetic acid. 2 different strains for ethanol (*Saccharomyces cerevisiae*)and xylitol (*Candida tropicalis*)production were selected. The maximum ethanol and xylitol productions increased from a conversion from batch co-culture to continuous co-culture reaching up to 80 % yield for ethanol and 68% yield for xylitol in a D.R. of 0.03 L/h.

Ylitervo et al. (2014) studied the effect of undissociatedacetic acid on ethanol production. Within the scope of study continuous cultivations of high and very high concentrations of yeast (~25 to 100–200 g/L) contained within a cross-flow membrane bioreactor were inhibited by addition of acetic acid (2.5 to 20.0 g/L) atpH 5.0, at a dilution rate of 0.5 h^{-1}. In acetic acid concentrations up to 15 g/L yeast was able to produce ethanol in a steady rate however production declined at acetic acid concentration of 20 g/L. The study concluded that through trapping the cells in the MBR the yeast cells had increased tolerance the acetic acid concentrations released by the conversion of lignocellulosic raw materials.

In their review for bioethanol production in MBR systems, Jain and Chaurasia (2014) have proposed that through combining pervaporation method in the fermentation stage, the produced ethanol may be removed simultaneously, resulting a simplification in downstream processing, as well as an increase in yeast cell robustness and hence improved ethanol yield.

In his doctorate thesis work Ishola (2014) studied lignocellulosic ethanol production and have developed a new method for an alternative of separate hydrolysis and fermentation andsimultaneous saccharification and fermentation methods; for those methods result in product inhibition and elevated contamination issues as well as preventing cell use. The proposed method involved usage of an MBR for the purpose of separating hydrolysis and fermentation stages so that they may take place in ideal conditions for each stage as well as enabling cell reuse. The study concluded that developed method may be used as an alternative to conventional methods of lignocellulosic ethanol production and that “high solids loading can be used to suppress bacterial infections during ethanol productions, as well as that phosphoric acid pretreatmentcan improve ethanol yield from lignocellulosic biomass”.

3. MATERIALS AND METHODS

3.1 Yeast and Bacteria Maximum Growth Rate Measurement

An understanding of the maximum growth rate of yeast and bacteria is of great importance when defining the dilution rate of a continuous fermentation process. Therefore, the growth rate of yeast at pH 5 and bacteria in pH 5 and 7 in a nutrient medium containing 30 g/l glucose, 10 g/l peptone, 5 g/l yeast extract and 0.35 g/l KH_2PO_4 was measured.

The microorganisms used in this research work were yeast strain *Saccharomyces cerevisiae* CBS 8066, provided by Centraalbureau voor Schimmelcultures (Delft, The Netherlands).Bacterial strain used in the current study was an *Enterobacter cloacae* complex, bacillus acetic acid producing bacterial contamination isolated form a xylose-glucose fermentation system at the University of Borås, Sweden, and was further identified at the Culture Collection University of Göteborg (CCUG). The identified *Enterobacter cloacae* complex consisted of nearly 30 different bacterial species with *Enterobacters* and in specific *E. cloacae* in dominance.

The cultivations were made in 250 ml Erlenmeyer flasks containing 100 ml of medium placed in a shaking water bath (Grant OLS 200, Grant instrument ltd, UK) set at 30°C/125 rpm. Frequent samplings were done at different time intervals. Samples were centrifuged, supernatant was removed and cells were suspended in PBS buffer and subjected to spectrophotometry Libra S60 (biochrom Ltd, Cambridge, England) (OD at 600 nm) against PBS blank. Readings were plotted against time and the slope of the graphs in the exponential growth phase was taken for defining the maximum growth rate.

3.2 Yeast and Bacteria Size Determination

As dimensions of both yeast and bacteria were essential in deciding the pore size of the membranes to be used for filtration purposes, their sizes were investigated. In order to evaluate dimensions of both yeast and bacteria, samples were taken under sterile conditions from yeast and bacteria cultures that were prepared for growth rate measurements. The samples were observed under light microscope following Gram staining(Beveridge, 2001) on Burker counting chamber, scaled and counted. Size distribution charts were prepared from the gathered data to calculate the average dimensions of yeast and bacteria.

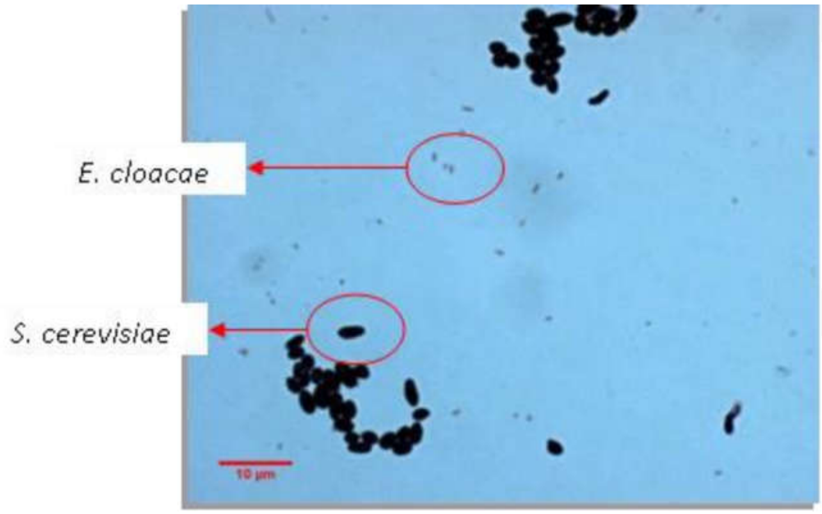

Figure 3-1: A light microscopy image obtained after Gram staining of yeast contaminated culture containing E. cloacae.

3.3 Experiments with MBR

The second generation IPC membranes panels used in the research work have double filtration layer and are typically casted onto a weft-type of polyester spacer-fabric support customized and developed by at the Flemish Institute of Technological Research (Vito NV, Belgium). The IPC membrane panels developed possess a significant strength in preserving structural integrity during backwash cycles as the membrane coatings show substantial adhesion properties to the 3D complex inter-tangled spacer-fabric (Doyen et al., 2010). The hollow area provided within the intertwined fabric is used for the withdrawal of the filtrate/permeate. Moreover, unlike many other commercial membrane panels available, IPCs come with an inbuilt air/gas channeling with 12 diffusers (6 on each side) of each 0.5 mm in diameter at the bottom of the panel. This provides the ease of reaching more efficient single panel aeration and membrane surface cleaning from foulants in comparison to universal aeration in MBRs. In 2nd generation IPCs panel aeration there is more control over the amount of air/gas provided for the cleaning of each panel, the energy invested in membrane sparging and cleaning through surface shear stress formation in more target oriented and homogeneous for all panels.

The 2nd generation IPC panels were integrated with a 1.5 l bench stirred tank reactor were the impellers were removed and the membrane was anchored to the middle shaft as well a 2 L tank in which two membranes were attached to the reactor lid enclosed with a cassette to increase membrane cleaning provided by aeration. The general schematic of the membrane bioreactor set up is illustrated in Figure 4.

Before each run, reactor is autoclaved and sterilized with chemical solutions after membrane is placed inside the reactor. Sterilization solutions consist of 2% NaOH, 1% H_3PO_4 and 200 ppm NaOCl. Each solution was kept in reactor for 30 minutes at 45 C°. Afterwards reactor was drained and rinsed with sterile water before the next solution was added.

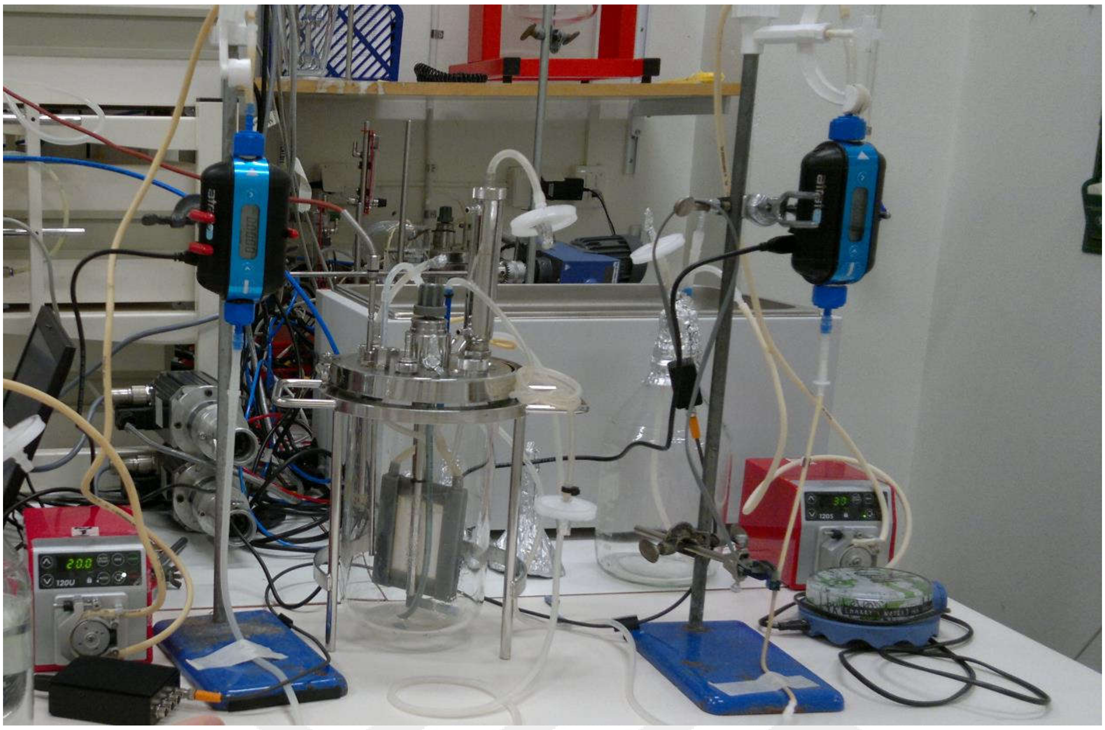

Figure 3-2 : MBR setup, sterilized prior to operation, reactor volume 1.5 L

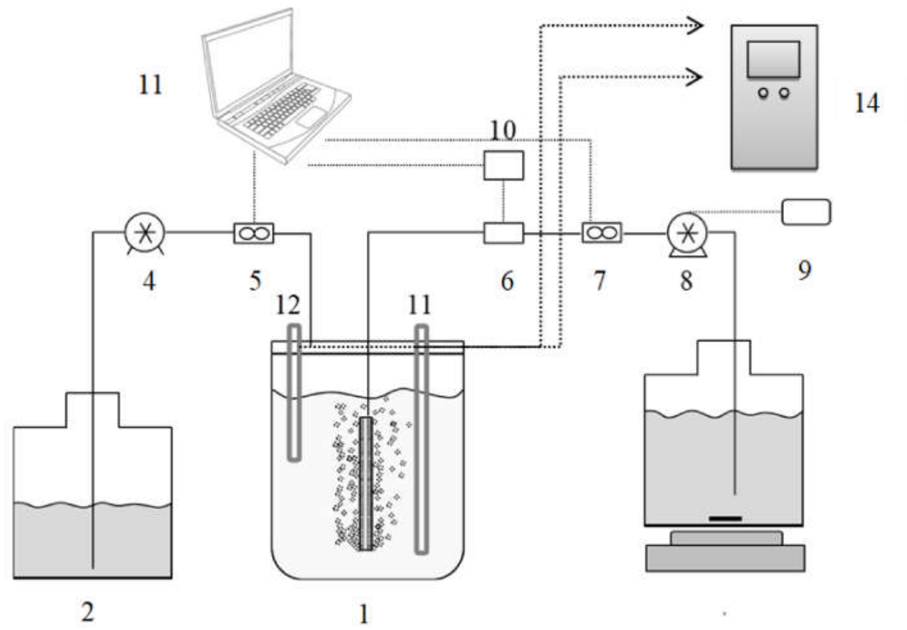

Figure 3-3 : Membrane Bioreactor Setup

1.	MBR	**8.**	PUMP
2.	FEED TANK	**9.**	RELAY
3.	PERMEATE TANK	**10.**	PRESSURE SENSOR READER
4.	PUMP	**11.**	COMPUTER
5.	FLOW METER	**12.**	**PH ELECTRODE**
6.	PRESSURE SENSOR	**13.**	**TEMPERATURE SENSOR**
7.	FLOW METER	**14.**	**CONTROL UNIT**

*Units marked with bold font are present in only double panel setup

In the applied MBRs the filtrate flow rate and also the transmembrane pressure (TMP) were monitored by means of a 710 Atrato ultrasonic flowmeter (Titan Enterprises Ltd., United Kingdom) and a Microfluidic pressure sensor MPS3 (Elveflow, France), respectively. The raw data provided from the measurement devices were recorded and processed in a connected computer. The reactor was fed from the feed tank and also the filtrate was removed from in-between membrane layers using Watson-Marlow 403U/R1peristaltic pumps (Watson Marlow, United Kingdom). In cases were backwashing was used, the cycle was controlled through Schneider Zelio logic relay (Schneider Electric Automation GmbH, Germany) attached to the permeate pump. Two different cycles of Backwash were tried in order to investigate the effect of backwashing on reversible fouling; 4-minute forward flow cycle; 30 seconds of backwash + 3.5 minutes of permeation and 3-minute cycle; 30 seconds of backwash + 2.5 minutes of permeation.

3.4 Reactor Runs with PBS as Feeding Solution

During all filtration screening and evaluation experiments in order to keep the viability of the cells during the filtration cycle PBS buffer was used as the main medium in the bioreactor;

- 8 g of NaCl
- 0.2 g of KCl
- 1.42 g of Na_2HPO_4
- 0.24 g of KH_2PO_4,

were dissolved in 1 L of distilled water and sterilized by autoclave. Same content was used for all runs with PBS.

The reactor was aerated with 200 l/h air for PBS runs.

Inoculum preparation

One colony was put inside 10 ml of PBS solution and placed in a shaking water bath at 30°C / 125 rpm for 30 min. 100 ml of nutrient medium was inoculated with 1 ml of yeast or bacteria inoculum. The cultures were grown in shake flasks for 29 hours at 30°C / 125 rpm. The media was centrifuged at 5000 xg for 5 minutes, the supernatant was removed and cells were re-suspended in PBS.

3.4.1 Yeast retention in an MBR

For the actual MBR experiments the pumps were set at 31 rpm for 0.75 l/h flow rate and 0.5 h^{-1}dilution rate with an aeration rate of 200 l/h.300 ml of inoculum was added to the reactor under sterile conditions. The zero hour samples were taken for HPLC analysis (1 ml). Moreover dry mass samples (5 ml, 2 replicates for each sample) were taken from shaking flasks and reactor for 0 hour. In addition, samples were taken for spectrophotometry (λ=600 nm) and plate inoculation at 0-5-10-20 mins and every 20 mins following that.

Run 1

A membrane with 1 μm pore size was used. For the permeate line 10^{-1} and non-diluted samples of 0.2 ml were used to inoculate each plate. Duplicates were made from each sample.

Run 2

A membrane with 2.4 μm pore size was used. Plates prepared from reactor 0 hour, shake flasks and permeate by the following dilution rates:10^{-5}, 10^{-6}, 10^{-5}, 10^{-6},Non diluted and 10^{-1}.

3.4.2 Bacteria filtration in an MBR

Membrane pore size of 1 and 2,4μm were used for inoculum sizes of 300, 100 and 50 ml of bacteria.

Runs were executed in;

- Dilution Rate of 0.66, 0.5, 0.38 and 0.25 h^{-1} in a reactor with single membrane panel with a backwash cycle of 3,5 minute permeation+ 30 seconds of backwash
- Dilution Rate 0.5, and 0.25 h^{-1} in a reactor with single membrane panel without backwash
- Dilution Rate of 0.63 and 0.5 h^{-1} in a reactor with double membrane panel with a backwash cycle of 3,5 minute permeation+ 30 seconds of backwash

Inoculum was added to the reactor under sterile conditions.

The zero hour samples were taken for HPLC analysis (1 ml).

Moreover dry mass samples (5 ml, 2 replicates for each sample) were taken from shaking flasks for 24 h and reactor for 0 hour. In addition, samples were taken for spectrophotometry (λ=600 nm) in all runs and plate inoculation for runs with single panel.

3.4.3 Bacteria filtration+ yeast retention in an MBR

Membrane pore size of 2,4μm was used for the filtration of 50 ml bacteria and 100 ml yeast. Runs were executed in;

- Dilution Rate of 0.5 h^{-1} in a reactor with single membrane panel with a backwash cycle of 3,5 minute permeation+ 30 seconds of backwash
- Dilution Rate of 0.5 and 0.75 h^{-1} in a reactor with double membrane panel with a backwash cycle of 3,5 minute permeation+ 30 seconds of backwash

Zero samples for spectrophotometry (λ=600 nm) were taken after the addition of bacteria inoculum following 10 minutes of mixing as well as after the addition of yeast. Dry mass samples (5 ml, 2 replicates for each sample) were taken from shaking flasks for 24 h and reactor for 0 hour.

3.5 Reactor Runs with Fermentation Medium;

For fermentation conditions, sterile synthetic medium consisting of Glucose, Peptone, yeast extract and K_2HPO_4 was used in two different concentrations;

- 30 g/l Glucose + 10 g/l Peptone + 6 g/l Yeast Extract + 3,5 g/l K_2HPO_4
- 20 g/l Glucose + 1 g/l Peptone + 1 g/l Yeast Extract + 3,5 g/l K_2HPO_4

Inoculum preparation

One colony was put inside 10 ml of PBS solution and placed in a shaking water bath at 30°C / 125 rpm for 30 min. 100 ml of nutrient medium was inoculated with 1 ml of yeast or bacteria inoculum. The cultures were grown in shake flasks for 29 hours at 30°C / 125 rpm and added to the reactor in sterile conditions.

Sampling;

- Samples for dry mass (5 ml, 2 replicates for each sample) and HPLC (1 ml for each flask) were taken from shaking flasks after 24 h cultivation.

Dry mass samples were centrifuged in 10 ml Falcon tubes at 3000 RCF for 5 minutes, the supernatant was poured and cells were re-suspended in 5 ml of Milli-q water. Samples were again centrifuged, 4 ml supernatant was removed by pipette. 1 ml of Milli-q water was added and samples were vortexed. The content was poured in glass tubes and put in 70 C° oven for 3 days.

- HPLC samples were centrifuged at 15 XG and the supernatant was examined.

The high performance liquid chromatography (HPLC) (Walters 2695, Walters Corporation, Milford, USA) used had a hydrogen-based column (Aminex HPX-87H, Bio-Rad, Hercules, USA) working at 60°C with 5 mM H_2SO_4 eluent flowing at 0.6 $ml.min^{-1}$ proper for detection and quantification of sugars and fermentation metabolites such as glucose, ethanol, glycerol and acetic acid.

- Samples for dry mass (5 ml, 2 replicates for each sample) and HPLC (1 ml) were taken from reactor and permeate tank at every 2 hours starting from 0 hour.

Dry mass samples were centrifuged in 10 ml Falcon tubes at 3000 RCF for 5 minutes, 4 ml supernatant was removed by pipette and cells were re-suspended in 4 ml of Milli-q water. Samples were again centrifuged, 4 ml supernatant was removed by pipette. 1 ml of Milli-q water was added and samples were vortexed. The content was poured in glass tubes and put in 70 C° oven for 3 days.

- HPLC samples were centrifuged at 15 XG and the supernatant was examined.
- Plates were inoculated with 50 μL of sterile samples taken from reactor and permeate line at 0, 5 and 10 h for single panel runs for dilutions of $10^{-1,}$ 10^{-3} and $10^{-5.}$

Filtration Conditions;

- Backwash was applied in all fermentation runs in two different cycles;
  4 minute cycle; 30 seconds of backwash + 3.5 minutes of permeation
  3 minute cycle; 30 seconds of backwash + 5.5 minutes of permeation
- Dilution rates between 1 and 0.11 h^{-1} were operated with flux rates between 145 and 16 l/m2/h
- Reactor was aerated with 250 l/h N_2 to keep the conditions anaerobic for fermentation purpose in all runs.

pH;

In all runs with single membrane panel pH was not controlled till 10^{th} hour. After 10^{th} hour sampling,0.5 ml 10 M NaOH was added to the reactor. The reactor and permeate tank was then left for fermentation for 24 hours.

In runs with double panel pH is controlled at 5 by automatic addition of 2 M NaOH through the control unit in some runs in order to investigate the effect of pH in MBR filtration. Other runs pH was manually controlled in the same manner of single panel runs.

4. RESULTS AND DISCUSSION

In order to evaluate the capabilities of the IPC membrane module in removing bacteria selectively while retaining high concentration of yeast in the bioreactor the following stages of work have been covered till present:

4.1 The Evaluation of The Bacterial Strain and Yeast Complex Growth and Metabolic Activity In Shake Flask Cultivation

In this regard the maximum growth rate of the target yeast and bacteria was separately measured at 30°C and pH (5 and 7) that are either favorable for the growth of the bacteria or present the optimum condition for fermentation. Estimation of the growth rate of both microorganisms was of essential importance to adjust continuous fermentation conditions such as the dilution rate in order to skip loss of nutrients while effectively removing bacteria.

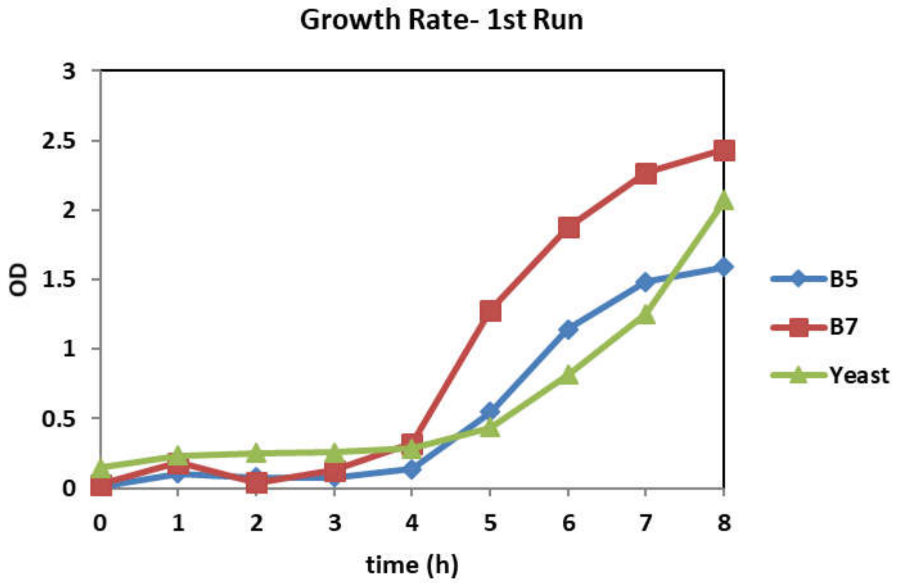

Figure 4-1 : Optical Density vs Time graph for Bacterial Cultivation at pH 5 (B5), Bacterial Cultivation at pH 7 (B7) & Yeast Cultivation at pH 5

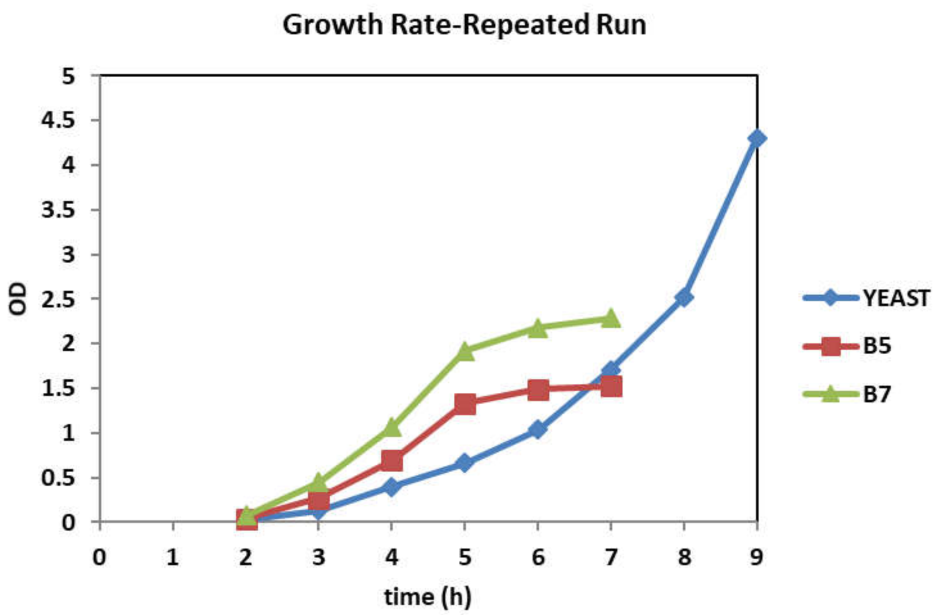

Figure 4-2 : Optical Density vs Time graph for Bacterial Cultivation at pH 5 (B5), Bacterial Cultivation at pH 7 (B7) & Yeast Cultivation at pH 5

Linear equations of each cultivation group were determined in order to calculate growth rate based on doubling time, the results are summarized in Table 2-1.

Table 4-1 : Growth rate calculations of Bacterial Cultivation at pH 5, Bacterial Cultivation at pH 7 & Yeast Cultivation at pH 5 based on doubling time

1st run	**Repeated Run**
Yeast (pH 5)	Yeast (pH 5)
$\mu = 0.434$	$\mu = 0.451$
Bacteria (pH 5)	Bacteria (pH 5)
$\mu = 0.464$	$\mu = 0.523$
Bacteria (pH 7)	Bacteria (pH 7)
$\mu = 0.899$	$\mu = 0.852$

The optimum condition for yeast fermentation (pH 5) does not comply with that of the *E. cloacae* (pH 7). This condition as presented in table and figures above give as different maximum growth rate to the bacterial complex during fermentation. As the dilution rate and relatively permeate flow rate in an MBR directly affects the growth and washout of bacterial contamination the maximum growth rate of the bacteria defines the permeate flow rate. As tested the maximum growth rate of bacteria in fermentation condition favorable for yeast is relatively the same as yeast.

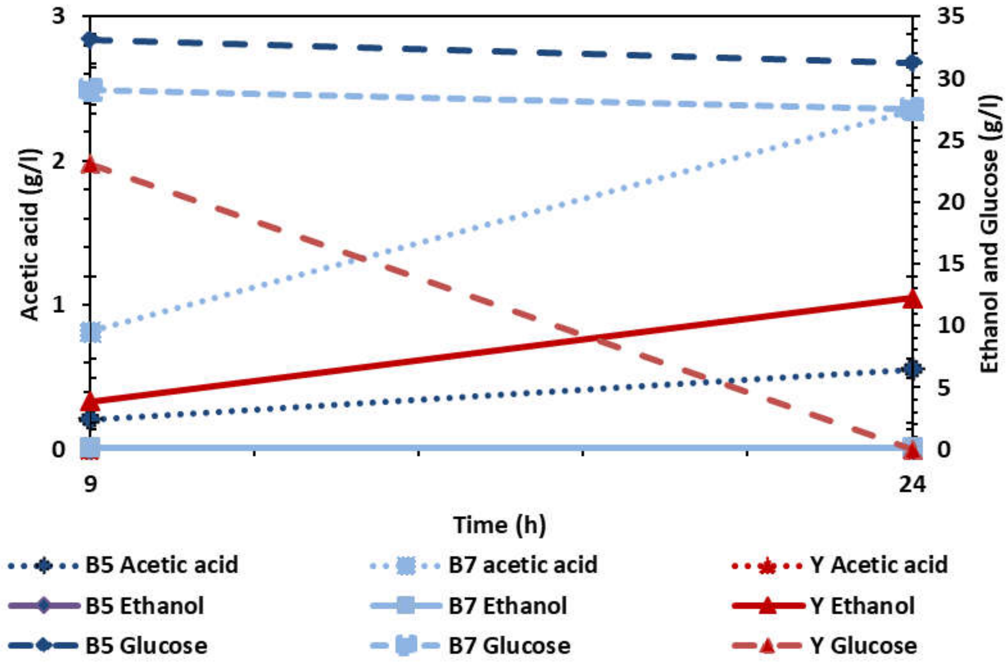

Figure 4-3 : Ethanol, Glucose & Acetic Acid concentrations observed during 24 hours of cultivation for Bacteria at pH 5 (B5), Bacteria at pH 7 (B7) & Yeast (Y) at pH 5

The HPLC results along with spectrophotometry measurements have both confirmed that the bacteria prefer to grow on pH 7 over 5 as the metabolic activity of bacterial complex seems to be more in the former than the latter pH. As seen in Fig 5, the acetic acid produced by bacteria at 24th hour of cultivation in pH 7 is more times more that the amount produced at pH 5. Therefore the results obtained from both growth and metabolite surveys comply with the results from previous studies by other groups (Subuhdi et al.,2012;Kumar et al.,2000;Khanna et al.,2010).

4.2 The Concomitant Cultivation of Yeast Growth and Bacteria in Shake Flask

The metabolic respond of both microorganisms separately and also in a mixture to a nutrient media were evaluated using sampling and HPLC analysis in order to find out the expected the type and range of metabolite produced during fermentation and also to screen the changes in metabolic activity of either microorganism in a medium inoculated with a mixture of both.

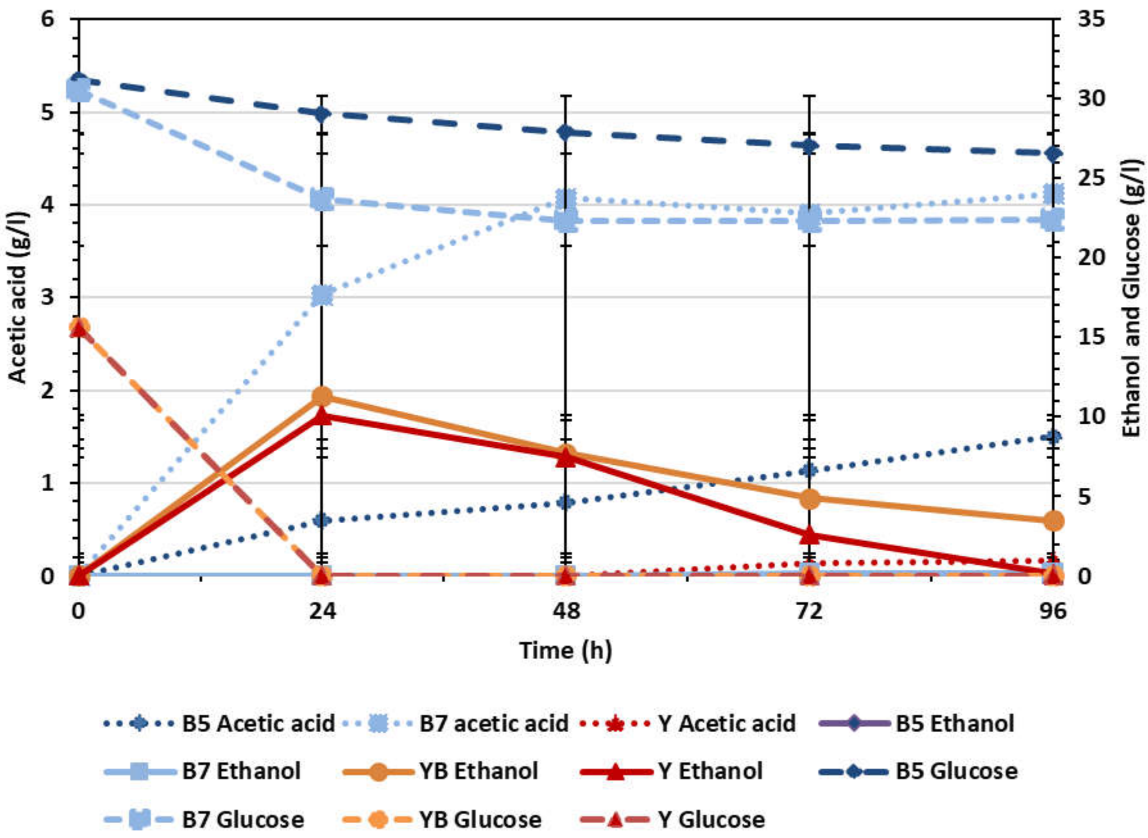

Figure 4-4 : Ethanol, Glucose & Acetic Acid concentrations observed during 96 hours of cultivation for Bacteria at pH 5 (B5), Bacteria at pH 7 (B7), Yeast (Y) at pH 5 and Bacteria and Yeast (YB) at pH 5

The metabolites produced by yeast and bacteria as well as glucose uptake complied with previous studies (Subuhdi et al.,2012;Khanna et al.,2010). Ethanol concentration in yeast cultures continued to increase until the glucose was finished at 24th after which is decreasing as it is utilized by the yeast as carbon source; which shows that yeast prefers glucose over ethanol as carbon source also in compliance with previous studies (Bekatorou et al. 2006)

On the other hand the ethanol production by yeast and YB cultures showed very similar trends; as the ethanol production by bacteria is negligible it can be concluded that ethanol production by yeast is not affected by the pH drop caused by bacteria. Yeast tolerance for pH between 3.5 to 6 is also observed in previous studies (Wasungu and Simard,1982;Eroshin et al.,1976; Buzas et al.,1988).

4.3 Reactor Runs with PBS as Feed Solution

4.3.1 Yeast retention in an MBR

As the retention of yeast in the membrane bioreactor is of prime importance in a bioethanol fermentation system aiming at high productivity, initially membrane properties meeting the needs in this regard were evaluated accordingly. Different membrane pore sizes and filtration set ups considering filtration flux, transmembrane pressure and dilution rate were examined and the optimum conditions applicable were addressed in order to have highest flux through the membrane while confining yeast in the bioreactor and preventing excessive fouling. The mentioned stage was performed by adding inoculums prepared in nutrient media in shaking flask and addition to the main MBR containing PBS buffer solution. The reactor runs for this stage are summarized in Table 4-2.

Table 4-2 : Experiments run with PBS to obtain the optimum conditions of yeast retention in an MBR

Pore size (µm)	Inoculum volume (ml)	Initial inoculum concentration in reactor (g/L)	Average Flux (L/m2/s)
1	300	0.6	112
2.4	300	0.6	109

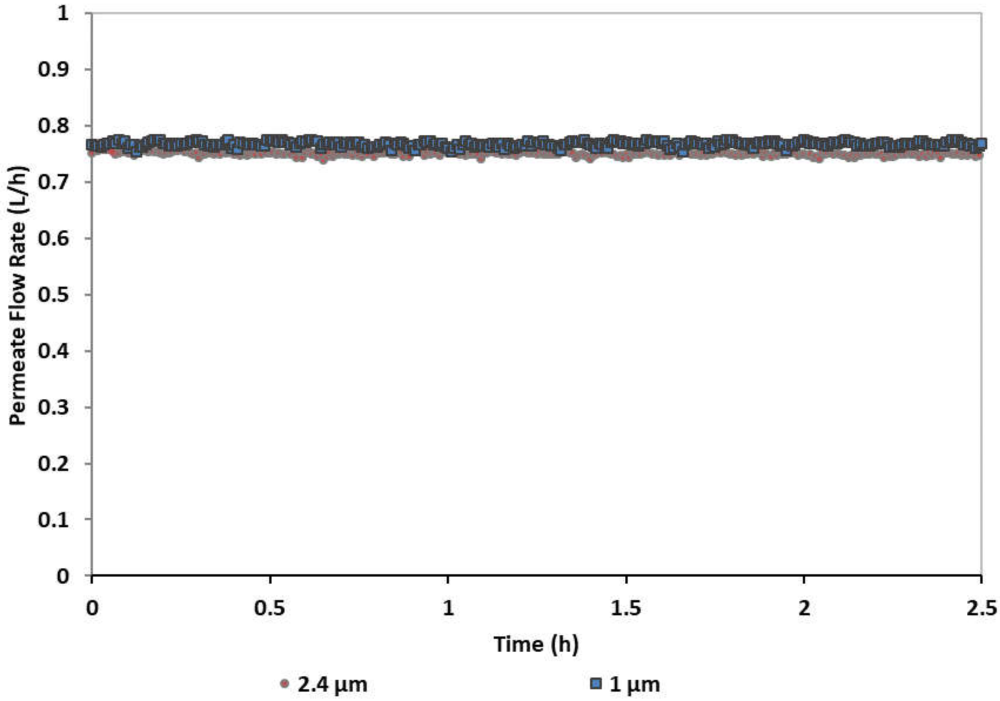

Figure 4-5 : Permeate line flow rate in yeast retention with pore size of 1µm and 2,4 µm

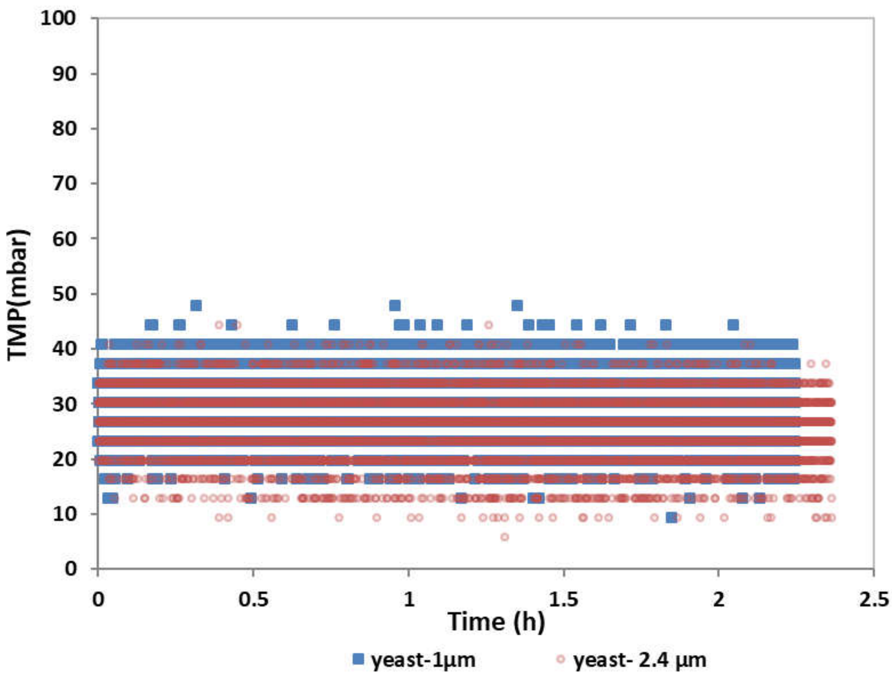

Figure 4-6 : TMP change during yeast retention with pore size of 1µm and 2,4 µm

As it can be seen from Figure 4-5&Figure 4-6 , the permeate flow rate and TMP was stable during both runs, this proves that this initial flow rate and filtration TMP the yeast suspension has not had extensive interaction with the membrane regarding cake layer formation and fouling. Also, after incubation (30 C°, 24h) no growth was observed at plates inoculated from permeate line that confirms the complete retention of yeast in the reactor.

This is further supported through the spectrophotometry readings taken from both the reactor and permeate tank (Figure 4-7); there is no turbidity increase in the permeate tank in either run, slight changes in the reactor samples are due to heterogenous samples.

Therefore it was concluded that 1 µm pore size is applicable to yeast retention however 2.4 µm pore size membrane was considered as the better choice to apply in the next step which is bacterial removal in an MBR as bigger pores ease the washout of bacteria.

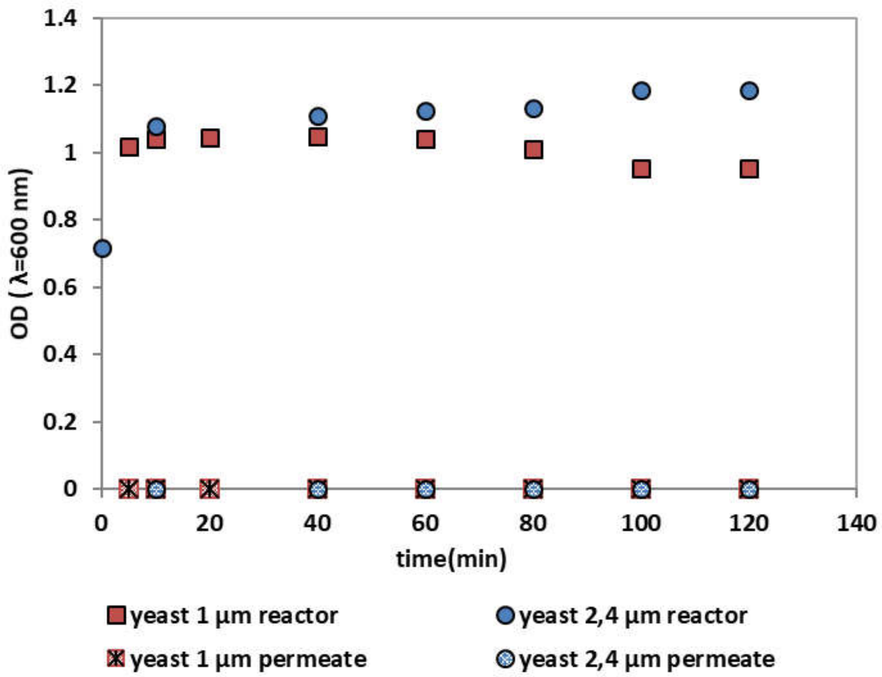

Figure 4-7 : Graph of optical density measurement in reactor and permeate samples; yeast retention with pore size of 1µm and 2,4 µm

4.3.2 Bacterial removal in an MBR

After the limits of yeast filtration were clarified, the conditions were applied to find the optimal extent of bacterial removal. As the size of the bacterial strain plays an important role in membrane fouling and also pore clogging several set ups were considered regarding membrane pore size, permeate flux and transmembrane pressure, through which the favorable filtration setting were gathered to have the highest percentage of initial bacterial inoculum removed from the system in the shortest cycle time. The following runs as shown inTable 4-3were executed to achieve this purpose.

Table 4-3 : Experiments run with PBS to obtain the optimum conditions of bacteria washout

Pore size (µm)	Inoculum volume (ml)	Initial inoculum concentration in reactor (g/L)	Average Flux (L/m2/s)	Backwash	Run time (h)
2,4	300	0.300	81	No	2,00
1	100	0.113	107	No	2,00
2,4	100	0.105	51	No	4,00
1	100	0.067	53	No	4,00
2,4	100	0.113	51	No	10,50
2,4	50	0.050	57	No	7,50
2,4	50	0.052	107	No	2,00
2,4	50	0.053	89	30 sec in 4 min	7,00
2,4	50	0.042	121	30 sec in 4 min	5,50
2,4	50	0.027	155	30 sec in 3 min	2,00
2,4	50	0.033	73	30 sec in 4 min	7
2,4	50	0.034	91	30 sec in 4 min	6,5
2,4	50	0.023	192	30 sec in 4 min	4

4.3.2.1 Pore size determination

During previous runs with yeast inoculum it was determined that both pore sizes of 1 µm and 2.4 µm were able to retain yeast, therefore they were both tested for capabilities of bacteria washout with an average flux of 52.5 l/m2/h with same bacteria inoculum size.

Even though no change was observed in permeate flow rate (Figure 2-1) , the pressure and optical density data (Figure 4-9&Figure 4-10) have both concluded that using a pore size of 1µm results in membrane fouling, while 2.4 µm is effective for bacteria washout since there is constant increase in pressure during filtration with 1µm pore sizes membrane however turbidity remains constantly around 0 in the permeate tank and decreases slightly in the reactor. It is concluded that the reason is that bacteria clogs the pores – hence O.D. drop in the reactor & pressure increase on membrane- unable to pass through. While 2.4 µm performs at stable pressure under the same filtration conditions with O.D. increasing in the permeate tank. Therefore 2.4 µm pore size was used for all following runs.

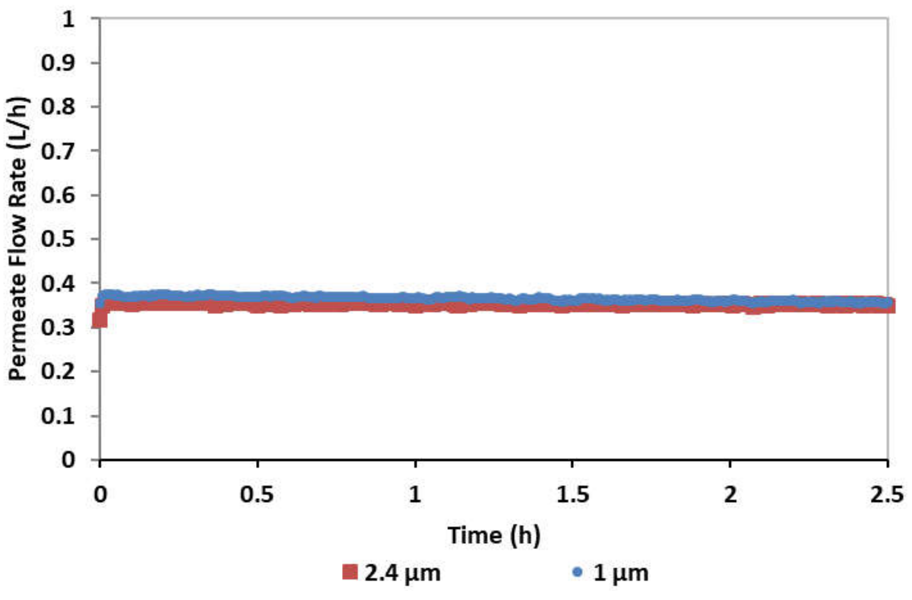

Figure 4-8 : Permeate line flow rate in bacteria filtration with a dilution rate of 0,5 h-1 and pore sizes of 1µm and 2,4 µm, filtration in single panel

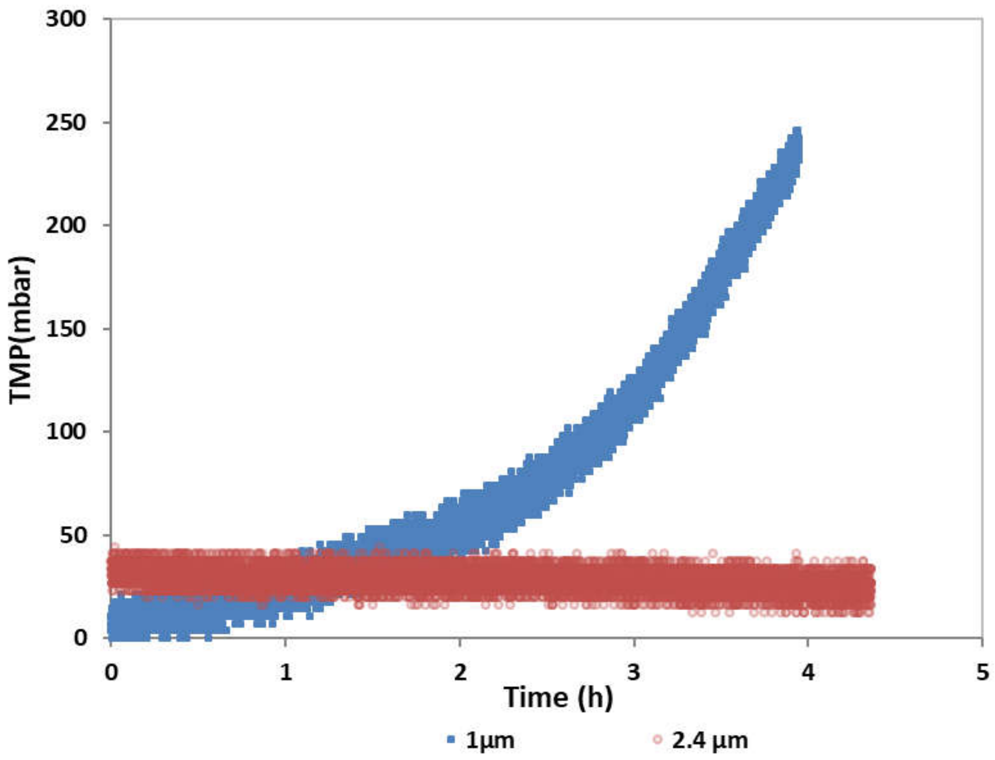

Figure 4-9 : TMP change during bacteria filtration with a dilution rate of 0,5 h-1 and pore sizes of 1µm and 2,4 µm, filtration in single panel

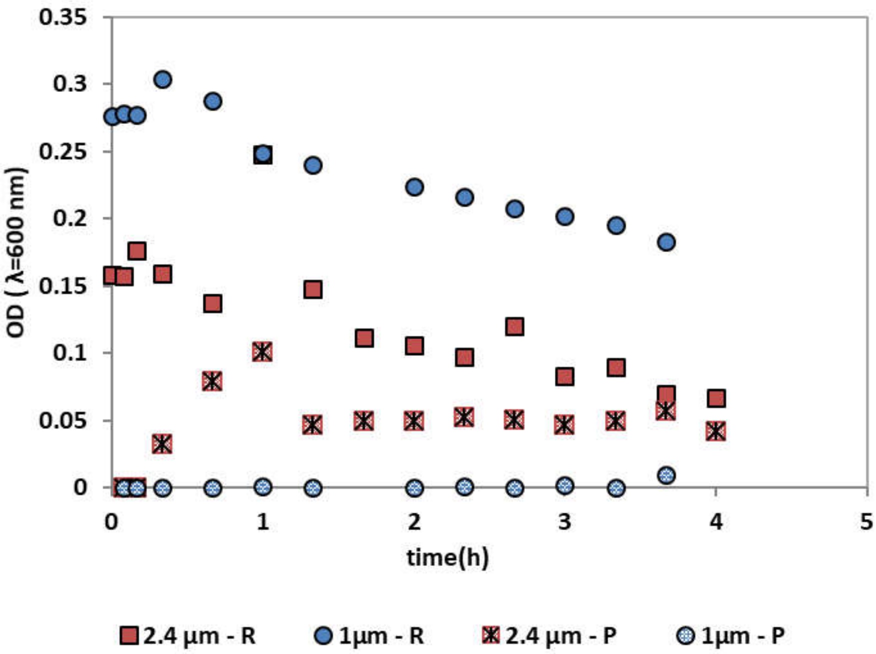

Figure 4-10 : Graph of optical density measurement in reactor and permeate samples; bacteria filtration with a dilution rate of 0,5 h-1 and pore sizes of 1µm and 2,4 µm, filtration in single panel

4.3.2.2 Inoculum size determination

Since it was established with the previous run that a pore size of 2,4 µm is sufficient to retain yeast in the reactor while achieving bacteria washout, ideal bacteria inoculum concentration was investigated as the next step. For this purpose, inoculum sizes of 100 ml and 50 ml (initial reactor concentrations; 0,1 g/l & 0,05 g/l) were compared for same Dilution Rate of 0.25 h^{-1}.

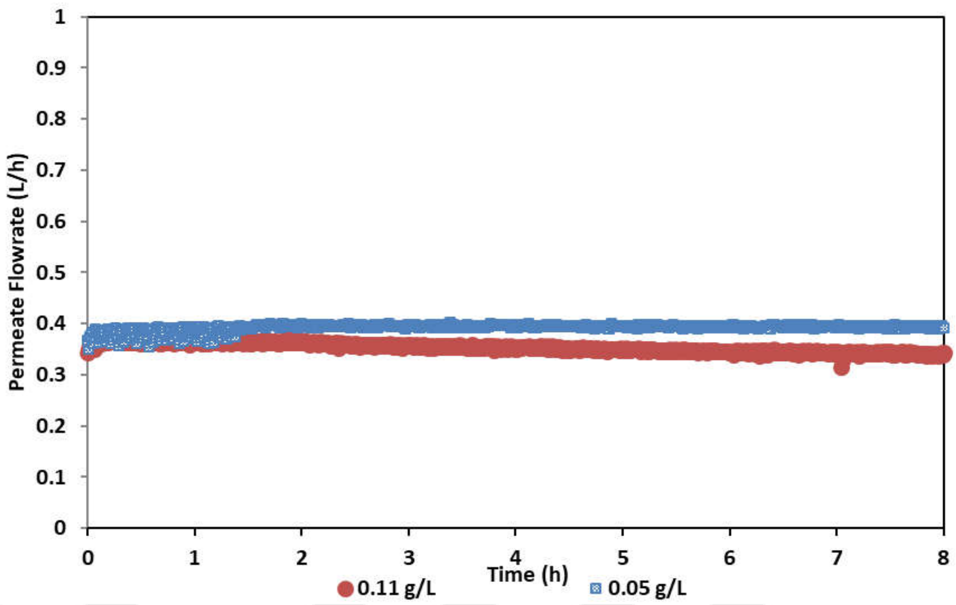

Figure 4-11: The changes in permeate flowrate (L/h) during the filtration of bacteria containing medium with inoculum size of 0.11 g/L and 0,05 g/L., filtration in single panel

In both cases a significant change was not observed in the permeate flow (Figure 4-11), however, while turbidity in the reactor dropped in both cases (Figure 4-12), the TMP constantly increased when the inoculum concentration is 0.1 g/l; signifying membrane fouling.

Therefore it was determined that an initial bacteria concentration of 0.1 g/l is too high to achieve continuous filtration of bacteria from the system. 50 ml (0,05 g/l) inoculum size was therefore chosen as the inoculum size to investigate filtration parameters in the following runs.

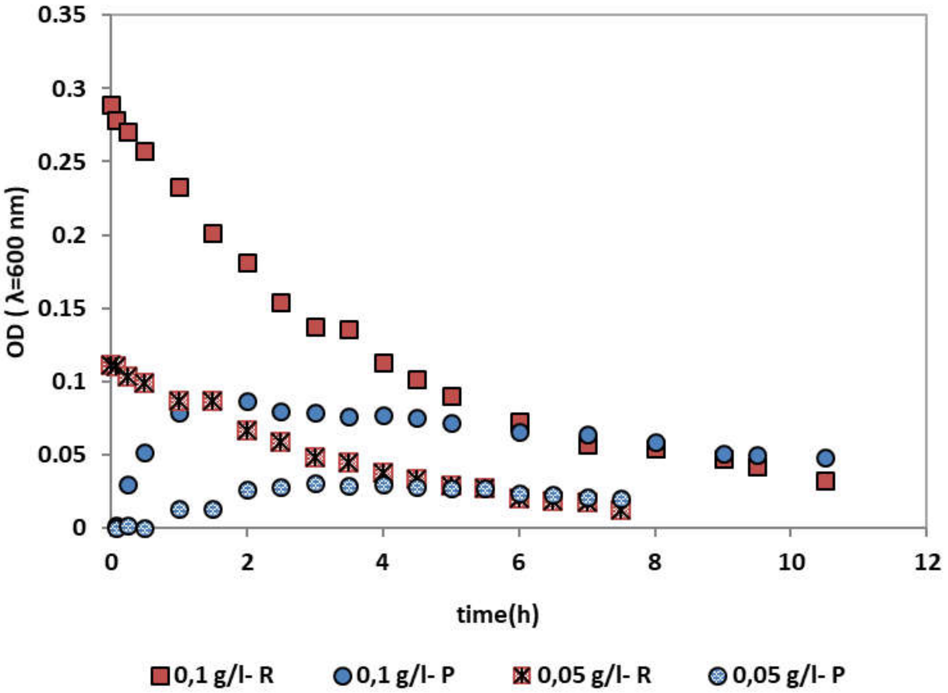

Figure 4-12 : The changes in the turbidity of the reactor media (R) and permeate media (P) during the filtration of bacteria containing media with inoculum size of 0.11 g/L and 0,05 g/L, filtration in single panel

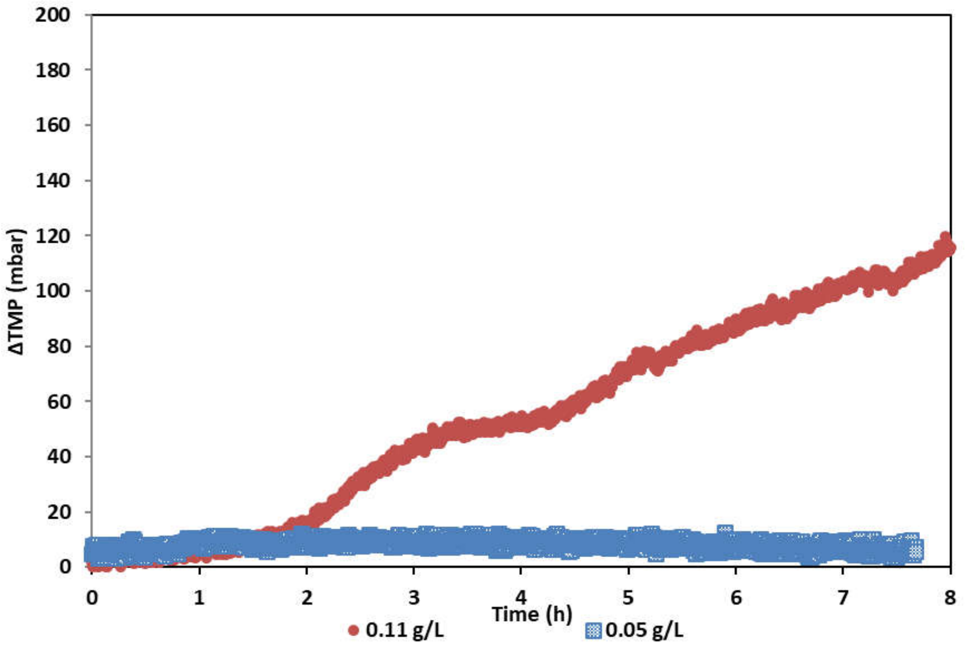

Figure 4-13 : The changes in TMP during the filtration of bacteria containing medium with inoculum size of 0.11 g/L and 0,05 g/L., filtration in single panel

4.3.2.3 Determination of Critical Dilution Rate

As inoculum size for bacteria had been established from previous runs an attempt was made to double the permeation flowrate. However, for the same inoculum size a D.R. of 0.5 h^{-1} proved too high; membrane was fouled and bacteria washout could not be achieved; TMP increased as membrane get clogged and reactor O.D. values stabilized before complete washout could be achieved (Figure 4-14&Figure 4-15).

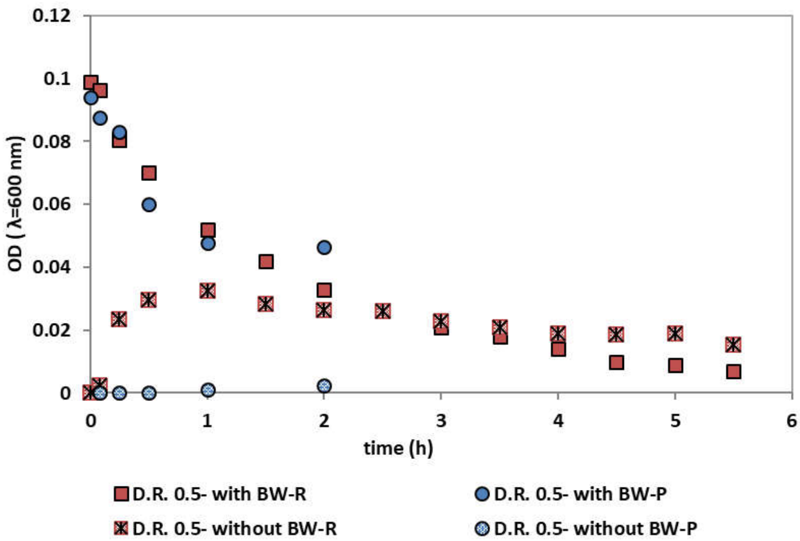

Figure 4-14 : The changes in the turbidity of the reactor media (R) and permeate media (P) during the filtration of bacteria containing media with and without backwash, filtration in single panel

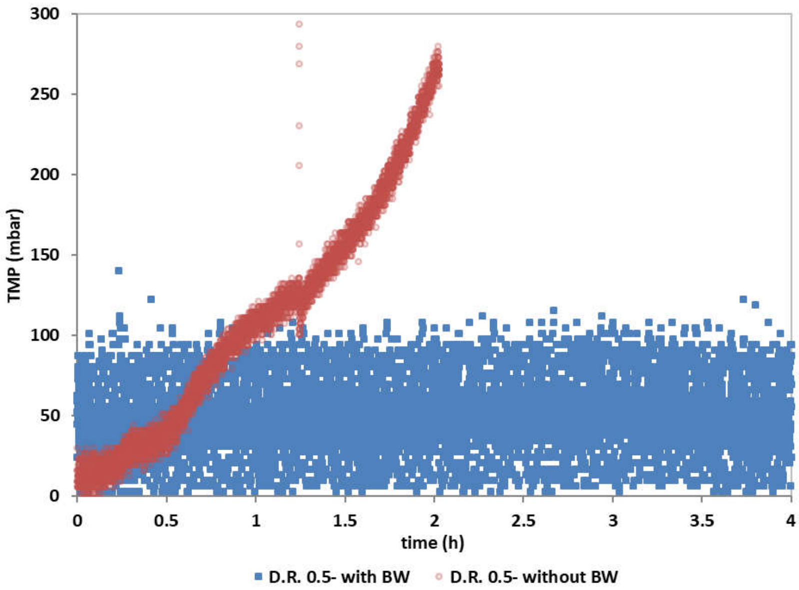

Figure 4-15 : The changes in TMP during the filtration of bacteria containing media with and without backwash, filtration in single panel

To have a D.R. of 0.5 h^{-1}it was decided to apply backwash to the system. A backwash cycle of 3.5 minutes of permeation + 0.5 minutes of backwash was chosen. Though the remaining conditions were unchanged, the run had yielded favorable results. The TMP and permeate flowrate had remained stable during the run while continuous bacterial removal was observed though optical density results (Figure 4-14&Figure 4-15&Figure 4-16).

Though backwash is generally used for membrane cleaning in this case it was determined to work as a preventative measure for fouling.

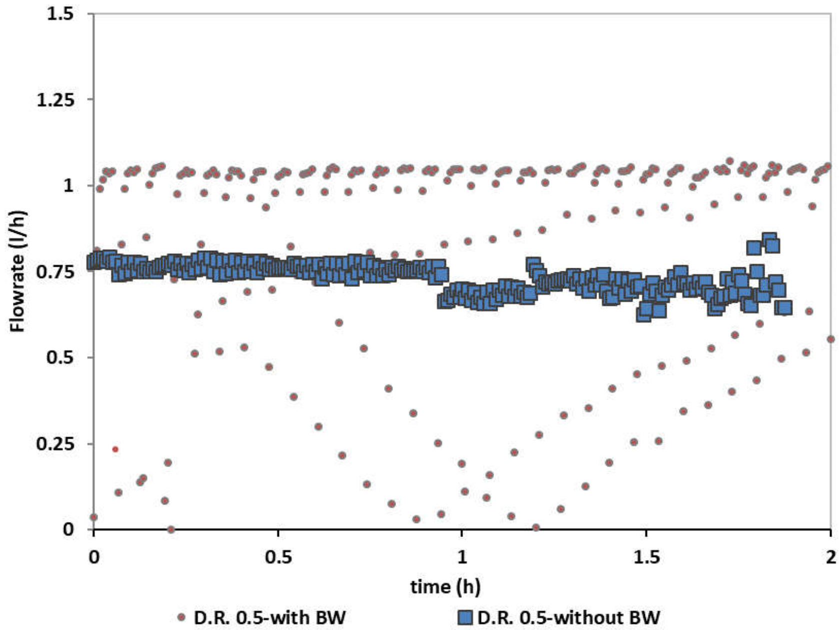

Figure 4-16 : The changes in the permeation flow filtration of bacteria containing media with and without backwash, filtration in single panel

To wash out the bacteria in a shorter time, dilution rate was increased from 0.5 1/h to 0.66 1/h while backwash cycle was changed as 2.5 minute forward wash and 0.5 minute backwash instead of former application of 3.5 minute forward wash and 0.5 minute backwash at this experiment. As Figure 4-17 shows that pressure increased by the effect of higher dilution rate but then stabilized at an acceptable pressure value. More frequent backwash cycle could not compensate the fouling of membrane caused by higher flow rate, pressure builds as bacteria is stuck on membrane surface and as the number of bacteria in the reactor is finite, stabilizes in time.

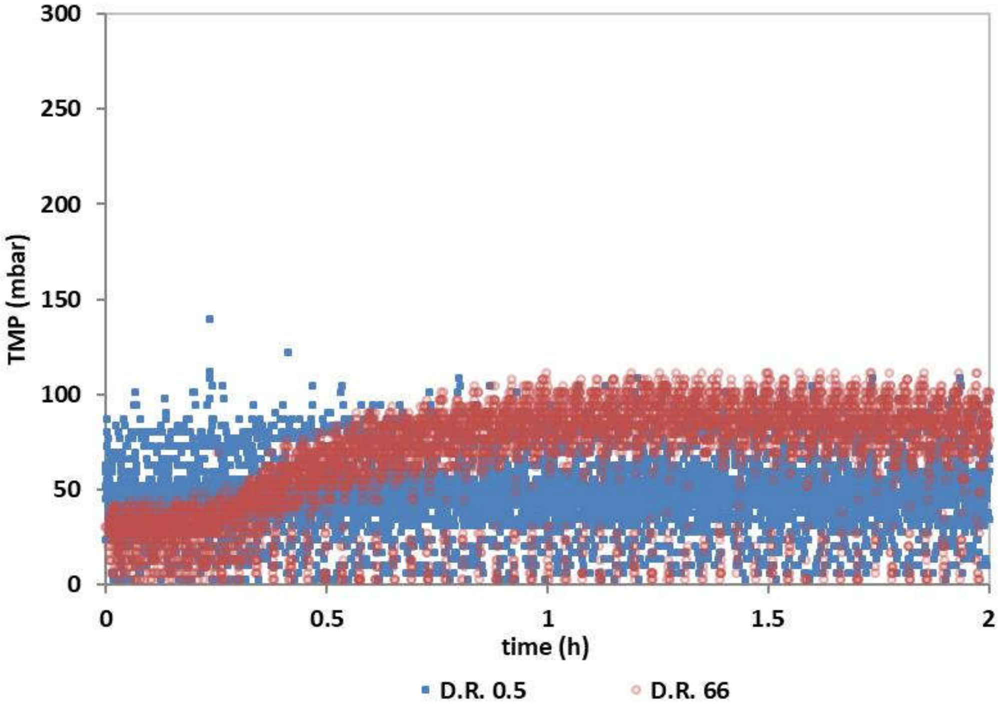

Figure 4-17 : The changes in TMP during the filtration of bacteria containing media with D.R. 0.5 and 0.66 h-1, filtration in single panel

As can be seen in optical density values versus time, bacteria removal rate significantly decreased after the first hour (Figure 4-18). As such when the previous experiments were considered, it was concluded that 0.5 1/h dilution rate gave the best results for this system due to high bacteria removal in a relatively shorter period. By this method, the pressure was kept constant, while 93 % of bacterial removal was achieved (Figure 4-17&Figure 4-18).

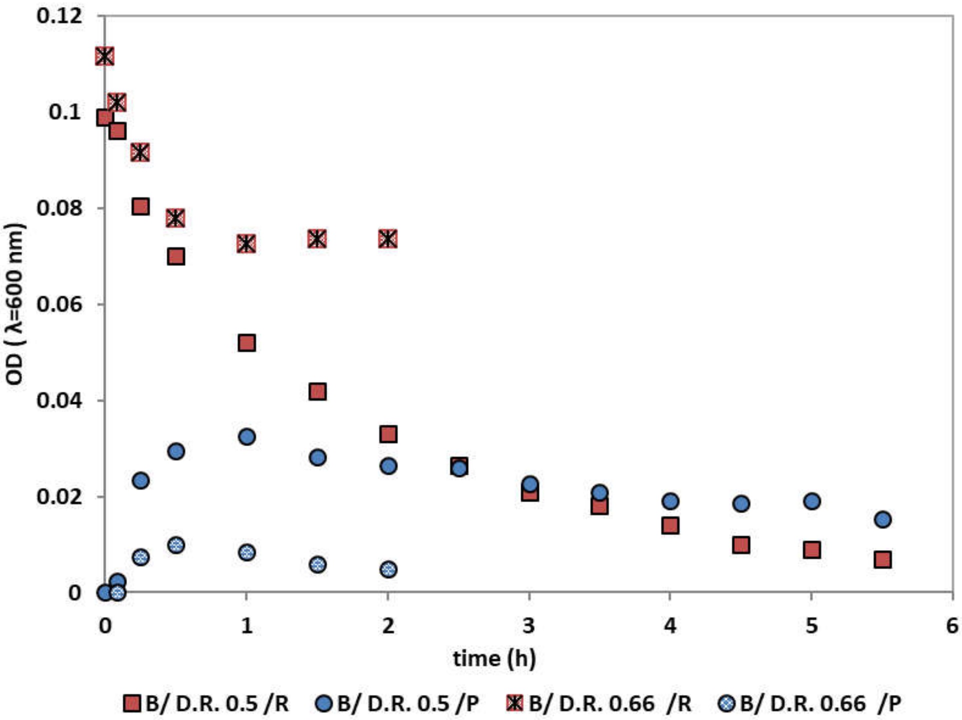

Figure 4-18 : The changes in the turbidity of the reactor media (R) and permeate media (P) during the filtration of bacteria containing media with D.R. 0.5 and 0.66 h-1, filtration in single panel

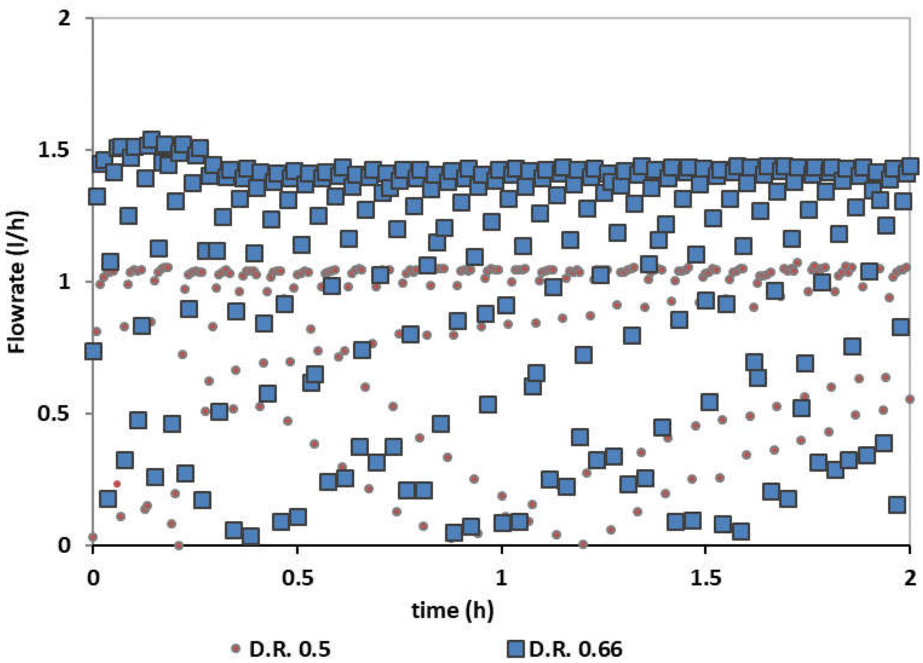

Figure 4-19 : The changes in permeate flow during the filtration of bacteria containing media with D.R. 0.5 and 0.66 h-1, filtration in single panel

Effect of membrane area was also investigated during bacteria filtration: bacteria filtration with the same inoculum size (0,033 g/l) and Dilution Rate (0.5 h-1) was executed with double and single membranes and speed of bacteria washout was observed.

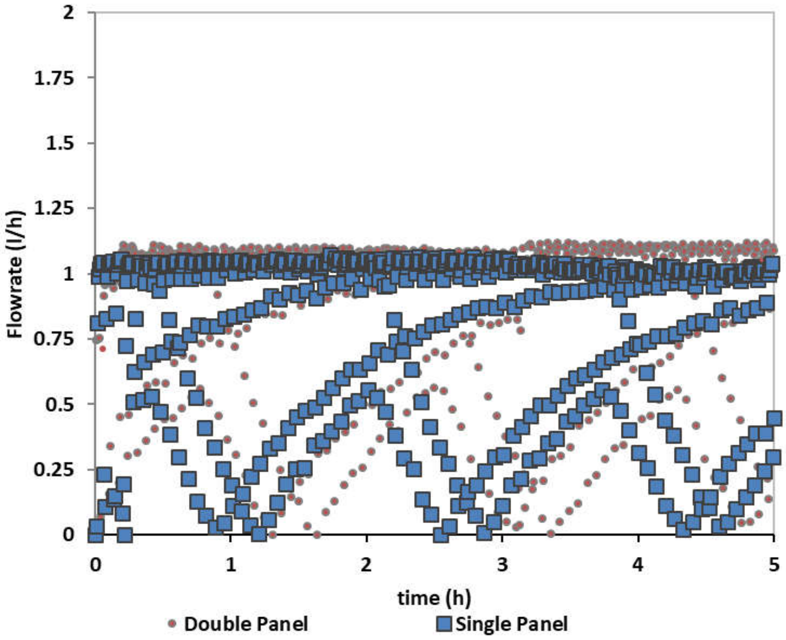

Figure 4-20 : The changes in permeate flow during the filtration of bacteria containing media with D.R. 0.5 h-1, filtration in single panel and double panel

Though the filtration area is doubled with two membranes, as the permeate flowrate is same (Figure 4-20) the permeation on each membrane panel is half of single panel. Therefore the suction provided by membranes are weaker in double membrane, which results in slower bacteria filtration as can be observed in optical density measurements (Figure 4-21); Optical density stabilizes in 6 hours in double panel and 5 hours in single panel.

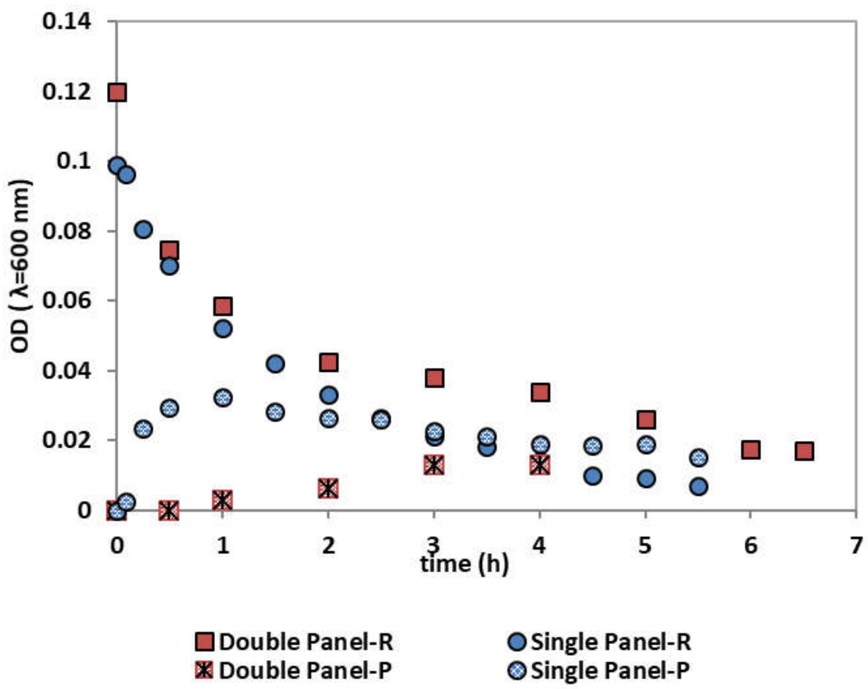

Figure 4-21 : The changes in the turbidity of the reactor media (R) and permeate media (P) during the filtration of bacteria containing media with D.R. 0.5 h-1, filtration in single panel and double panel

4.3.3 Filtration behavior of a mixture of bacteria and yeast

As the presence of both microorganism in a system comes along with new filtration challenges, the needs for efficient bacterial removal in presence of yeast was also investigated. It was observed that the presence of yeast hinders effective bacterial removal by increasing the full washout time. This issue was also confronted and remediated in order to have better understanding of the process in a true fermentation process.

At this experiment, yeast and bacteria inoculums were put inside the reactor in PBS to observe the bacterial removal while the yeast was kept inside without microbial growth of species. Dilution rate was 0.5 h^{-1} and backwash method is applied with 4 minute cycle (3.5 minute forward wash and 0.5 minute backwash). Optical density was measured after the bacteria inoculum was included and mixed for 10 minutes in order to determine the turbidity caused by bacteria inoculum only.

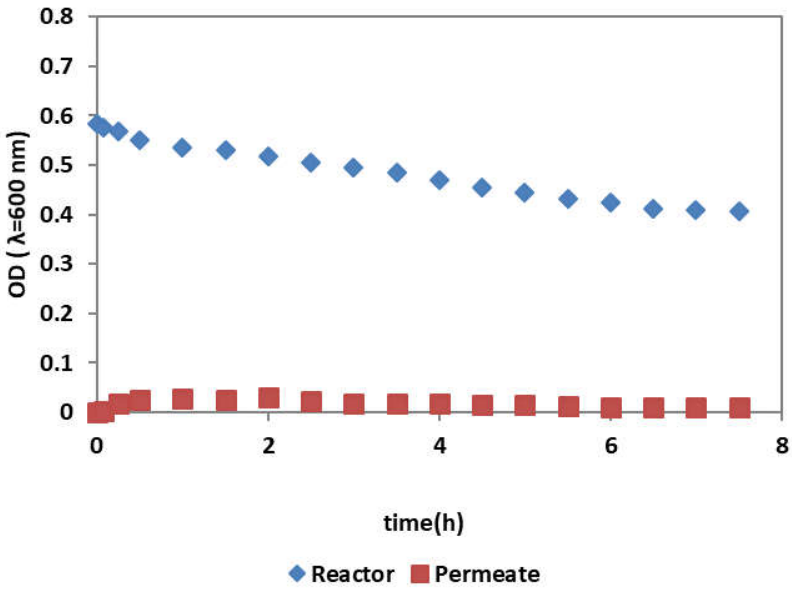

Figure 4-22 : The changes in the turbidity of the reactor media (R) and permeate media (P) during the filtration of bacteria and yeast containing media with D.R. 0.5 h-1, filtration in single panel

As the Figure 4-22 indicates, concentration in the reactor stabilizes over time; as the initial bacteria concentration was measured, this coincides to almost 100% bacterial removal. Also, Figure 4-23 shows the TMP change during the experiment and the change is acceptable for this process. There was a slightly change on pressure but it stabilized after a while; indicating at bacteria removal along with turbidity measurements.

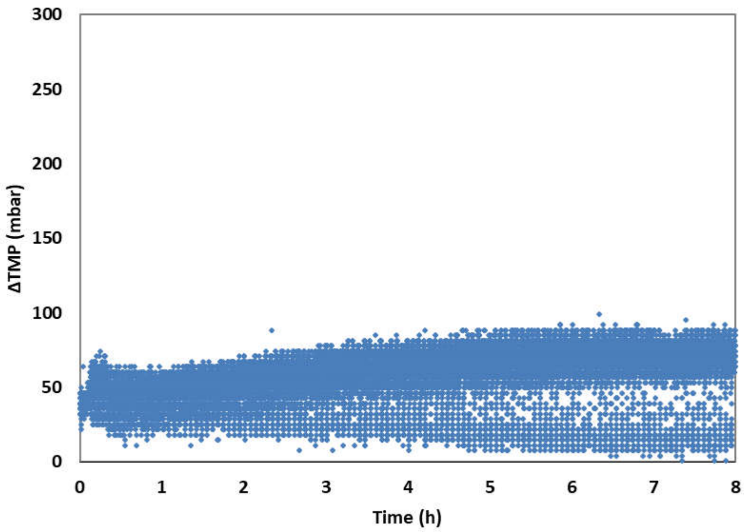

Figure 4-23 : The changes in TMP during the filtration of bacteria and yeast containing media with D.R. 0.5 h-1, filtration in single panel

The effect of membrane area in bacteria filtration was also investigated. For this purpose yeast and bacteria inoculum was filtrated in single and double panel with a flux of 150 $l/m^2/h$. The turbidity of bacteria inoculum in the reactor was measured separately before the addition of yeast inoculum. In both cases full washout of bacteria achieved according to turbidity measurements (Figure 4-24), however it was significantly faster with double panels, as in both cases the flux was the same. In double membrane the effective filtration area was double while the permeation on each membrane was the same with single panel filtration (Figure 4-25).

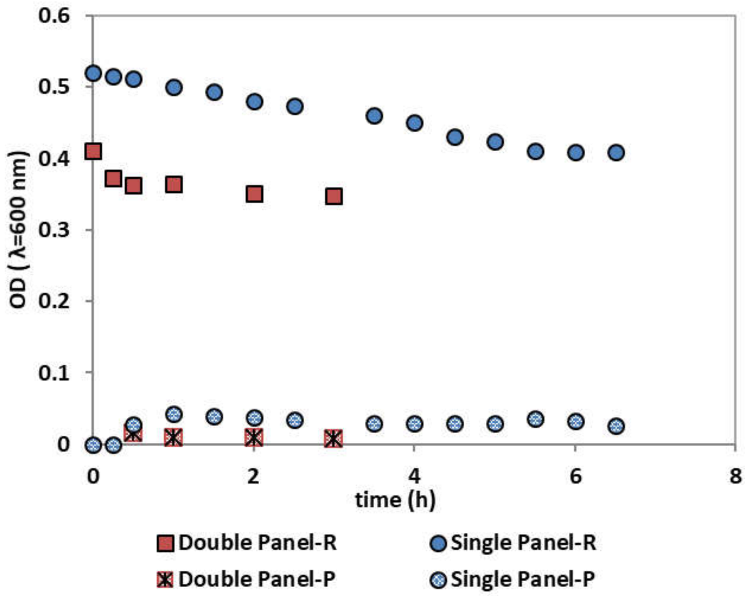

Figure 4-24 : The changes in the turbidity of the reactor media (R) and permeate media (P) during the filtration of bacteria and yeast containing media with a flux of 150 l/m2/h, filtration in single and double panel

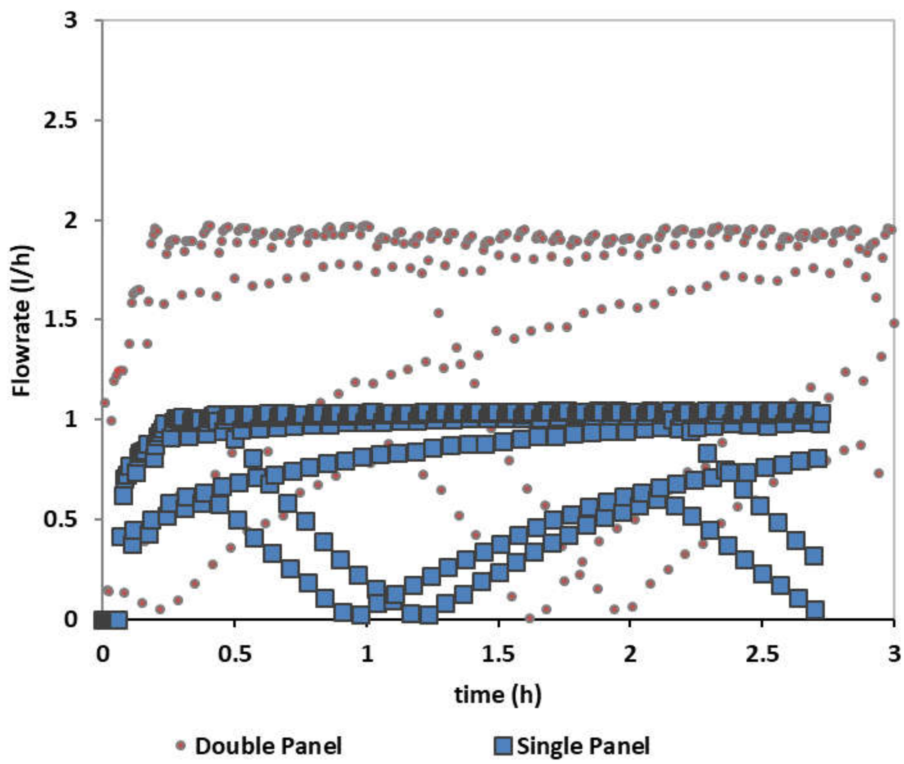

Figure 4-25 : The changes permeate flowrate during the filtration of bacteria and yeast containing media with a flux of 150 l/m^2/h, filtration in single and double panel

4.4 Fermentation of Nutrient Media Inoculated With Yeast, Bacteria and Both Microorganisms

In order to evaluate the metabolic activity of yeast and bacteria in a nutrient media containing glucose, peptone, yeast extract and salts, 1.5 l of nutrient broth was separately inoculated with either yeast, bacteria of both microorganisms together. Thereafter the changes in the metabolites and substrate content of the media were screened using HPLC analysis and samples were withdrawn at certain time intervals to measure the cell concentration in the medium. Each culture was cultivated for 10 h and sampling occurred in 2 h intervals.

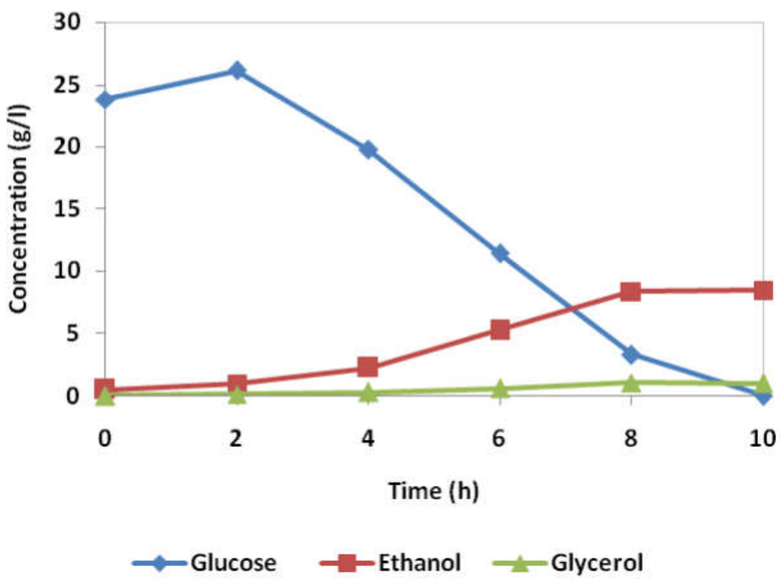

Figure 4-26 : Ethanol, Glucose & Glycerol Concentrations versus time during reactor fermentation of yeast

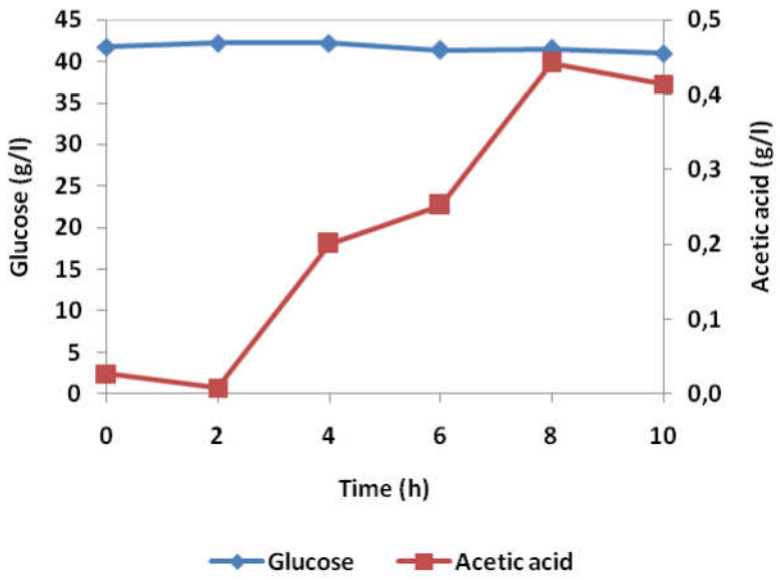

Figure 4-27 : Glucose &Acetic acid Concentrations versus time during reactor fermentation of bacteria

As it can be seen in Figure **4-26**, after 10 h of cultivation the yeast has been capable to consume all the glucose content of the medium and produce ethanol and glycerol while there is an imperceptible consumption of glucose to the bacterial culture while after 10 h in has been capable of producing around 0.4 g/l of acetic acid. As it was observed in the shake flask cultivations, the yeast fermentation conditions are not favorable for desirable activity of bacteria and in these conditions the bacterial culture has slow growth and relatively small amount of acetic acid production (Figure 4-27).

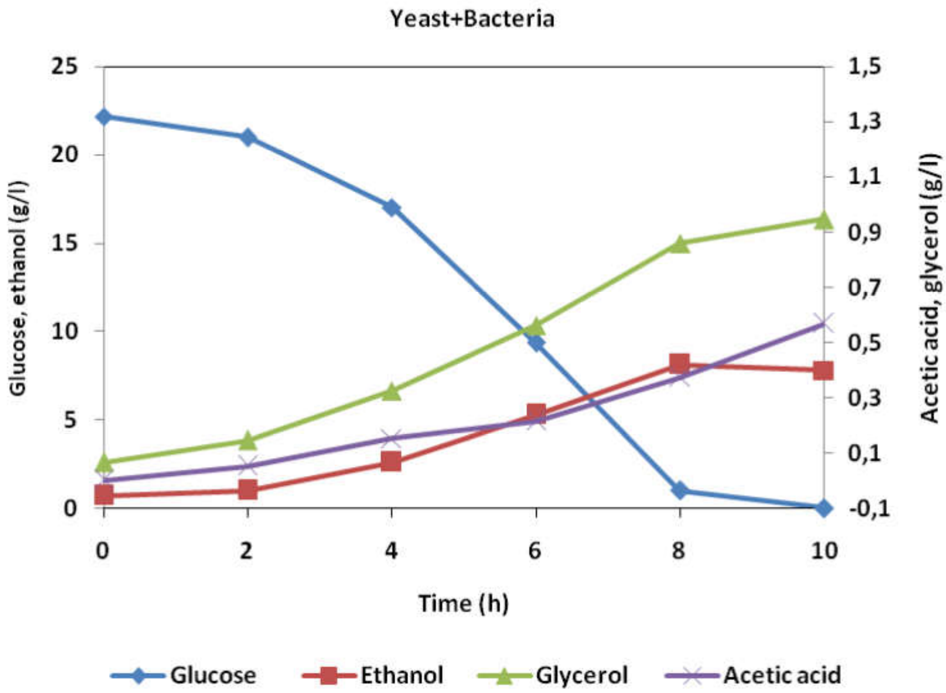

Figure 4-28 : Ethanol, Glucose, Glycerol and Acetic acid Concentrations versus time during concomitant reactor fermentation of yeast and bacteria

When the bacterial complex and yeast were co-cultured in the reactor, the sugar consumption rate increased as expected while more acetic acid was released in the media by the bacterial strains metabolic activity. It was observed that in the presence of yeast of higher concentration from the beginning the bacterial complex can easily contaminate the system while the changes in the acetic acid content of the media can be a proof of that (Figure 4-28).

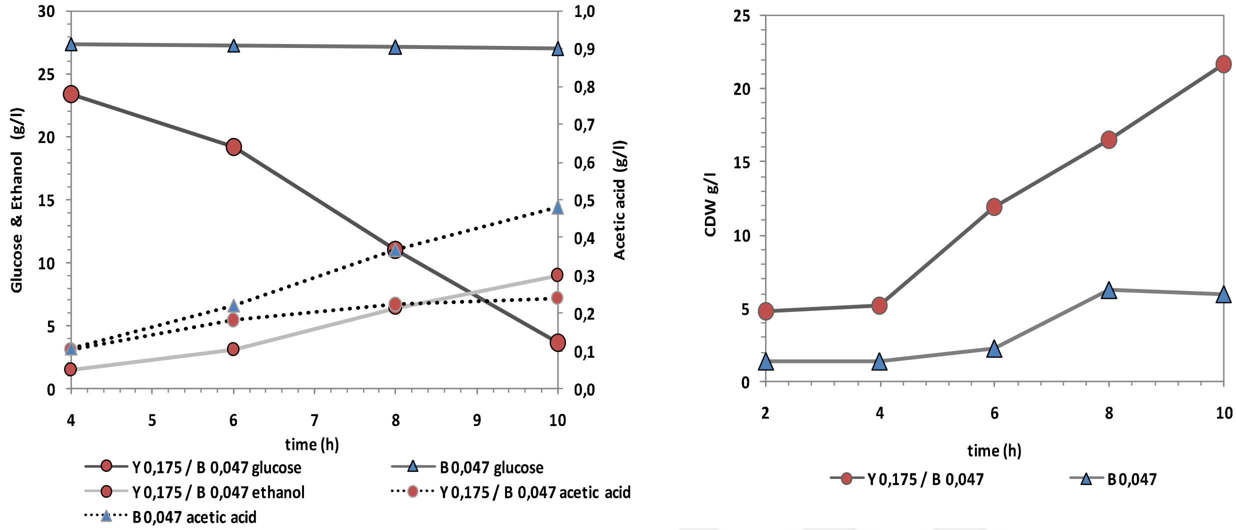

Figure 4-29 : Ethanol, Glucose & Glycerol Concentrations (on the right) and dry biomass concentrations (on the left) versus time during reactor fermentation of yeast and bacteria with initial inoculum of yeast 0,175 g/l + bacteria 0.047 g/l &bacteria 0.047 g/l.

Through dry mass data it is observed that in batch fermentation conditions bacteria goes under exponential growth phase between 6-8 hours, therefore the dry mass increase in Y+B cultivation after 8th hour must be attributed to yeast (Figure 4-29). When acetic acid concentrations are compared, it is seen that presence of yeast inhibits the metabolic activities of bacteria; in the absence of yeast the acetic acid concentration in the reactor is doubled even though bacteria has a low tolerance for an acidic environment (Figure 4-29).

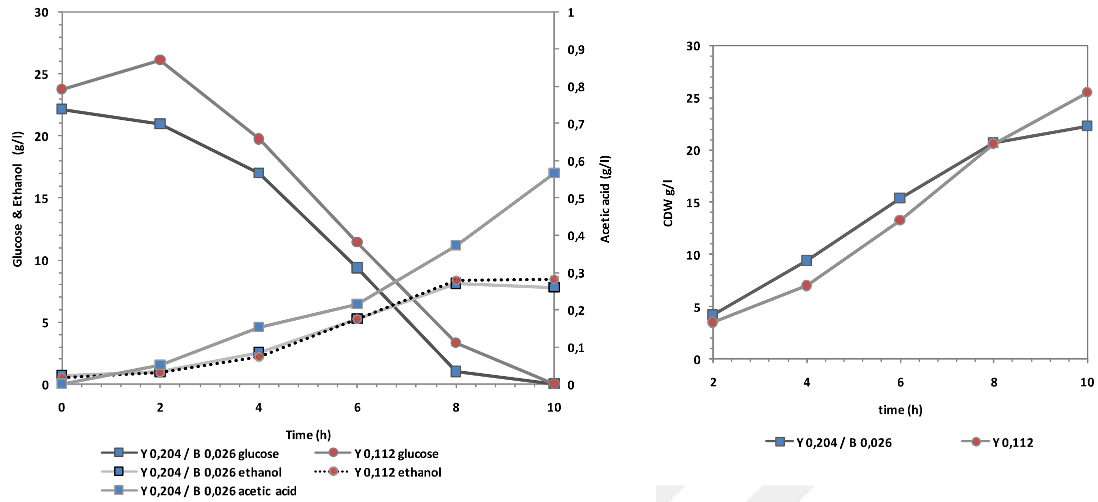

Figure 4-30 : Ethanol, Glucose & Glycerol Concentrations (on the right) and dry biomass concentrations (on the left) versus time during reactor fermentation of yeast and bacteria with initial inoculum of yeast 0,204 g/l + bacteria 0.026 g/l &yeast 0.112 g/l.

In Figure 4-30 it is observed that even though initial yeast inoculum sizes are not equal glucose is finished within 10 hours in both cases; ethanol production also follows the same trend in both cases. Dry mass data follows the same trend in both cases -though as expected initially it is higher inside the reactor with higher initial yeast inoculum, the final dry mass is higher in reactor with no bacteria inoculum as the glucose is finished more quickly in the one with bacteria.

We have concluded from observing the data in both Figure 4-29&Figure 4-30 that yeast has tolerated the slightly acidic environment at pH 4, as both ethanol production and glucose uptake is not disturbed by the presence of bacteria in a batch fermentation for 10 hours which comply with the results obtained by other researches (Buzas et al.,1988; Azhara, Abdullaa, Jamboa, & Marbawia, 2017).

4.5 Reactor Runs with Fermentation Medium

4.5.1 Effect of feed composition on membrane during bacteria filtration

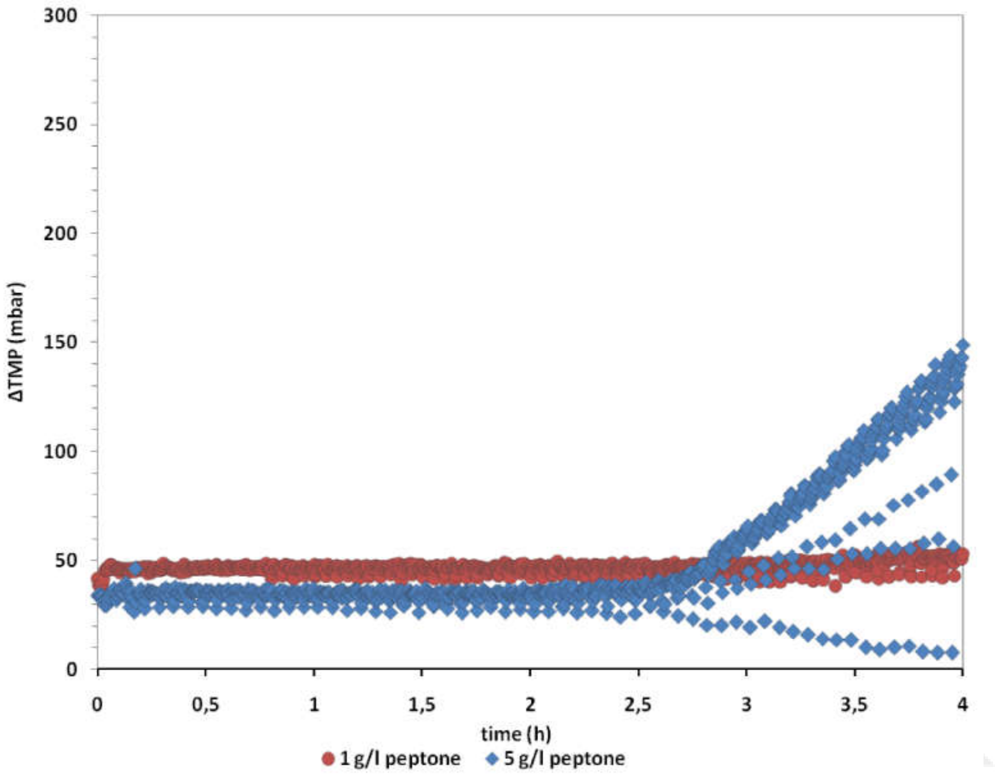

Figure 4-31 : Change in TMP; comparison based on composition of feed solution with peptone concentration of 1 g/l (D.R. 0,25 h^{-1}) and 5g/l (D.R. 0,5 h^{-1})

During the first run with bacteria+ yeast fermentation in the reactor, the feed composition was the same as the nutrient medium used in shake flask cultivations. However as it can be seen in Figure 4-31 this composition has yielded an earlier rise in TMP compared to the next run (1 g/l peptone) which had the same inoculum of bacteria and yeast (0,03 g/l & 0,18 g/l). Permeate flow also showed decrease and irregularity with high nutrient concentration (Figure **4-32**).

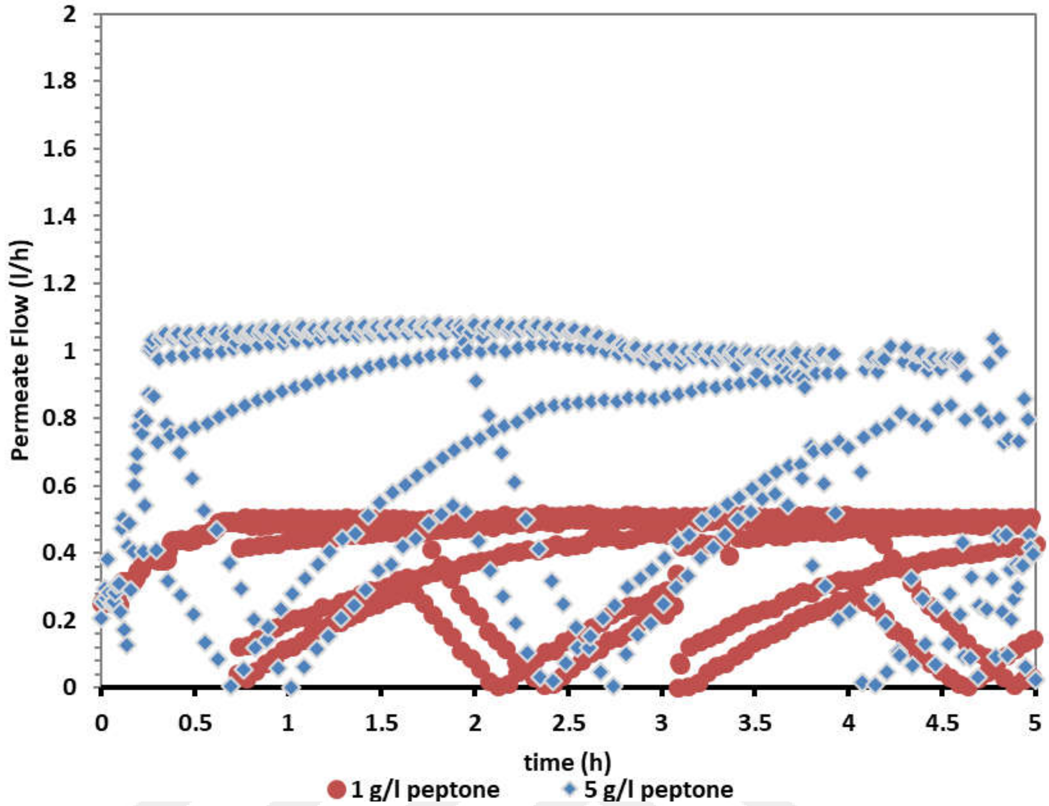

Figure 4-32 : Change in Permeation flow: comparison based on composition of feed solution with peptone concentration of 1 g/l (D.R. 0,25 h-[1]) and 5g/l (D.R. 0,5 h-[1])

Bacteria prefers peptone as nitrogen source (Khaled et al.,2015) as such higher peptone concentration of 5 g/l is more favorable to bacteria, as can be seen in acetic acid concentration (Figure 4-33) compared to reactor run with same inoculum size and half the dilution rate.

It is also assumed that peptone is prone to clog the membrane pores due to its physical properties. Accordingly for the following runs, peptone, yeast extract and glucose concentrations in the feed solution were reduced from;

30 g/l Glucose + 10 g/l Peptone + 6 g/l Yeast Extract + 3,5 g/l K_2HPO_4, to
20 g/l Glucose + 1 g/l Peptone + 1 g/l Yeast Extract + 3,5 g/l K_2HPO_4

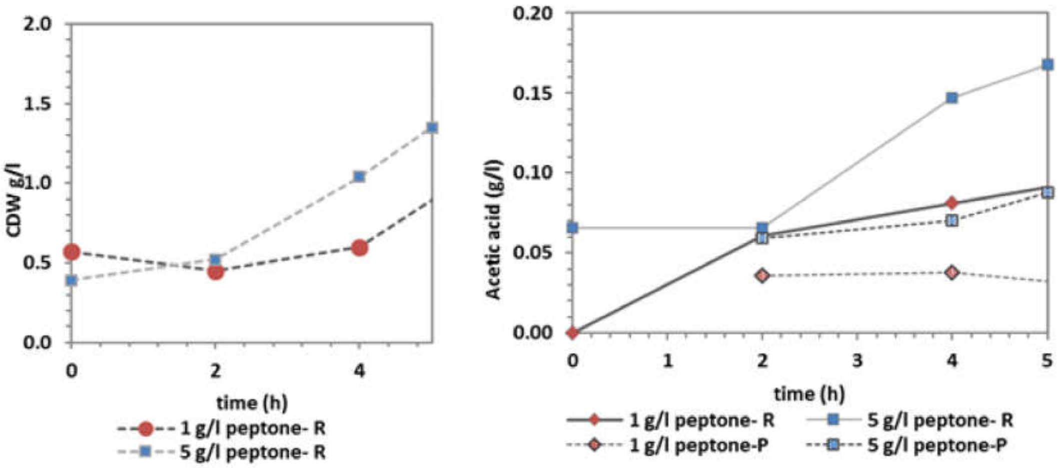

Figure 4-33 : Biomass concentration in the reactor and permeate tank (on the right) and acetic acid concentrations inside the reactor and permeate tank (on the left) based on composition of feed solution with peptone concentration of 1 g/l (D.R. 0,25 h^{-1}) and 5g/l (D.R. 0,5 h^{-1})

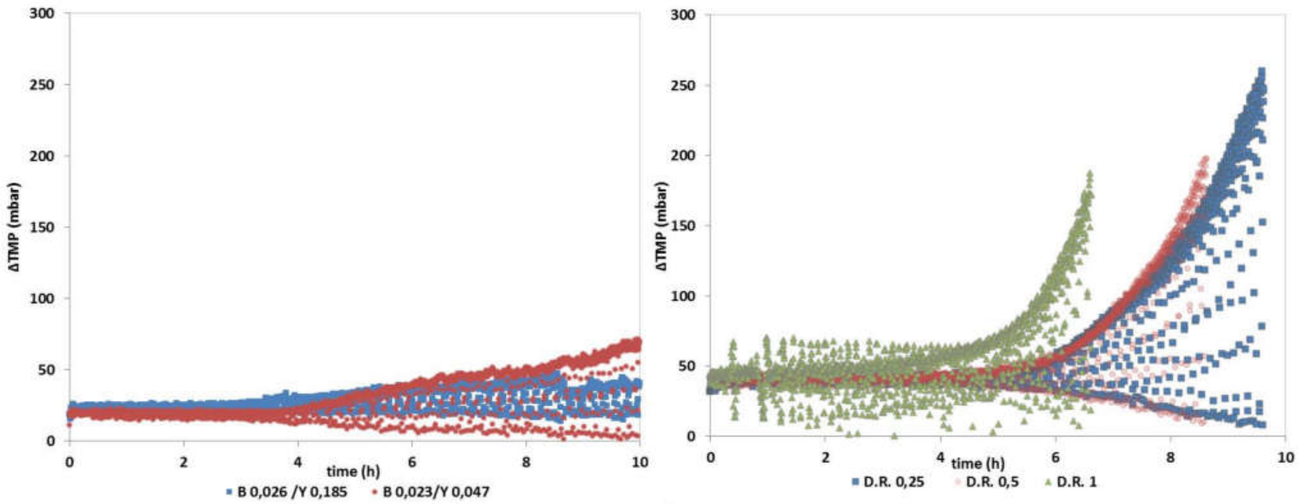

Figure 4-34 : Change in TMP comparison based on initial inoculum concentration in the reactor; with bacteria 0,26 g/l & yeast 1,85 g/l , bacteria 0,23 g/l & yeast 0,47 g/l filtration with a dilution rate of 0,25 h^{-1}in single panel (left) & TMP based on Dilution Rate; bacteria filtration of an inoculum size of 0,001 g/l with D.R. 0,25 h^{-1} , D.R. 0,5 h^{-1}, D.R. 1 h^{-1} (right) in double panel.

Ideal washout parameters for bacteria had previously been established during MBR runs with PBS as feed solution. However since there was no increase in the number of cells in PBS solution, bacteria washout was more straightforward compared to MBR fermentation. When bacteria washout was tried in MBR fermentation condition it was observed that bacteria alone inoculum is not feasible for a continuous filtration as it can be observed in Figure 4-34; when filtration was applied on a bacteria only inoculum with a cell concentration of 0,001 g/l with Dilution Rates of 1, 0.5 and 0.25 $h^{-1}$the final TMP values were all significantly higher than the bacteria filtration with yeast addition (inoculum; bacteria 0,26 g/l & yeast 0,47 g/l) with a dilution rate of 0.25 h^{-1}. Furthermore when two runs of equal flux (Figure 4-36) with yeast addition (bacteria 0,26 g/l & yeast 1,85 g/l , bacteria 0,23 g/l & yeast 0,47 g/l filtration with a dilution rate of 0,25 h^{-1}) are compared; it is observed that though initial bacteria concentration is higher in reactor run with bacteria 0,26 g/l inoculum, the acetic acid concentration is lower (Figure 4-35) which is due to the glucose consumption and ethanol production of the higher initial yeast concentration that hinders bacteria growth. It is also assumed that yeast is preventing the membrane from fouling by staying on the membrane surface and forcing the bacteria to pass through the pore vertically.

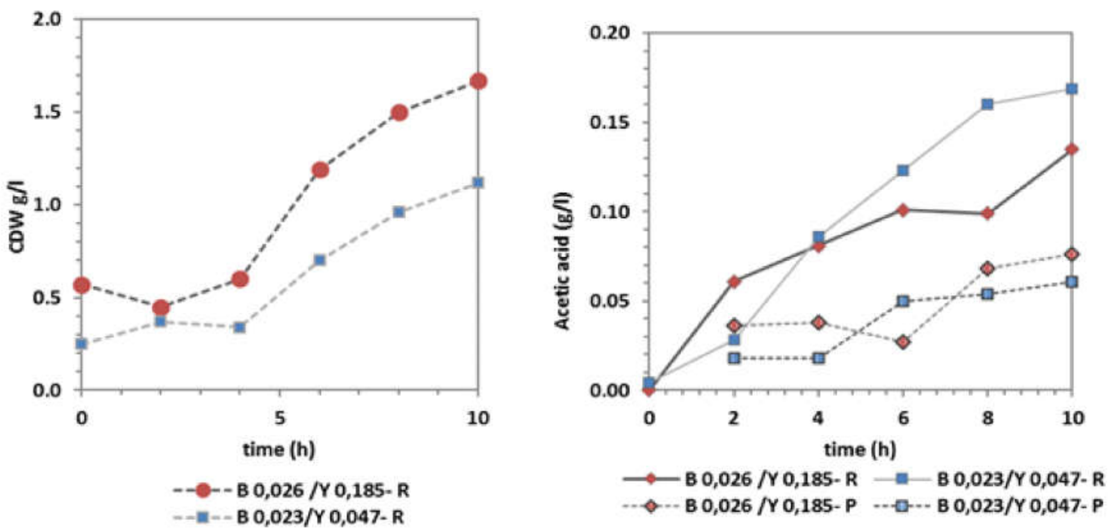

Figure 4-35 : Biomass concentration in the reactor and permeate tank (on the right) and acetic acid concentrations inside the reactor and permeate tank (on the left) based on concentration of initial yeast inoculum.

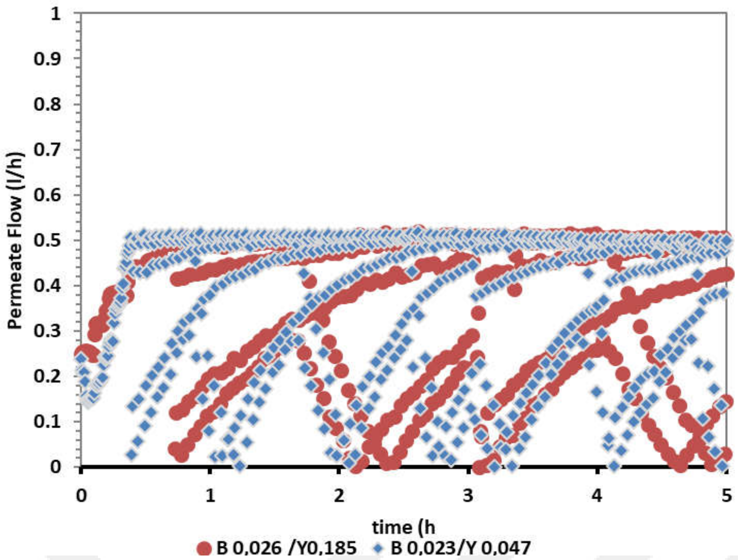

Figure 4-36 : Permeate flow comparison based on initial inoculum concentration in the reactor; with bacteria 0,026 g/l & yeast 0,185 g/l and bacteria 0,023 g/l & yeast 0,047 g/l; bacteria filtration with a dilution rate of 0,25 h-1 in single panel.

It was further attempted to washout the bacteria without yeast by decreasing the initial inoculum size of bacteria. However though the inoculum size was reduced as far as 0,001 g/l bacteria with a filtration rate of 0,25h^{-1}a continuous process could not be achieved due to eventual rise of pressure on membrane (Figure 4-37).

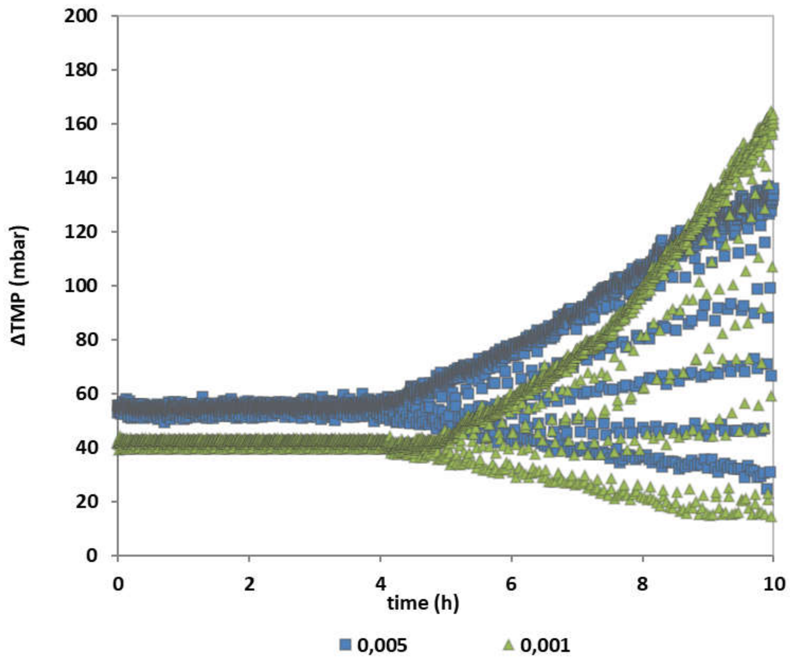

Figure 4-37: Change in TMP comparison based on initial inoculum concentration in the reactor; with bacteria 0,05 g/l &bacteria 0,01 g/l; bacteria filtration with a Dilution Rate of 0,25 h^{-1}in single panel.

Though a continuous washout could not be achieved the fermentation+ filtration runs with bacteria only inoculum has been a good source of data to investigate the metabolic behavior of bacteria in MBR process. It was determined that within MBR fermentation conditions bacteria goes into exponential growth phase after 4^{th} hour, till then bacteria washout is achieved, and there is no increase in TMP (Figure 4-37&Figure 4-39).Permeate flowrate does not seem to be affected by the rise of TMP in either cases (Figure 4-38).

In the run with bacteria concentration of 0,001 g/l the membrane starts clogging after 5th hour, dry mass concentration in the reactor drops till 8th which can be assumed as cake formation on the membrane layer plus continued bacteria filtration as the dry mass in the permeate tank remains stable till the 6th hour and slights raises between 6th and 8^{th} (Figure 4-37). This is also justified by acetic acid concentration in the reactor as seen in Figure 4-39 which raised significantly between 6th and 8th hour (same trend is seen in B 0,005 g/l).

In the run with bacteria concentration of 0,005 g/l the biomass concentration appears stable in the reactor while the acetic acid concentration rises after 6th hour so we can assume the bacteria is duplicating on the membrane surface and forming a biofilm; hence continuous pressure increase on membrane after 4th hour (Figure 4-37&Figure 4-39).

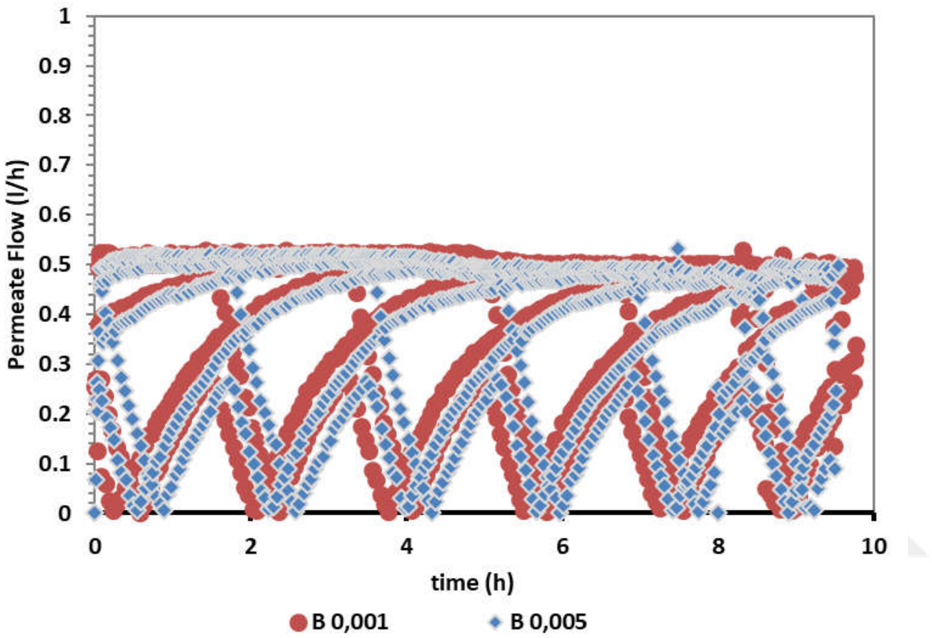

Figure 4-38: Permeate flow comparison based on initial inoculum concentration in the reactor; with bacteria 0,001 g/l &bacteria 0,005 g/l; bacteria filtration with a dilution rate of 0,25 h^{-1} in single panel.

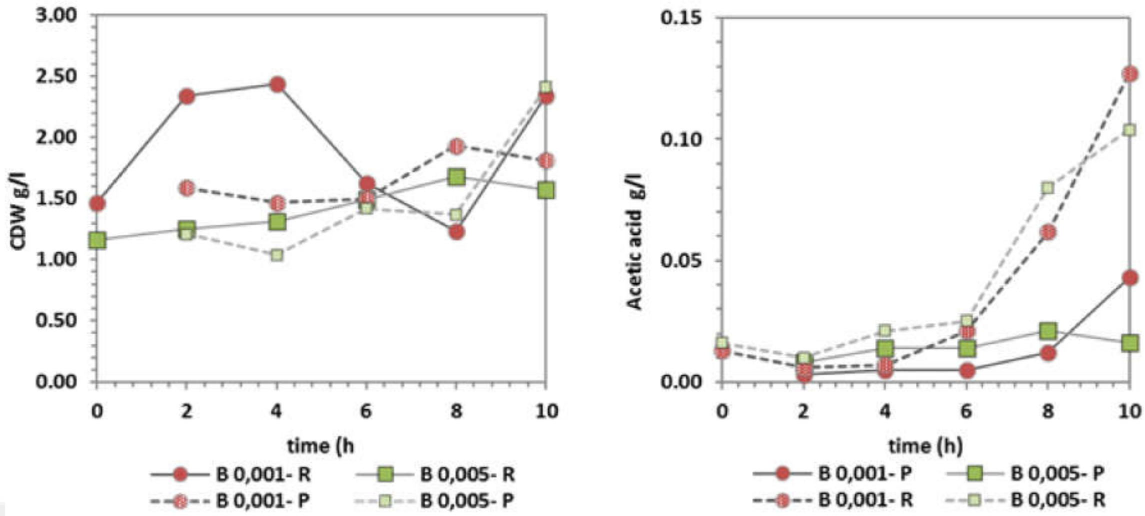

Figure 4-39 : Biomass concentration in the reactor and permeate tank (on the right) and acetic acid concentrations inside the reactor and permeate tank (on the left) based on initial inoculum concentration in the reactor; with bacteria 0,001 g/l &bacteria 0,005 g/l; bacteria filtration with a dilution rate of 0,25 h-1 in single panel.

4.5.2 Determination of critical dilution rate

It was established from previous runs that a continuous filtration process could not be achieved without the presence of yeast. Therefore all the following runs were executed with a higher content of yeast and smaller bacteria inoculum.

Different concentrations of inoculum were also tried along with different dilution rates to find the ideal parameters for a continuous MBR fermentation process. Dilution rates between 0.5 and 0.11 h^{-1}were tried with double membrane panel filtration (Figure 4-40).

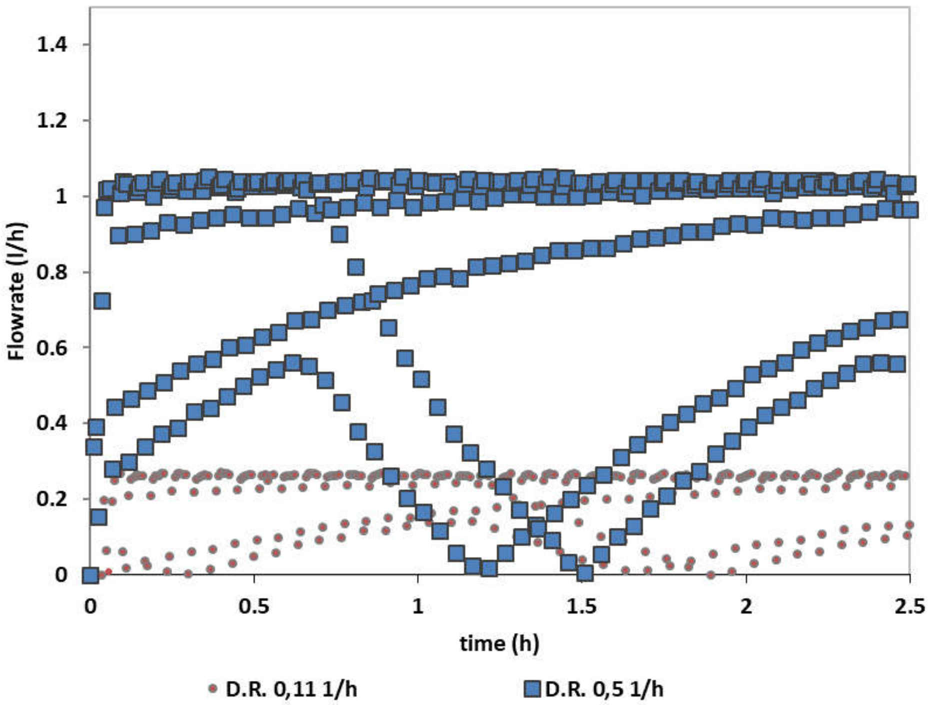

Figure 4-40: Permeate flow comparison based on Dilution Rate with D.R. 0,5 h^{-1} and D.R. 0,11 h^{-1}(inoculum; bacteria 0.001 g/l + yeast 0.036 g/l), filtration in double panel

Based on TMP values it was determined that a dilution rate of 0.11 h^{-1} results in stable pressure on membrane and therefore continuous washout of bacteria from the system (Figure 4-41).

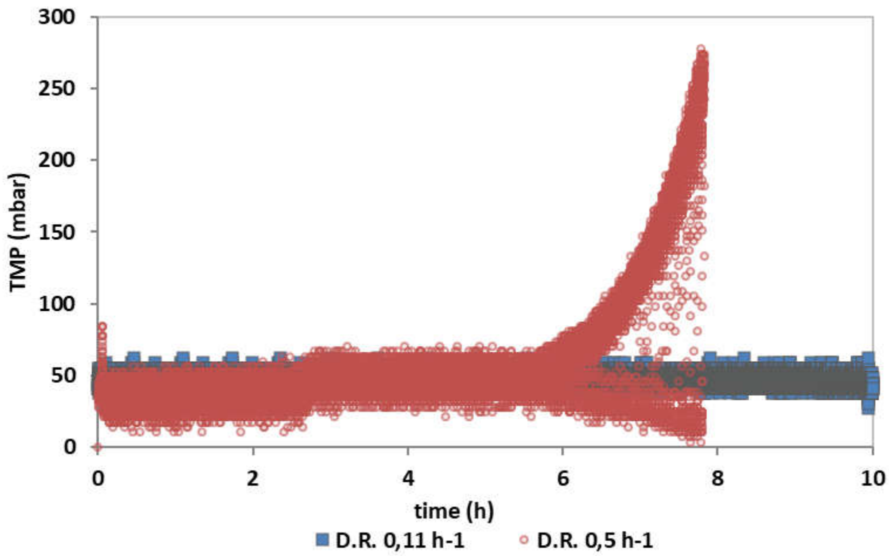

Figure 4-41: Change in TMP; comparison based on Dilution Rate with D.R. 0,5 h^{-1} and D.R. 0,11 h^{-1}(inoculum; bacteria 0.001 g/l + yeast 0.036 g/l), filtration in double panel

However although continuous washout is achieved the acetic acid concentration in the reactor could not be stabilized in D.R. 0.11 h^{-1}(Figure 4-42) and pH in the reactor kept decreasing (Figure 4-43) until it reached pH 4 in which the bacteria metabolism slows down as bacteria prefers higher pH (Kumar et al.,2000;Khanna et al., 2010). The trend is also apparent at acetic acid concentration values as they both decrease in the reactor after 8th hour in D.R. 0.11 despite the dramatic increase between 6th and 8th (Figure 4-42).

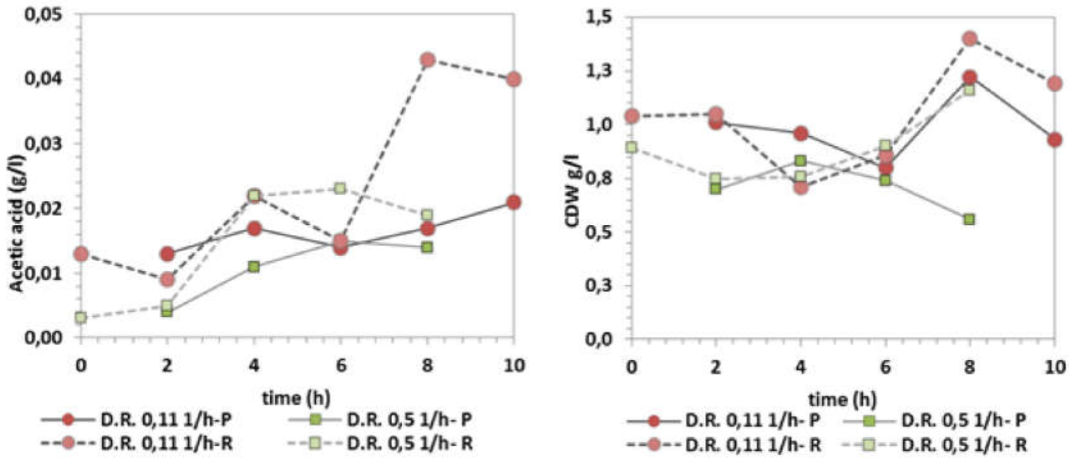

Figure 4-42 : Biomass concentration in the reactor and permeate tank (on the right) and acetic acid concentrations inside the reactor and permeate tank (on the left) based on based on Dilution Rate with D.R. 0,5 h-1 and D.R. 0,11 h-1(inoculum; bacteria 0.001 g/l + yeast 0.036 g/l), filtration in double panel

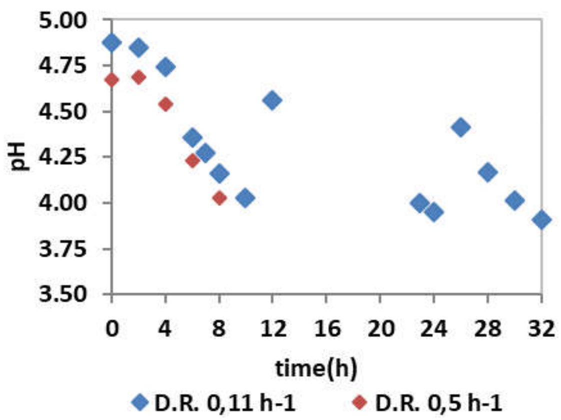

Figure 4-43 : Change in pH during MBR run with bacteria 0.001 g/l + yeast 0.036 g/l inoculum concentration, D.R. 0.5 and 0,11 h-1filtration in double panel; pH was adjusted with NaOH addition at 10th and 24th hour

In order to prove that the Dilution Rate of 0.11 $h^{-1}$is applicable to a continuous MBR process for contaminated fermentation medium the same D.R. was applied to fermentation condition with different initial yeast and bacteria inoculum concentrations and changes in TMP, flowrate, glucose consumption and ethanol and acetic acid concentration as well as dry mass was observed;

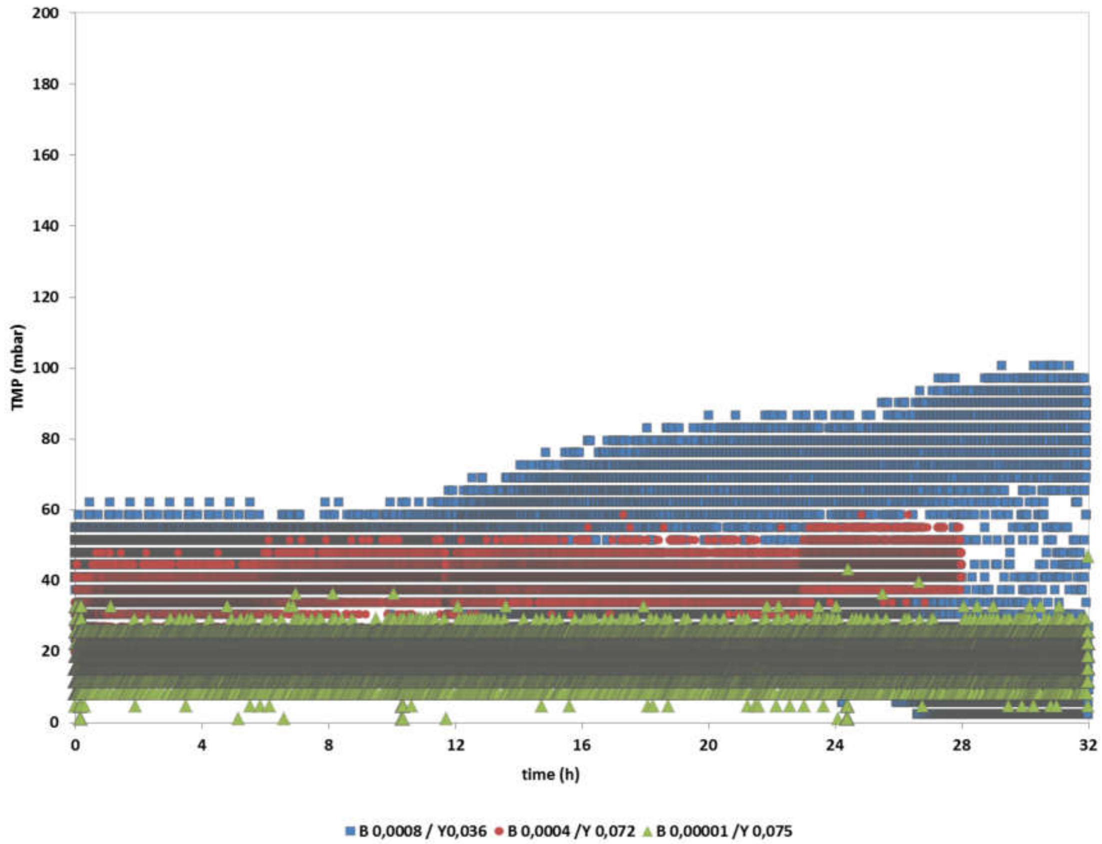

Figure 4-44 : Change in TMP; comparison based on inoculum; bacteria 0.0008 g/l + yeast 0.036 g/l, bacteria 0.0004 g/l + yeast 0.072 g/l & bacteria 0.0001 g/l + yeast 0.075 g/l, D.R. 0,11 h^{-1}filtration in double panel

The TMP stayed stable in all runs with D.R. 0.11 $h^{-1}$and therefore was concluded as suitable for a MBR ethanol fermentation system contaminated with E. *cloacae* (Figure 4-44).

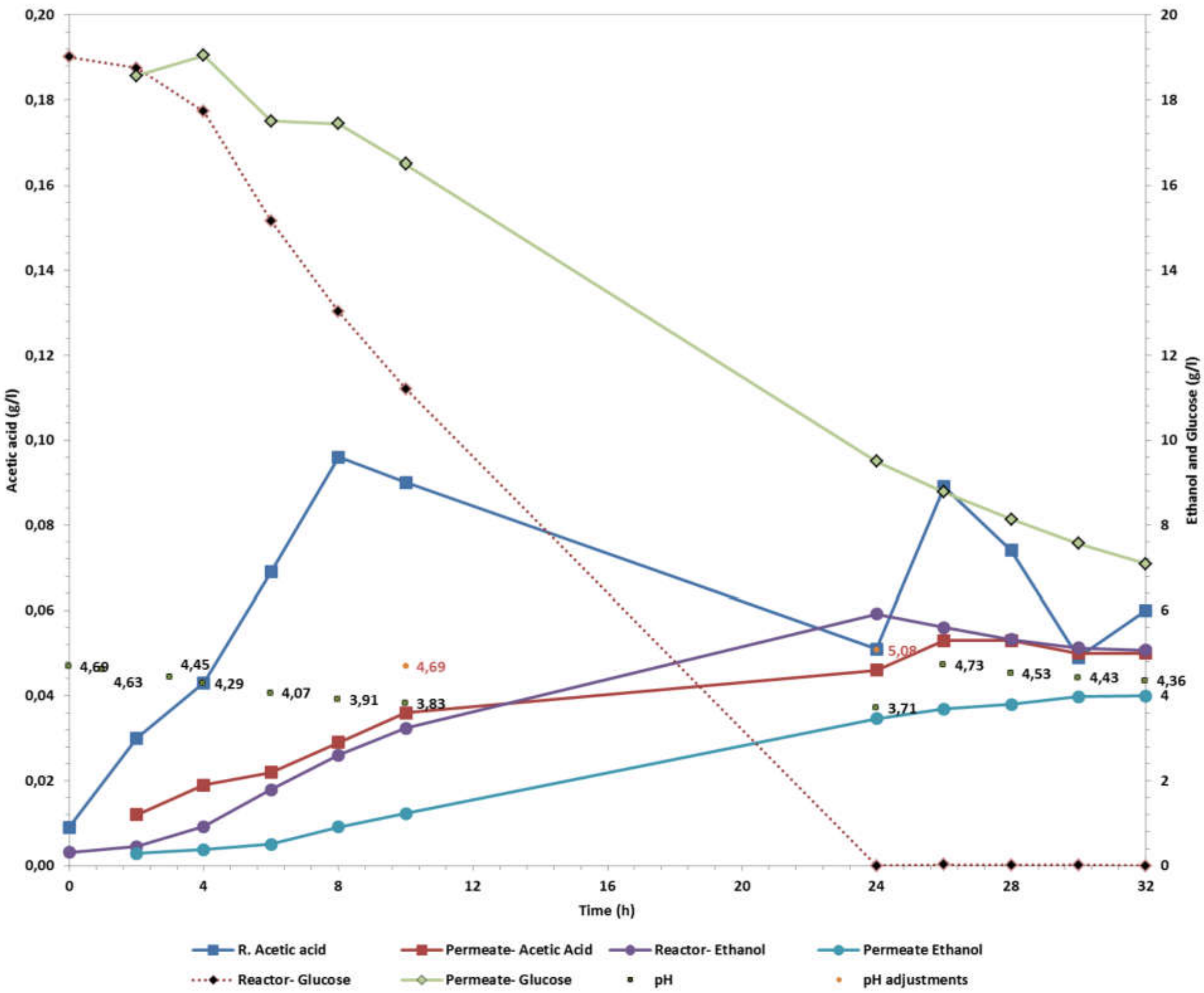

Figure 4-45 : Glucose, Ethanol and Acetic acid concentrations in the reactor and permeate tank & pH reading and adjustments from the reactor; Dilution Rate 0,11 h^{-1} (inoculum; bacteria 0.001 g/l + yeast 0.075 g/l), filtration in double panel

When the metabolic activities of yeast and bacteria are observed it can be seen that with an initial inoculum of 0.075 g/l, in 24 hours yeast completely consumed glucose inside the reactor, as previously observed in experiments ran with shake flasks. However the pH drop till 10^{th} hour does not stop the ethanol production which continues till the glucose is consumed completely in 24^{th}hour (Figure 4-45). It can also be deducted from the drop in acetic acid concentration following 8^{th} hour that while bacteria is severely affected from a pH drop below 4, yeast is not and can continue to consume glucose and produce ethanol (Kumar et al.,2000; Khanna et al..2010; Buzas et al. 1988;Azhara, Abdullaa, Jamboa, & Marbawia, 2017)

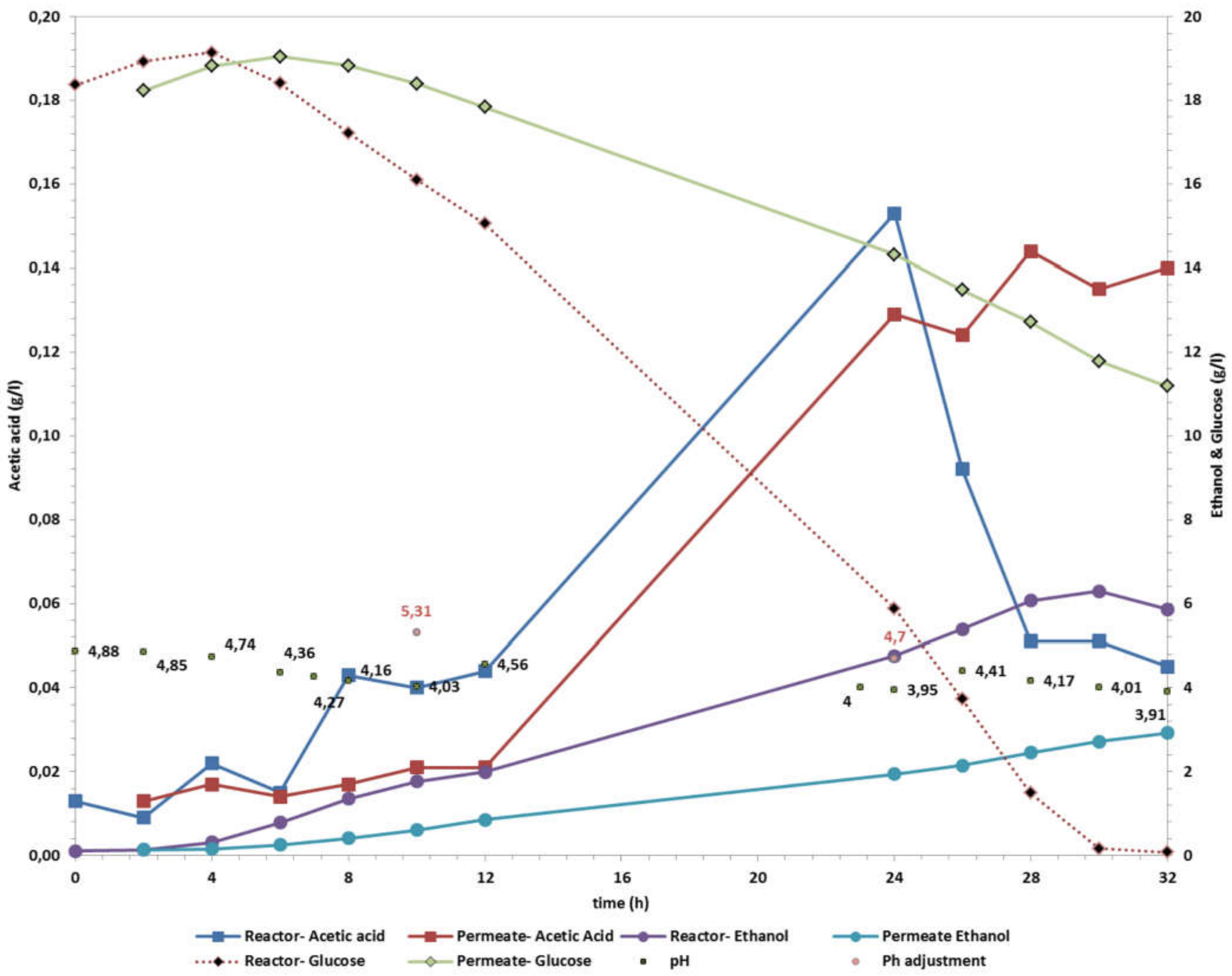

Figure 4-46 : Glucose, Ethanol and Acetic acid concentrations in the reactor and permeate tank & pH reading and adjustments from the reactor; Dilution Rate 0,11 h^{-1} (inoculum; bacteria 0.0008 g/l + yeast 0.036 g/l), filtration in double panel

On the other hand in the run with bacteria 0.0008 g/l + yeast 0.036 g/l as inoculum the glucose was consumed at 30th hour due to smaller initial inoculum of yeast as well as slower acclimation of yeast to MBR conditions, as the glucose starts decreasing after 4th hour (Figure 4-46). We can also observed that ethanol fermentation is not stopped due to presence of bacteria as concentration of ethanol continues to rise steadily in reactor and permeate till the 30th hour even though pH drops as far as 3,9 (Figure 4-46).

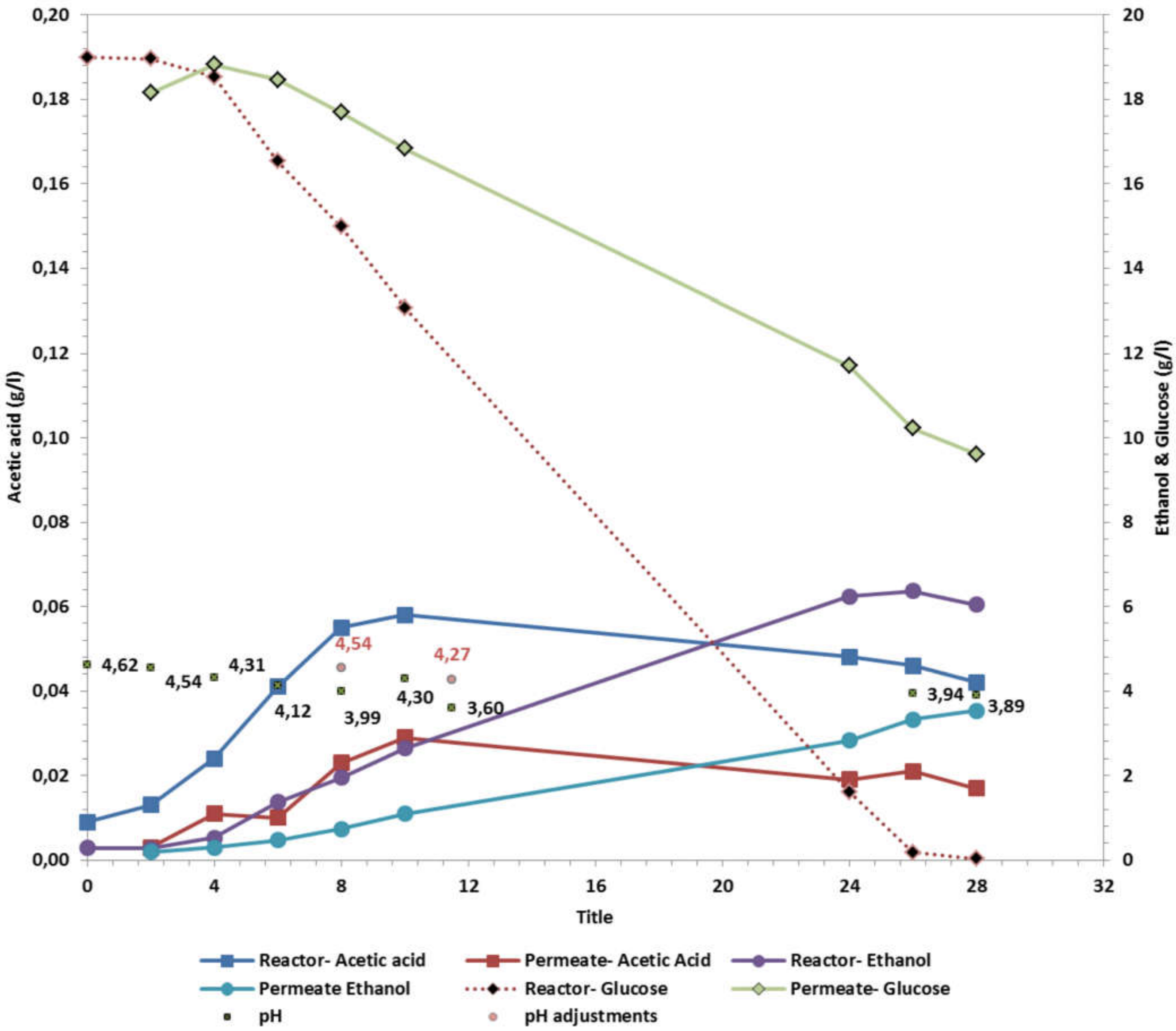

Figure 4-47 : Glucose, Ethanol and Acetic acid concentrations in the reactor and permeate tank & pH reading and adjustments from the reactor; Dilution Rate 0,11 h^{-1} (inoculum; bacteria 0.0004 g/l + yeast 0.072 g/l), filtration in double panel

In the run with bacteria 0.0004 g/l + yeast 0.072 g/l as inoculum the glucose was consumed at 26th hour and as observed before the ethanol fermentation did not stop till glucose was fully consumed (Figure 4-47). This run had the smallest rise of acetic acid at 24th hour which can be explained by the drop in pH below 4 (Figure 4-46).

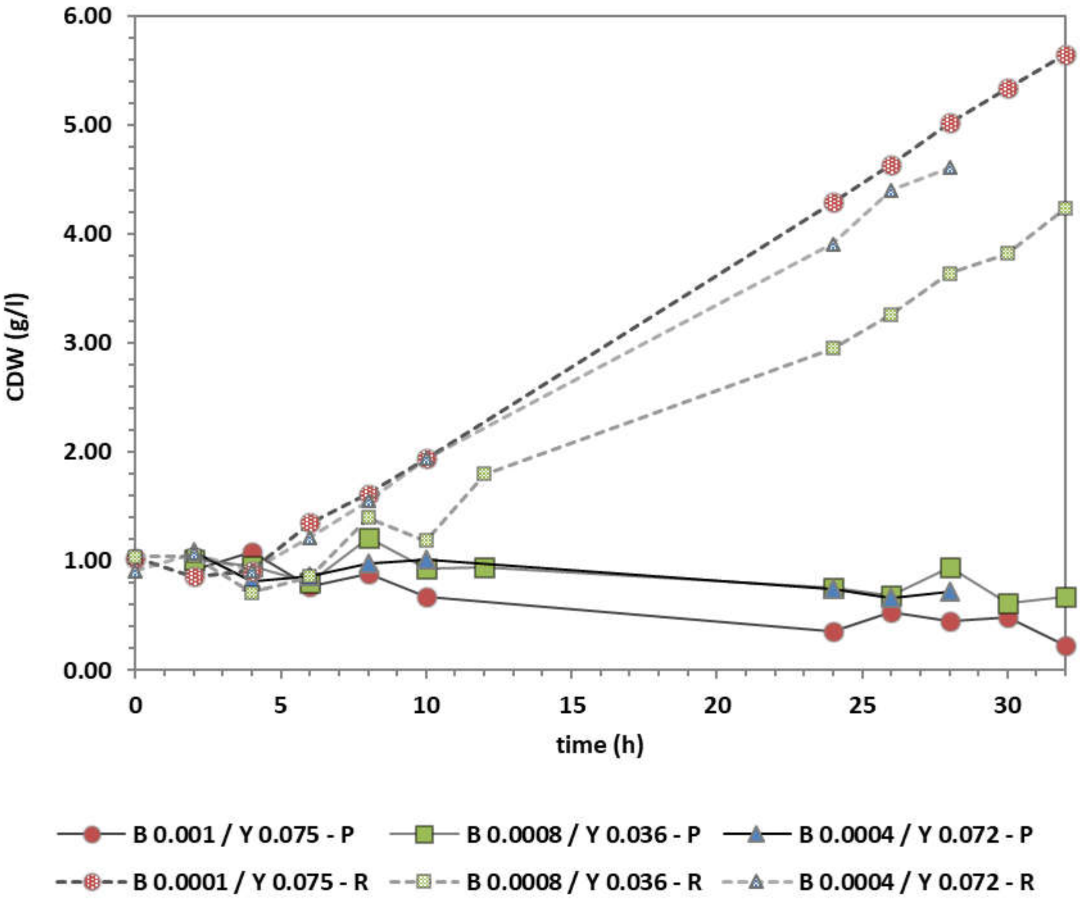

Figure 4-48: Change in Cell Dry Weight (g/l); comparison based on inoculum; bacteria 0.0008 g/l + yeast 0.036 g/l, bacteria 0.0004 g/l + yeast 0.072 g/l & bacteria 0.0001 g/l + yeast 0.075 g/l, D.R. 0,11 h^{-1}filtration in double panel

In all runs with 0.11 h^{-1} yeast could continue to increase in CDW till the run is stopped (Figure 4-48), therefore it can be concluded that the MBR conditions allow yeast cultivation. As expected, highest growth was observed with the highest initial yeast inoculum, though ethanol production at 28^{th} hour is less compared to other runs; (bacteria 0.0004 g/l + yeast 0.072 & bacteria 0.0008 g/l + yeast 0.036) due to earlier consumption of glucose in the reactor.

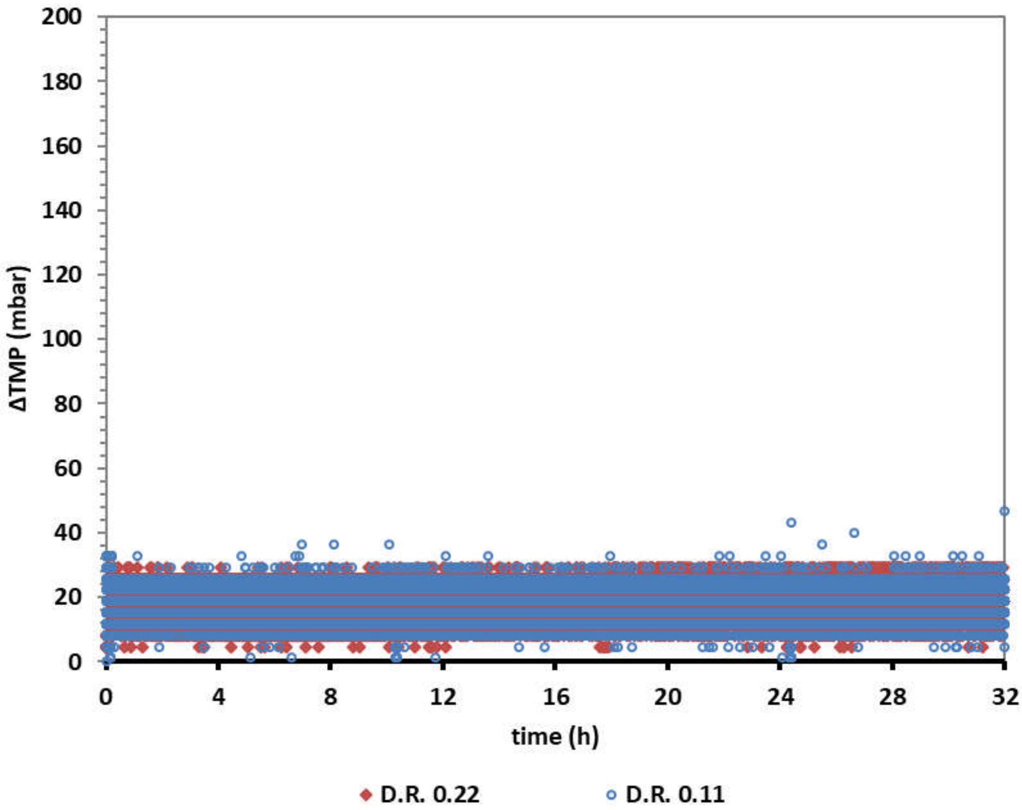

Figure 4-49 : Change in TMP; inoculum; bacteria 0.00001 g/l, yeast 0.075 g/l, filtration in double panel with D.R. 0.11 and 0.22 h^{-1}

As all runs that had no pH control with D.R. 0.11 h^{-1}had given successful results an attempt was made to increase the D.R. by double. However though an increase in pressure was not observed in the course of this run (Figure 4-49) there was no turbidity in the permeate tank after 24 hours of filtration, (Figure 4-50) hence no cell growth.

Figure 4-50 : Permeate tank after 24 hours of filtration; D.R. 0.22 h^{-1}, inoculum; bacteria 0.00001 g/l, yeast 0.075 g/l, filtration in double panel.

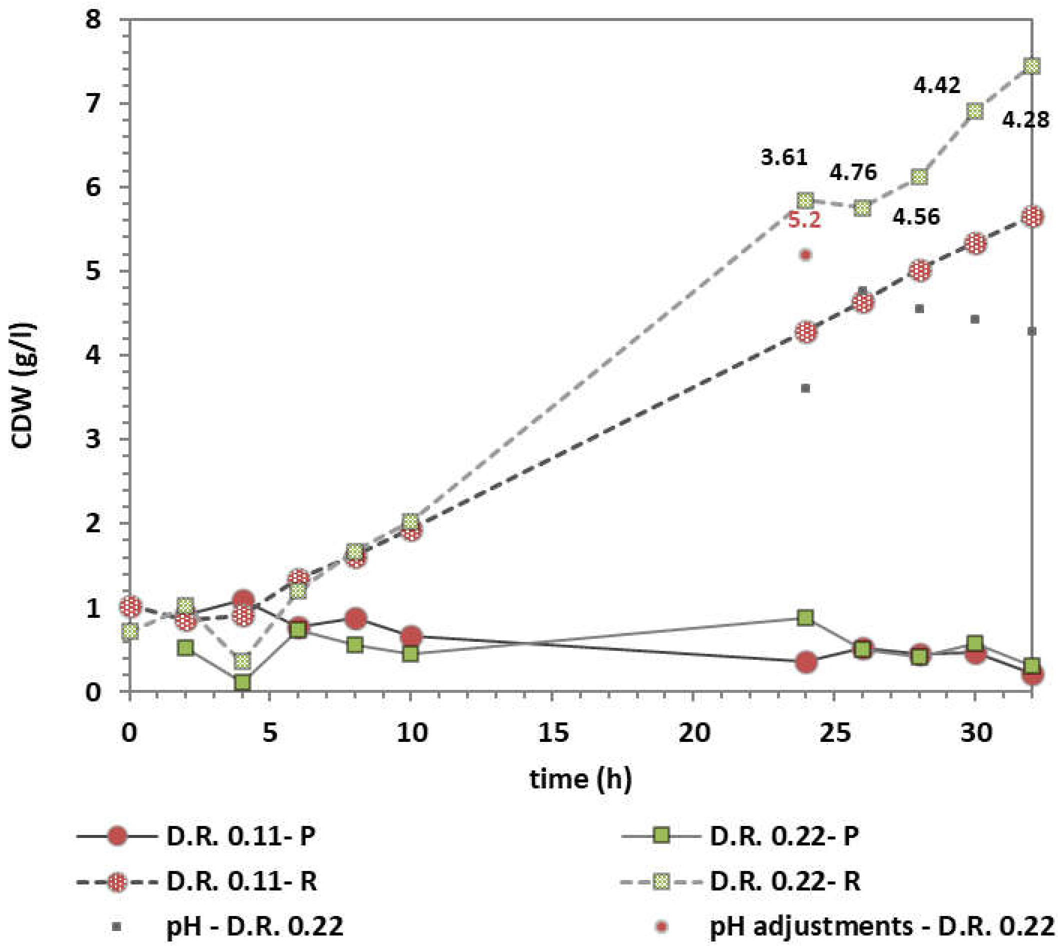

Figure 4-51 : Change in Cell dry weight; inoculum; bacteria 0.00001 g/l, yeast 0.075 g/l, filtration in double panel with D.R. 0.11 and 0.22 h^{-1}

A comparison in dry mass data between yeast + bacteria filtration with D.R. 0.11 h^{-1} and D.R. h^{-1}(Figure 4-51) also points to the conclusion that bacteria could not be washed out in D.R. 0.22 h^{-1}. Though the dry mass is steadily increasing in the reactor after 24 hours of fermentation when the D.R. is 0.11 $h^{-1,}$ dry mass is subjected to pH adjustments in D.R. 0.22 h^{-1} since bacteria cells are greatly affected by changes in pHfrom 5 to 4 (Kumar et al.,2000; Khanna et al..2010) (Figure 4-51).

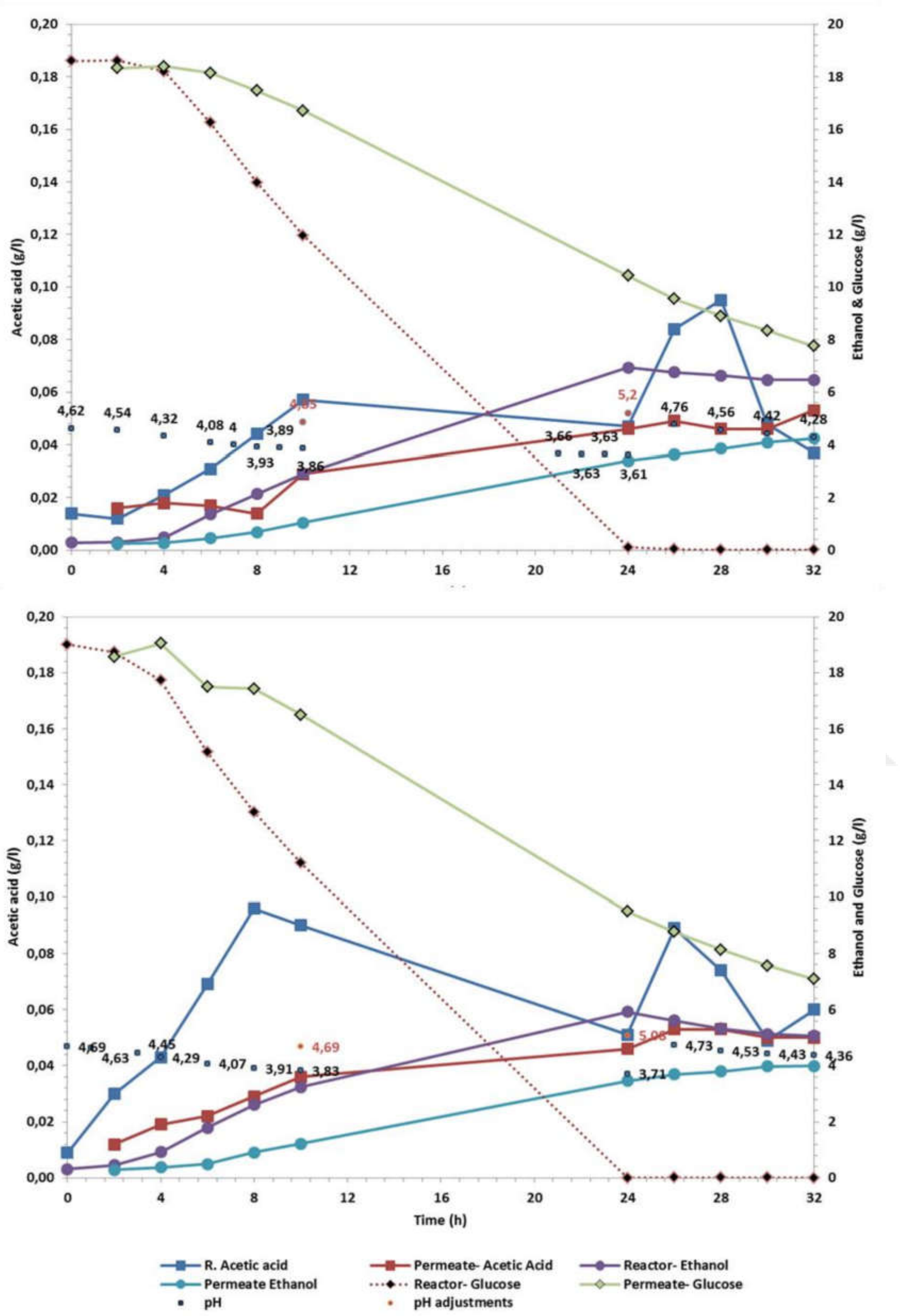

Figure 4-52 : Glucose, Ethanol and Acetic acid concentrations in the reactor and permeate tank & pH reading and adjustments from the reactor; inoculum; bacteria 0.0004 g/l + yeast 0.072 g/l, Dilution Rate 0,22 h^{-1}(top) and D.R. 0.11h^{-1}(bottom) filtration in double panel.

Measurements of metabolic activity further prove that bacteria could not be washed out; even though the D.R. is double in D.R. 0.22 h^{-1} the acetic acid concentration in reactor at 24th hour is higher than that of D.R. 0.11h^{-1} (5.2 g/l vs 5.08 g/l) (Figure 4-52). Nevertheless when the final ethanol concentrations in both runs at 32nd hour are compared, it is observed that D.R. 0.22 h-1 provides higher concentration of ethanol, which due to the fact that the reactor is fed more glucose when D.R. is 0.22 h^{-1} (Figure 4-52).

4.5.3 Effect of pH control at 5

As rate and inoculum concentration that is required to have a working MBR process was established from earlier runs the effect of pH was investigated as the next step by controlling the pH of the reactor medium at 5 through automatic addition of 2 M NaOH.

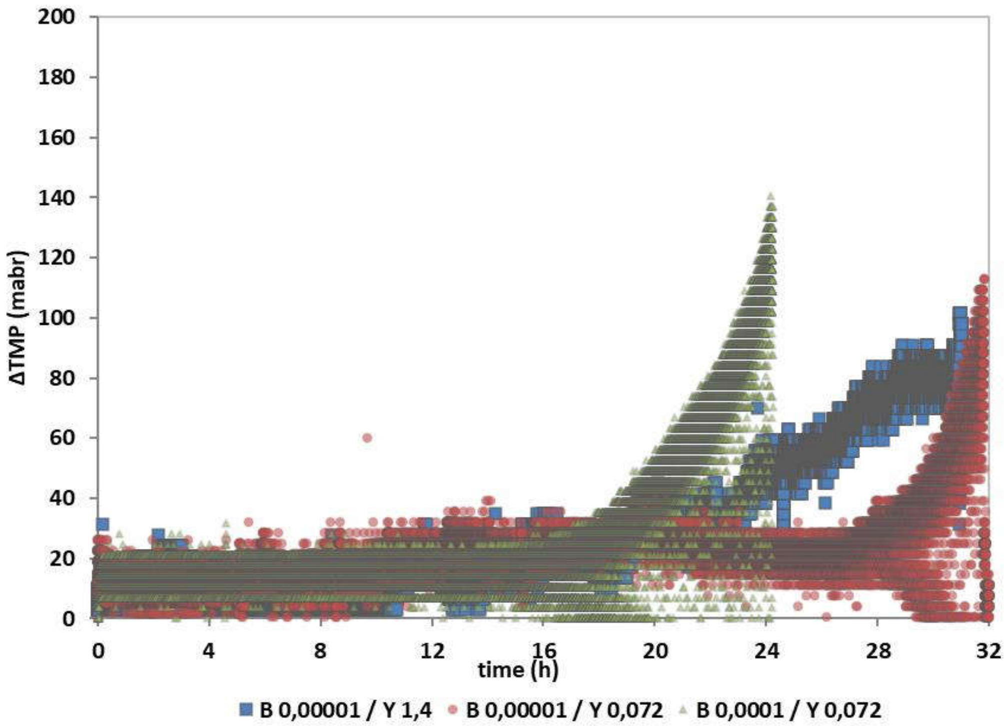

Figure 4-53 : Change in TMP; comparison based on inoculum, filtration in double panel with D.R. 0.11 h^{-1}inoculum; bacteria 0.00001 + yeast 1.4 g/l, bacteria 0.00001 + yeast 0.072 g/l, bacteria 0.0001 + yeast 0.072 g/l, pH is controlled at 5

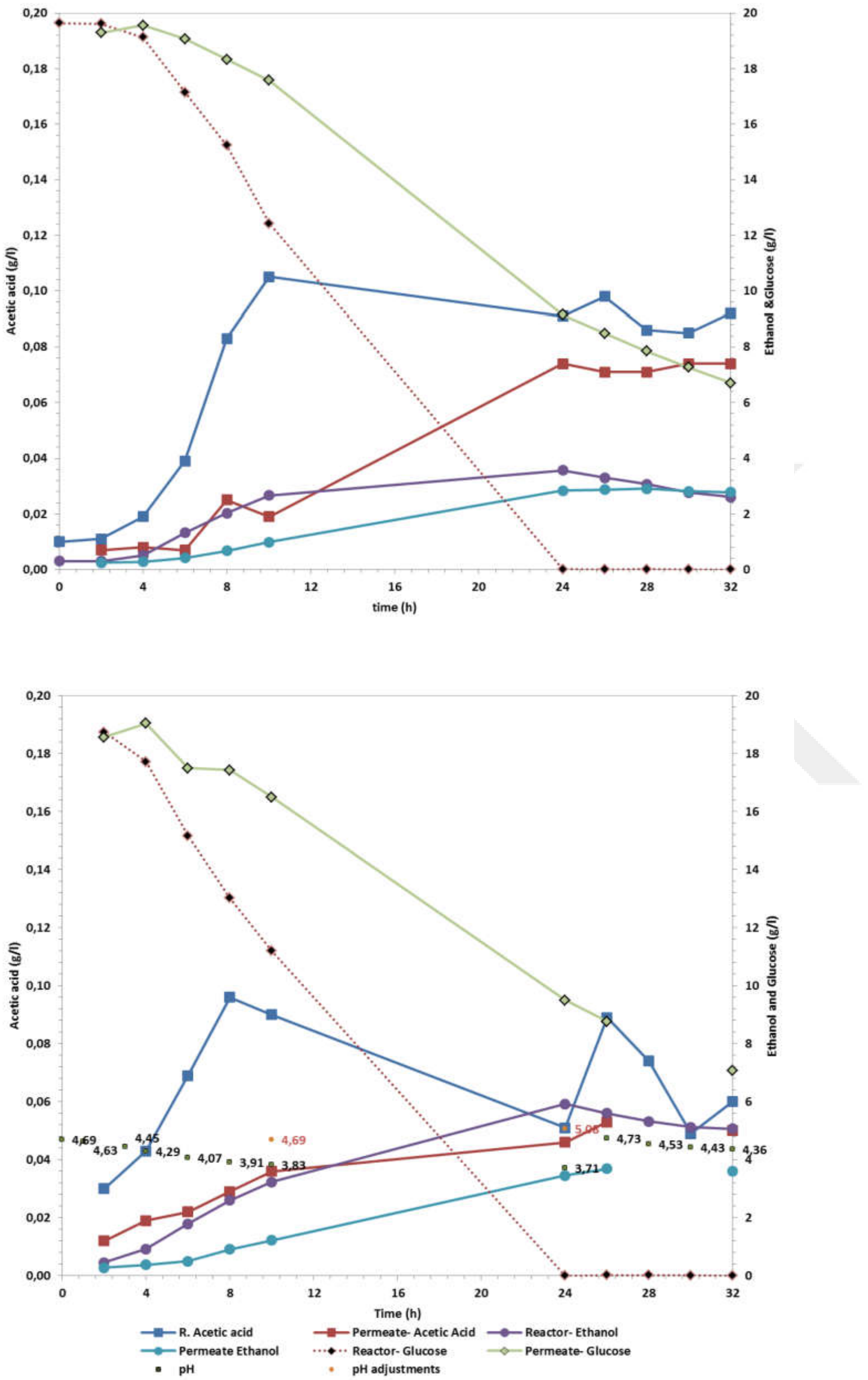

Figure 4-54 : Glucose, Ethanol and Acetic acid concentrations in the reactor and permeate tank & pH reading and adjustments from the reactor; Dilution Rate 0,11 h^{-1} , inoculum; bacteria 0.00001 g/l + yeast 0.072 g/l), filtration in double panel with automatic pH control at 5 (above) and without automatic pH control (below).

As bacteria prefer higher pH (Kumar et al.,2000; Khanna et al..2010) a continuous process could not be achieved when pH is controlled at 5; doubling rate of the bacteria increased and the pressure on membrane eventually rose and could not stay stable in all runs executed with pH control at 5 (Figure 4-53).

It was observed that when pH is controlled at 5 ethanol concentration in reactor and permeate tank also decreased probably due to higher level of acetic acid produced by bacteria (Figure 4-54). This assumption is also supported by dry biomass data (Figure 4-55) as there is higher cell growth when the pH is controlled manually which must be attributed to yeast growth since bacteria prefers higher pH.

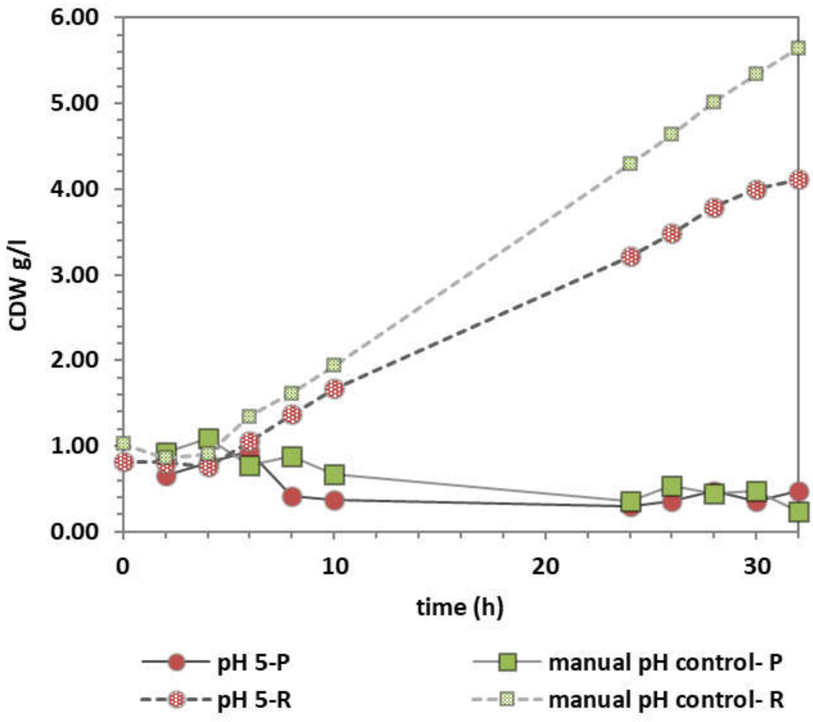

Figure 4-55 : Biomass concentration in the reactor and permeate tank comparison based on pH; Dilution Rate 0,11 h^{-1} , inoculum; bacteria 0.00001 g/l + yeast 0.072 g/l), filtration in double panel with automatic pH control at 5 and without automatic pH control.

Based on comparisons above it can be concluded if the pH is kept at 4 at the beginning of the fermentation process the contamination can be washed out entirely without hindering ethanol production.

4.5.4 Effect of membrane area

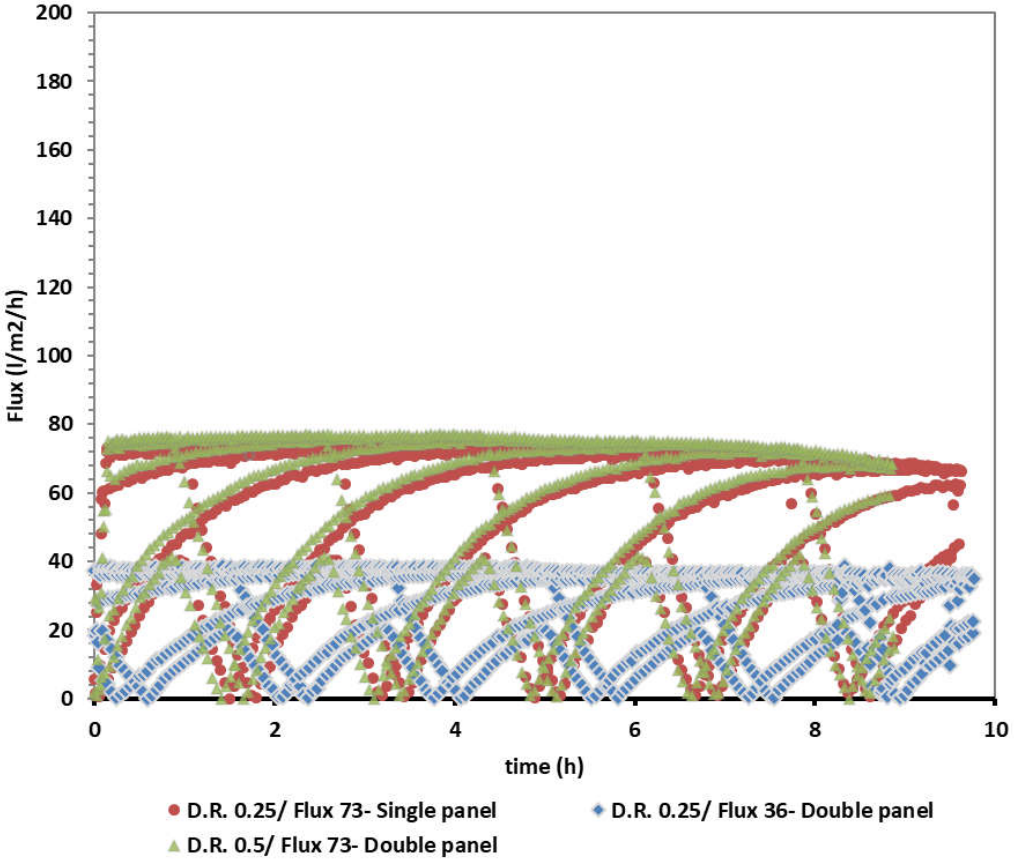

Figure 4-56 : Change in permeate flow, comparison based on membrane area; Single panel filtration with D.R. 0.25 h^{-1} and flux 73 $l/m^2/h$, Double panel filtration with D.R. 0.25 h^{-1} and flux 36 $l/m^2/h$ &Double panel filtration with D.R. 0.5 h^{-1} and flux 73 $l/m^2/h$

Effect of membrane area on bacteria washout was also investigated since 2 different reactors were used during the experimentation phase; one that could accommodate a single membrane panel and one with double membrane panels. Experiments were conducted with by keeping either the flux or the dilution rate same (Figure 4-56).

Despite having different membrane cleaning, pressure values showed that increase in membrane area does not facilitate improved bacteria washout. All runs showed the same trend; the membrane started failing once the bacteria went into exponential growth phase at 4^{th}hour (Figure 4-57). However reactor run with half flux rate (D.R. 0.25 h^{-1} Flux 36 $l/m^2/h$) showed a slower trend in TMP increase (Figure 4-57).

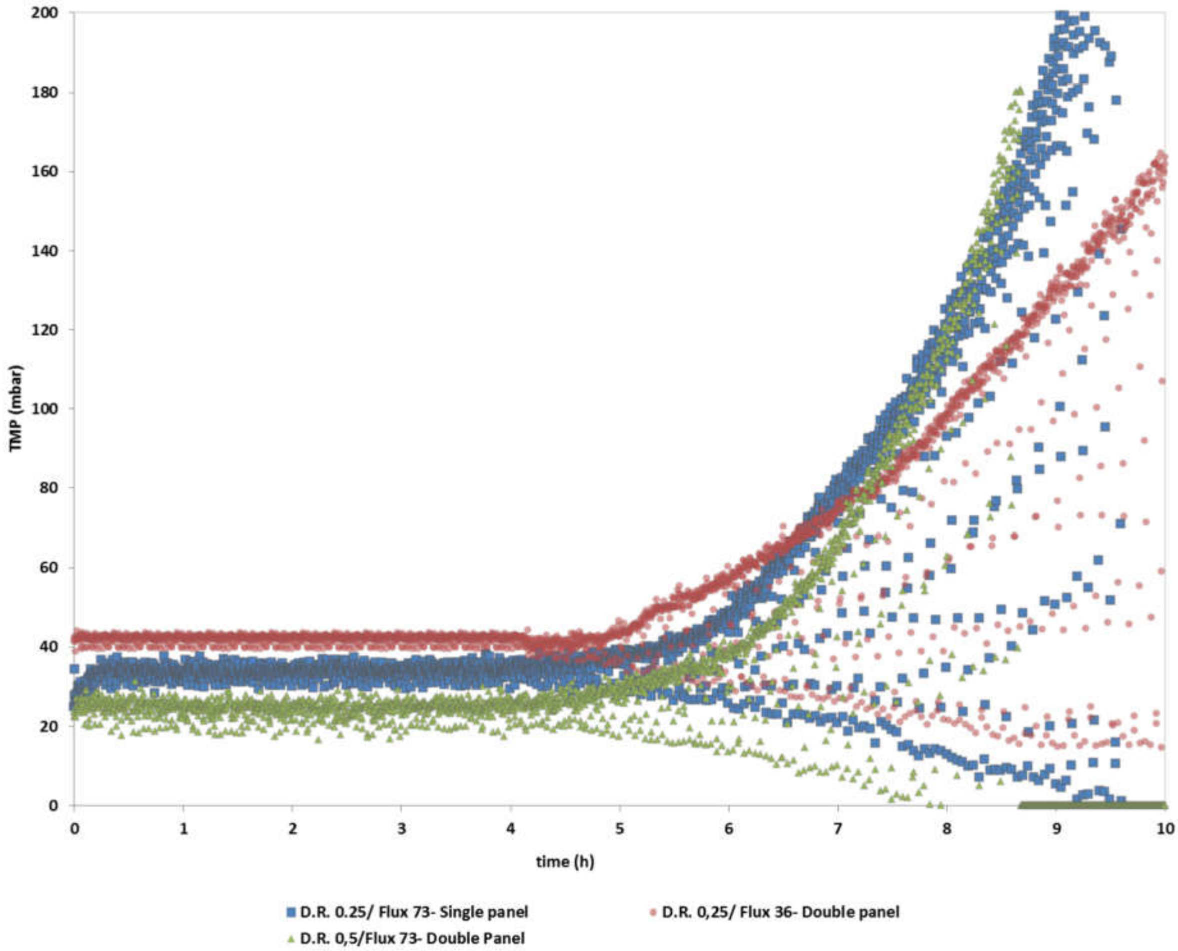

Figure 4-57: Change in TMP ;inoculum: bacteria 0.001 g/l comparison based on membrane area; D.R. 0.25 h^{-1}Flux 73 $l/m^2/h$ filtration in single panel, D.R. 0.25 h^{-1}Flux 36 $l/m^2/h$ filtration in double panel & D.R. 0.5 h^{-1}Flux 36 $l/m^2/h$ filtration in double panel

Acetic acid concentration values on the other hand showed variation with membrane area: the acetic acid concentration rose highest in D.R. 0.25 h^{-1}in single panel filtration despite having the same permeate flow rate with D.R. 0.25 h^{-1}in double panel (

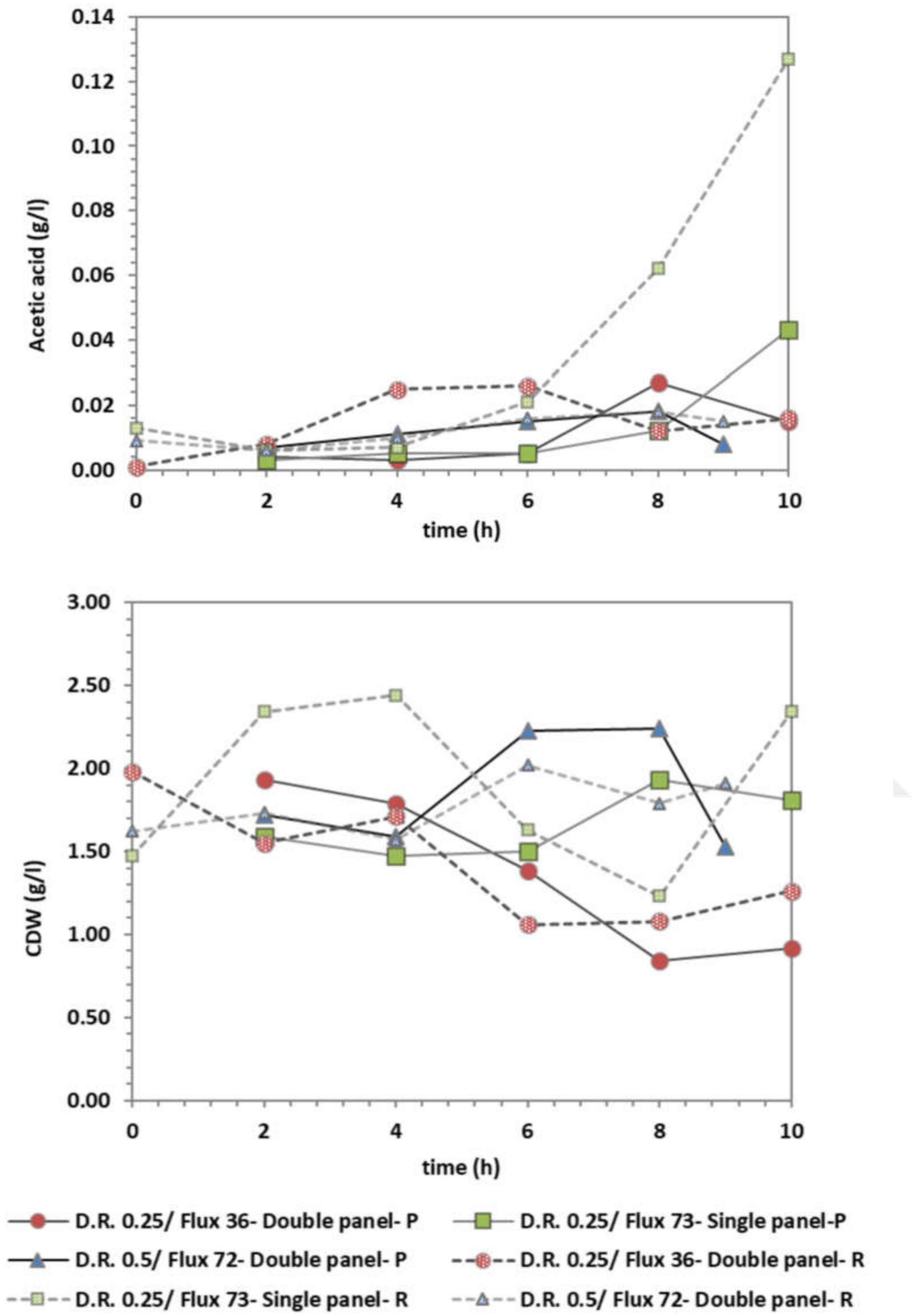

Figure 4-58). The reactor dry biomass values in D.R. 0.25 h^{-1} in single panel showed the exact same trend with D.R. 0.25 h^{-1} in double panel signaling similar speed in cell washout; however despite having a higher bacteria concentration in 0 h, the final

bacteria concentration of D.R. 0.25 h^{-1}in single panel is higher that D.R. 0.25 h^{-1}in double panel which may signify improved washout (Figure 4-58).

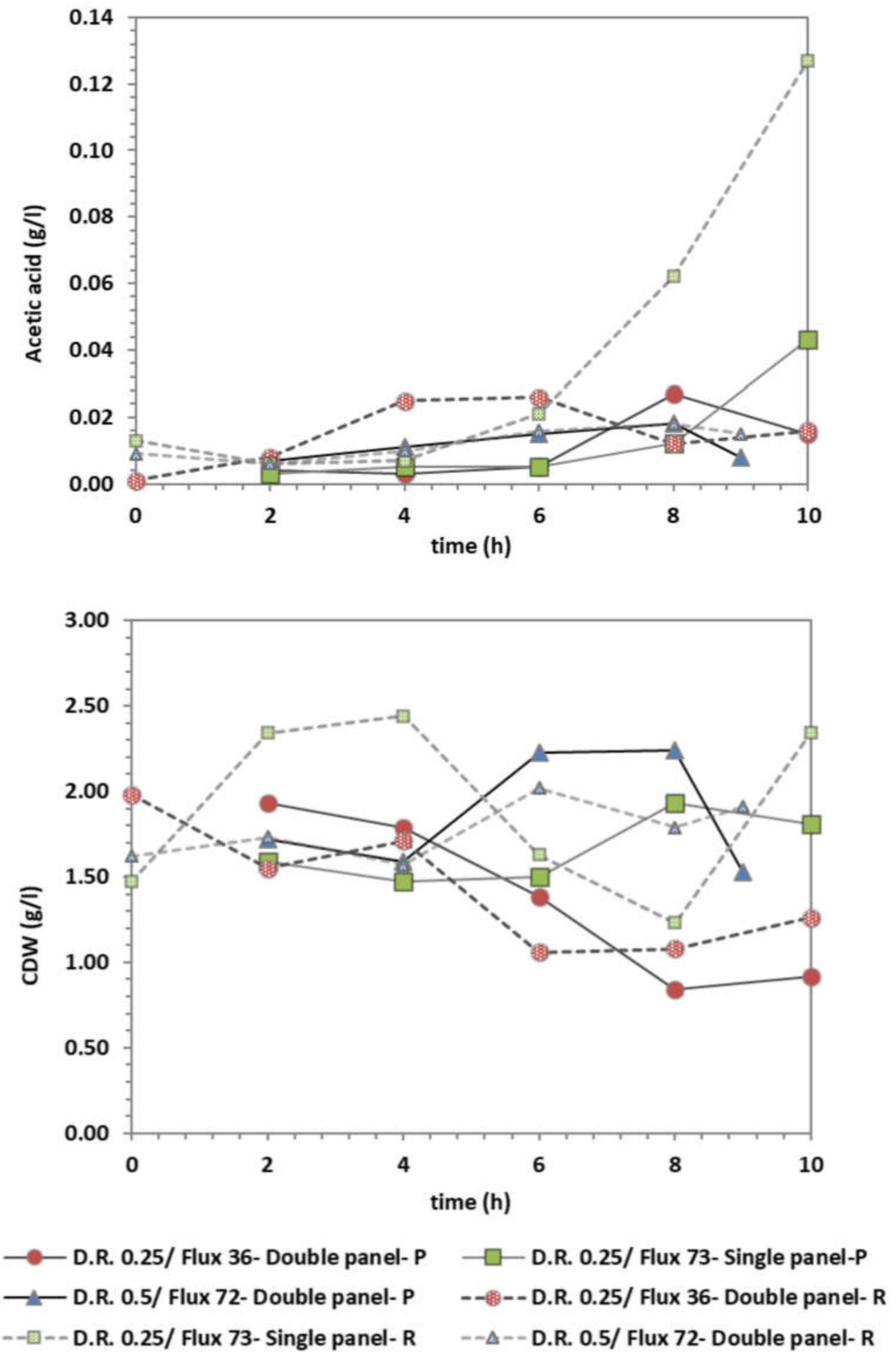

Figure 4-58: Biomass concentration in the reactor and permeate tank (below) and acetic acid concentrations inside the reactor and permeate tank (above) comparison based on membrane

area; D.R. 0.25 h^{-1}Flux 73 $l/m^2/h$ filtration in single panel, D.R. 0.25 h^{-1}Flux 36 $l/m^2/h$ filtration in double panel & D.R. 0.5 h^{-1}Flux 36 $l/m^2/h$ filtration in double panel

5. CONCLUSION

A major issue hindering efficient industrial fermentation of sugar based feed stock to ethanol is unwanted bacterial contamination(Khullar et al., 2013; Zia et al., 2011). However in industrial scale fermentation a totally contamination free process is hardly achieved as complete sterilization and maintaining sterility of the feedstock and instruments involved is very costly and laborious. In this regard, in order to remediate the contamination issue and control bacterial growth in fermentation systems, conventional anti-microbial compounds are applied(Chang et al., 1997; Limayem et al., 2011; Liu et al., 2015; Viegas et al., 2002). However, none of the anti-bacterial agents have proven to be fully effective for long term bacterial control and system disinfection.

The aim of this project was to achieve bacterial decontamination in an MBR set up during bioethanol fermentation by S. *cerevisiae. Enterobacter cloacae,* a rod shaped acetic acid producing bacteria isolated from a xylose-glucose fermentation system at the University of Boras was chosen as the contaminating agent. 2^{nd} generation integrated permeate channel (IPC) poly ethersulfune (PES) membrane cassettes were used in the immersed MBR set up during experimentation.

As the growth rates of both microorganisms were critical to determine the ideal Dilution Rate during the MBR, bacteria and yeast cultures were cultivated in shake flask and biomass growth was observed through spectrophotometry. The results yielded that both organisms are able to grow in pH 5 while the bacteria grows almost double in pH 7 compared to pH 5. The metabolic activities of both organisms were also observed through HPLC analysis. The results have also indicated bacteria’s preference to more alkaline environment as acetic acid production in pH 7 was determined to be 4 times the amount produced at pH 5.

In order to test the membranes ability to simultaneously retain yeast and washout bacteria Phosphate Buffer Saline solution was used as reactor medium and a reactor with a working volume of 1.5 L were used at the first step. IPC membranes with pore sizes 1 and 2.4 μm were applied based on the size determination of yeast and bacteria though light microscopy. Membrane with 2.4 μm pore size was observed to successfully retain yeast and allow bacteria washout, where as membrane with 1 μm pore size did not allow bacteria passage and therefore was eliminated from further experimentation steps.

In the next stages different permeation flow rates were tested for bacteria washout in bacteria alone medium and medium containing bacteria and yeast. In the experiments executed with PBS solution it was determined that almost 100% bacteria removal could be achieved in medium inoculated with yeast and bacteria cultures with a Dilution Rate of 0.5 $h^{-1}$and a 4 minute backwash cycle (3,5 minute permeation+ 30 seconds of backwash).

However fermentation conditions presented new challenges as both bacteria and yeast cells duplicated within the reactor. Even though a number of inoculums sizes and dilution rates were attempted, bacteria alone filtration could not be achieved under fermentation conditions as the membrane was fouled once the bacteria went to exponential growth phase. However when the reactor was inoculated with yeast and bacteria culture,the pressure on the membrane surface decreased significantly which is attributed to the inhibitory effect of the ethanol production as well as sugar consumption by yeast; it is also possible that yeast protects the membrane surface, allowing the bacteria to pass through only vertically hence preventing surface fouling.

Once the size of initial yeast inoculum was determined the critical dilution rate was investigated and a dilution rate of 0.11 h^{-1} was determined to be ideal as higher D.R. resulted in eventual increase on TMP preventing a continuous MBR system. Glucose, ethanol and acetic acid concentrations in the reactor also showed continuous increase in ethanol concentration until the glucose in the reactor is eliminated. Acetic acid concentrations also gave favourable results for continuous ethanol production, as bacteria metabolism stopped once the pH has dropped to 4. On the other hand, the drop in pH to 4 did not affect the ethanol production by yeast. However, when the pH was regulated at 5, pressure on the membrane raise to a critical level- even though the initial bacteria inoculum size was 10^{-1} of the previous system. Therefore, it was concluded that system may be used continuous bioethanol production if the pH is regulated at 4.

6. FUTURE WORKS

- The data obtained through latest experimental runs proved to be promising to achieve continuous bioethanol fermentation process in an MBR system. It is assumed that should the pH is kept at 4 until the contaminationis washed out, the process may be operated indefinitely. Therefore the same system could be tried out with a higher contaminant inoculum while the pH is automatically regulated at 4; it may also be possible to increase the dilution rate.

- Another option could be to increase the membrane area in proportion to D.R.; it was observed in experimental runs that doubling the D.R. simultaneously with membrane area yielded to quicker bacteria washout while retaining a stable pressure on membrane.

- This system could also be tested with an additional contaminant; as most of the bacterial contaminants are lactic acid producing bacteria it would be appropriate to test whether the system is operable when multiple types of bacteria is present during fermentation.

REFERENCES

Albers, E., Lidén, G., Larsson, C. & Gustafsson, L. (1998). Anaerobic redox balance and nitrogen metabolism in Saccharomyces cerevisiae.*Recent Research Developments in in Microbiology, 2,*253-279.

Amigun, B., Sigamoney, R., von Blottnitz, H.(2008). Commercialization of biofuel industryin Africa: a review. *Renew Sustain Energy Rev 12* (3), 690-711.

Amorim, H.V. & Lopes, M.L.(2005) Ethanol production in a petroleum dependent world. *Sugar J.67*,11–14.

Amorim, H.V., Lopes, M.L., Oliveira, J.V.C., Buckeridge M. & Goldman, G.H. (2011). Scientific challenges of bioethanol production in Brazil. *Appl Microbiol Biotechnol. 91*(5), 1267–1275.

Andrietta, M.G.S., Andrietta, S.R., Steckelberg, C.. and Stupiello E.N.A. (2007). Bioethanol – Brazil, 30 years of Proalcool. *International Sugar Journal, 109*, 195-200.

Attfield, P.V. (1997). Stress tolerance: The key to effective strains of industrial baker's yeast. *Nature Biotechnology, 15*, 1351-1357.

Azhar, S.H.M. , Abdulla, R. , Jambo S. A. , Marbawi, H. , Gansau, J. A. , Faik, A. A. M., Rodrigues, K. F., (2017).Yeasts in sustainable bioethanol production: A review. *Biochemistry and Biophysics Reports, 10,* 52–61.

Azhara, S. H., Abdullaa, R., Jamboa, S. A., &Marbawia, H. (2017). Yeasts in sustainable bioethanol production: A review. *Biochemistry and Biophysics Reports*,*10*, 52–61.

Bai, F.W., Anderson, W.A., & Moo-Young,M. (2008). Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv., 26,*89-105.

Ballesteros I., Negro M.J., Oliva J.M., Cabanas A., Manzanares P., Ballesteros M. (2006). Ethanol production from steam-explosion pretreated wheat straw. *Applied Biochemistry and Biotechnology, 130*(1-3), 496-508.

Bassi, A.P., da Silva, J.C., Reis, V.R.,&Ceccato-Antonini, S.R., (2013**).** Effects of single and combined cell treatments based on low pH and high concentrations of ethanol on the growth and fermentation of Dekkerabruxellensis and Saccharomyces cerevisiae. *World J MicrobiolBiotechnol*, *29*, 1661–1676

Basso,L.C., Basso, T. O., Rocha, S.N.,(2011). Ethanol Production in Brazil: The Industrial Process and Its Impact on Yeast Fermentation, Biofuel Production-Recent Developments and Prospects. Dr. Marco Aurelio Dos Santos Bernardes (Ed.), doi: 10.5772/17047

Bekatorou, A., Psarianos, C., &Koutinas, A.A.,(2006). Production of Food Grade Yeasts. *Food Technology and Biotechnology. 44* (3), 407–415.

Bhatia S.C.(2014) Ethanol. In: Bhatia S.C., editor. Advanced renewable energy systems. Part I. Woodhead Publishing India PVT LTD; New Delhi, 523–572.

Buzas, Z., Dallmann., K., &Szajani., B.,(1988). Influence of pH on the Growth and Ethanol Production of Free and Immobilized Saccharomyces cerevisiae Cells. *Biotechnology and Bioengineering,. 34*, 882-884

Campbell C.H. and Laherrere J.H.(March 1998). The end of cheap oil. *Scientific American*. Retrieved from https://nature.berkeley.edu/er100/readings/Campbell_1998.pdf

Chae, S.R., Ahn, Y.T., Kang, S.T. &Shin, H.S. (2006) Mitigated membrane fouling in a vertical submerged membrane bioreactor (VSMBR). *Journal of Membrane Science, 280*, 572-581.

Chang, I.S., Kim, B.H.,& Shin, P.K. (1997). Use of sulfite and hydrogen peroxide to control bacterial contamination in ethanol fermentation. *Applied and Environmental Microbiology, 63*(1), 1-6.

Charoenchai, C., Fleet, G., &Henschke, P.A. (1998) Effects of temperature, pH and sugar concentration on the growth rates and cell biomass of wine yeasts. *American Journal of Enology and Viticulture, 49,* 283–288.

Cheng, N.G. Hasan, M. & Kumoro, A.C. (2009). Production of ethanol by fed-batch fermentation.*Pertanika J. Sci. Technol.17*, 399–408.

Cot, M., Loret, M.O., François, J., &Benbadis, L.,(2007) Physiological behaviour of Saccharomyces cerevisiae in aerated fed-batch fermentation for high level production of bioethanol.*FEMS Yeast Research, 7*, 22–32.

Demirbas A. (2007) Producing and Using Bioethanol as an Automotive Fuel. *Energy Sources, Part B: Economics, Planning, and Policy, 2*(4), 391-401, doi:10.1080/15567240600705466

Diao, L., Liu, Y., Qian, F., Yang, J., Jiang, Y. & Yang, S. (2013). Construction of fast xylose-fermenting yeast based on industrial ethanol-producing diploid Saccharomyces cerevisiae by rational design and adaptive evolution. *BMC Biotechnology*, *13*, 1-9.

Díaz-Montaño, D. M. (2013). Biomass Now – Sustainable Growth and Use. In Matovic, M. D. (Ed), *Continuous Agave Juice Fermentation for Producing Bioethanol*. (pp.209-230) Retrieved from https://www.intechopen.com/books/biomass-now-sustainable-growth-and-use

Doyen, W., Mues, W., Molenberghs, B., Cobben, B. (2010). Spacer fabric supported flat-sheet membranes: A new era of flat-sheet membrane technology. *Desalination, 250*(3), 1078-1082.

Dunn, I.J., et al.,(2003). Biological Reaction Engineering. 2nd Edition ed. New York: WileyVCH

Eroshin, V. K., Utkin, I. S., Ladynichev, S. V., Samoylov, V. V., Kuvshinnikov, V. D. and Skryabin, G. K.,(1976). Influence of pH and temperature on the substrate yield coefficient of yeast growth in a chemostat. *Biotechnology and Bioengineering*.,*18*, 289–295.

Feldmann, H.,(2010). Yeast: Molecular and Cell Biology 2nd edition. Wiley-Blackwell.

Ferreira, L.V., Amorim, H.V., Basso, L.C.,(1999). Fermentation of endogenous trehalose and glycogen by saccharomyces cerevisiae.*Ciência e Tecnoogia de Alimentos (Campinas), 19*(10), 29-32.

Galbe M. & Zacchi G.(2002). A review of the production of ethanol from softwood.*Applied Biobiology and Biotechnology, 59*, 618-628.

Goldemberg, J.(2008). The Brazilian biofuels industry. *Biotechnology for Biofuels1*(6) doi: 10.1186/1754-6834-1-6.

Gutiérrez-Correa, M. and Villena G.K., (2010). Chapter 7. Characteristics and Techniques of Fermentation Systems. *"Comprehensive Food Fermentation and Biotechnology"*. Vol. 1., New Delhi: Asiatech Publisher.

Hines, M., Holmes, C., Schad, R.,(2010). Simple strategies to improve bioprocess pure culture processing. *Pharmaceutical Engineering, 30*(3), 10-28.

Ishola, M.M. (2014). *Novel Application of Membrane Bioreactors in Lignocellulosic Ethanol Production: Simultaneous Saccharification, Filtration and Fermentation (SSFF).* (Doctorate Thesis). University of Borås. BORAS

Jain, A. & Chaurasia, S.P.(2014). Bioethanol Production in Membrane Bioreactor (MBR)System: A Review. *International Journal of Environmental Research and Development, 4* (4), 387-394.

Jain, A. and Chaurasia, S.P. (2014).Bioethanol Production in Membrane Bioreactor (MBR) System: A Review. *International Journal of Environmental Research and Development, 4* (4),387-394.

John R.P., Anisha, G.S., Nampoothiri, K.M., Pandey, A. (2011) Micro and macroalgal biomass: a renewable source for bioethanol.*Bioresource Technology, 102* (1), 186–193.

Johnston, M. & Kim J.H. (2005). Glucose as a hormone: receptor-mediated glucose sensing in the yeast Saccharomyces cerevisiae. *Biochemical Society Transactions 33* (1), 247-252. doi: 10.1042/BST0330247

Kandler, O.(1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek Journal of Microbiology, 49*(3), 209-224.

Kasemets., K., Nisamedtinov, I., Laht, T.M., Abner, K., Paalme, T., (2007). Growth characteristics of Saccharomyces cerevisiae S288C in changing environmental conditions: auxo-accelerostat study. *Antonie van Leeuwenhoek,92*(1), 109–128

Khaled, M.,Khleifat, Sharaf, E.F., & Al-limoun., M.O., (2015). Biodegradation of 2-Chlorobenzoic Acid by Enterobacter cloacae: Growth Kinetics and Effect of Growth Conditions. *Bioremediation Journal, 19*(3), 207-217, doi: 10.1080/10889868.2015.1029113

Khanna, N., Kotay, S.M,, Gilbert, J.J., Das, D.,(2010). Improvement of biohydrogen production by Enterobacter cloacae IIT-BT 08 under regulated pH.*Journal of Biotechnology, 152*(1-2), 9-15.

Khullar, E., Kent, A.D., Leathers, T.D., Bischoff, K.M., Rausch, K.D., Tumbleson, M.E., Singh, V.,(2013). Contamination issues in a continuous ethanol production corn wet milling facility. *World Journal of Microbiology and Biotechnology, 29*(5), 891-898.

Kumar N, Das D. (1999). Studies on molecular hydrogen production by Enterobacter cloacae IIT-BT 08. *Paper presented at 9th European Congress on Biotechnology, Brussels, Belgium*, 11–15

Kumar N., Das D. (2000). Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08. *Process Biochemistry, 35*, 589–593.

Lim, A. L. and Bai, R. (2003) Membrane fouling and cleaning in microfiltration of activated sludge wastewater. *Journal of Membrane Science,216,* 279-290.

Limayem, A., Hanning, I.B., Muthaiyan, A., Illeghems, K., Kim, J.W., Crandall, P.G., O'Bryan, C.A., &Ricke, S.C. (2011). Alternative antimicrobial compounds to control potential Lactobacillus contamination in bioethanol fermentations. *Journal of environmental science and health. Part. B, Pesticides, food contaminants, and agricultural wastes, 46*(8), 709-714.

Liu, M., Bischoff, K.M., Gill, J.J., Mire-Criscione, M.D., Berry, J.D., Young, R., &Summer, E.J. (2015). Bacteriophage application restores ethanol fermentation characteristics disrupted by Lactobacillus fermentum. *Biotechnology for Biofuels, 8*(132).doi 10.1186/s13068-015-0325

Lopes, M. L., Paulillo, S. C. de L., Godoy, A., Cherubin, R. A., Lorenzi, M. S., Giometti, F. H. C., … Amorim, H. V. de. (2016). Ethanol production in Brazil: a bridge between science and industry. *Brazilian Journal of Microbiology*, *47*(Suppl 1), 64–76. http://doi.org/10.1016/j.bjm.2016.10.003

Macedo I.C., Seabra J.E.A. &Silva J.E.(2008). Greenhouse gases emissions in the production and use of ethanol from sugarcane in Brazil: the 2005/2006 averages and a prediction for 2020. *Biomass Bioenergy, 32* (4), 582-595.

Mahboubi, A., Ylitervo, P., Doyen, W., De Wever, H.& Taherzadeh, M. J. (2016) Reverse membrane bioreactor: Introduction to a new technology for biofuel production. *Biotechnology Advances, 34,* 954-975.

Mahboubi, A., Ylitervo, P., Doyen, W., De Wever, H., Molenberghs, B.& Taherzadeh, M. J. (2017) Continuous bioethanol fermentation from wheat straw hydrolysate withhigh suspended solid content using an immersed flat sheet membrane bioreactor. *Bioresource Technology 241*, 296–308.

Mosby's Medical Dictionary, 8th edition. (2009). Retrieved June 23 2017 from http://medical-dictionary.thefreedictionary.com/Enterobacter+cloacae

Neto O.and Yokoya, F. (1997). Effects of nutritional factors on growth of Lactobacillus fermentum mixed with Saccharomyces cerevisiae in alcoholic fermentation. *Revista de Microbiologia, 28*(1), 25-31.

Nigam P.S. and Singh A., (2011) Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science 37*(1), 52–68.

Olympio, K.P.K., Gonçalves, C., Günther, W.M.R.& Bechara, E.J.H. (2009). Neurotoxicity and aggressiveness triggered by low-level lead in children: a review. *Pan Am J Public Health, 26*(3), 266–275.

Orantes, J., Wisniewski, C., Heran, M.& Grasmick, A. (2004) Filtration performance of different membrane module configurations in a submerged membrane reactor, operated under total biomass retention condition. *In: IWA Specialised Conference on Water Environment-Membrane Technology.Seoul-Korea.*

Petrovska, B., Winkelhausen, E. & Kuzmanova, S. (1999). Glycerol production by yeasts under osmotic and sulfite stress. *Canadian Journal of Microbiology, 45,*695-699.

Reddy, B., Ramesh, S., Ashok Kumar, A., Wani, S., Ortiz, R., Ceballos, H., et al.(2008). Bio-fuelcrops research for energy security and rural development in developingcountries. *Bioenergy Research, 1*(3), 248-258.

Reed G. and Peppler H. J .(1973). Yeast Technology. UniversalFoods Corp., Milwaukee, WI,.

Sánchez, Ó.J. & Cardona, C.A. (2008).Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology, 99*, 5270-5295

Sims, R. Taylor, M. Saddler, J. & Mabee, W. (2008)From 1st to 2nd generation biofuel technologies – Anoverview of current industry and R&D activities, in IEA Bioenergy. *OECD/IEA.*

Skinner K.A. and Leathers T.D. (2004). Bacterial contaminants of fuel ethanol production. *Journal of Industrial Microbiology &Biotechnolog,31*(9), 401-408.

Snoek I.S. and Steensma H. (2007). Factors involved in anaerobic growth of Saccharomyces cerevisiae, *Yeast 2007*, *24*, 1–10.

Staniszewski, M., Kujawski, W., Lewandowska, M.,(2007) Ethanol production from whey in bioreactor with co-immobilized enzyme and yeast cells followed by pervaporative recovery of product – Kinetic model predictions,*Journal of Food Engineering, 82*(4), 618–625.

Subudhi, S., Nayak, T., Kumar, N.R., Vijayananth, P., Lal, B., (2012). Impact of regulated pH on proto scale hydrogen production from xylose by an alkaline tolerant novel bacterial strain, Enterobacter cloacae DT-1. *International Journal of Hydrogen Energy, 38*(6), 2728-2737.

terSchure, E.G., van Riel, N.A., Verrips, C.T.,(2000). The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae.*FEMS Microbiology Reviews, 24*(1), 67-83.

Viegas, M.C., Andrietta, S.R., Andrietta, M.G.S. (2002). Use of tower reactors for continuous ethanol production. *Brazilian Journal of Chemical Engineering, 19*(2), 167-173.

Wang, F., Jiang, Y., &Guo, W. (2016). An environmentally friendly and productive process for bioethanol production from potato waste. *Biotechnology for Biofuels9* (50), doi: 10.1186/s13068-016-0464-7.

Wasungu K. M. and Simard R. E. (1982). Growth Characteristics of Bakers' Yeast in Ethanol. *Biotechnology and Bioengineering, 24*, 1125-1134.

Wheals, A.E., Basso, L.C., Alves, D.M.G. & Amorim, H.V. (1999). Fuel ethanol after 25 years. *Trends Biotechnol. 17,* 482–487.

Woo, J.M., Yang, K.M., Kim. S.U., Blank, L.M., Park, J.B. (2014). High temperature stimulates acetic acid accumulation and enhances the growth inhibition and ethanol production by Saccharomyces cerevisiae under fermenting conditions. *Applied Microbiology and Biotechnology, 98* (13), 6085-6094.

Wurst M., Sigler K ., Knotkova A., (1980). Gas Chromatographic Determination of Extracellular Metabolites Produced by Baker's Yeast during Glucose-Induced Acidification. *Folia Mierobiol. 25*(4), 306-310.

Ylitervo, P., Franzén, C.H. & Taherzadeh, M.J. (2014) Continuous Ethanol Production with a Membrane Bioreactor at High Acetic Acid Concentrations.*Membranes,4*, 372-387.

Zabed, H., Faruq, G., Sahu,J.N., Azirun, M.S., Hashim, R. &Boyce, A.N.(2014).Bioethanol production from fermentable sugar juice, *Scientific World Journal,* 1–11.

Zahed, O., Jouzani, G.S., Abbasalizadeh, S., Khodaiyan, F., Tabatabaei, M.(2016). Continuous co-production of ethanol and xylitol from rice straw hydrolysate in a membrane bioreactor.*Folia Microbiol, 61*(3), 179–189.

Zaldivar, J., Nielsen, J. & Olsson, L. (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Applied Microbiology and Biotechnology*, *56*, 17-34.

Zia, M.A., Asghar, M., Bhatti, H. (2011). Improving bio-ethanol yield: Using virginiamycin and sodium flouride at a Pakistani distillery. *African Journal of Biotechnology, 10*(53), 11071-11074.

URL-1:<https://www.afdc.energy.gov/data/10331>date retrieved06.11.2017

CURRICULUM VITAE

Name Surname :Beray Çaylı

Place and Date of Birth : KIRKLARELİ 29.06.1989

E-Mail : beraycayli@hotmail.com

EDUCATION :

- **B.Sc.** :2013, ITU, Civil Engineering Faculty, Department of Environmental Engineering

PROFESSIONAL EXPERIENCE:

- 2014- 2016ISTANBULKhatib&Alami – Tima Mühendislik Müşavirlik JV