The Pennsylvania State University

The Graduate School

Department of Agricultural and Biological Engineering

ENHANCED HUMAN LYSOZYME PRODUCTION BY

KLUYVEROMYCES LACTIS K7 IN BIOFILM REACTOR

A Dissertation in

Agricultural and Biological Engineering

by

Duygu Ercan

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2014

The dissertation of Duygu Ercan was reviewed and approved* by the following:

Ali Demirci
Professor of Agricultural and Biological Engineering
Dissertation Advisor
Chair of Committee

Virendra Puri
Distinguished Professor of Agricultural and Biological Engineering

Jeffrey Catchmark
Associate Professor of Agricultural and Biological Engineering

Paul Patterson
Professor of Poultry Science

Paul Heinemann
Professor of Agricultural and Biological Engineering
Head of the Department of Agricultural and Biological Engineering

*Signatures are on file in the Graduate School

ABSTRACT

Lysozyme is an antimicrobial agent in terms of enzymatic activity, positive charges, and antimicrobial peptides. Therefore, it has applications in food and pharmaceutical industries. Currently, egg white lysozyme is the most commercially available form of lysozyme. However, it has disadvantages compared to human lysozyme, such cause of allergic reactions and its lower enzymatic activity. Therefore, human lysozyme gene was expressed in many host cells. *Kluyveromyces lactis* K7 is a genetically modified microorganism, which can produce human lysozyme, has effective secretory capacity, which can provide high production and easiness for recovery processes. For high production, reactor design, fermentation conditions and strategies should be considered. Biofilm type reactor can enhance the production by high cell density in the reactor. In this study, a biofilm reactor with plastic composite support (PCS) was used in different fermentation modes to enhance the production of human lysozyme by *K. lactis* K7.

First, the best composition of PCS, which included polypropylene (50% (*w/w*)), soybean hulls (35%), soybean flour (5%), yeast extract (5%), and dried bovine albumin (5%), salts (2 g/kg of sodium acetate, 1.2 g/kg of $MgSO_4 \cdot 7H_2O$, and 0.06 g/kg of $MnSO_4 \cdot 7H_2O$), was selected for human lysozyme production. Then, a novel biofilm bioreactor configuration was implemented with selected PCS tubes. The optimum growth conditions for biomass and lysozyme productions in a biofilm reactor were determined as 27°C, pH 6, 1.33 vvm for biomass production and 25°C, pH 4 with no aeration for lysozyme production (Chapter 3). After, the evaluation of different pH and aeration shift strategies, 141 U/ml of human lysozyme production was achieved with a gradual pH decrease by auto-acidification at 25°C without aeration control (Chapter 3).

Then, the fermentation medium composition was evaluated. NaCl and NH_4Cl inhibited the biomass and human lysozyme production. Yeast nitrogen base was selected as the best nitrogen source compared to yeast extract and corn steep liquor. As a result of medium optimization, the production increased to 173 U/ml using the fermentation

medium including 16% lactose, 1.2% casamino acid, and 0.8% yeast nitrogen base. Biofilm reactor provided both higher production and production rate (173 U/ml and 4.07 U/ml/h) compared to the suspended-cell fermentation (110.3 U/ml and 2.00 U/ml/h) because of the increase in the cell density in the reactor (Chapter 4). Then, fed-batch fermentation with 90 g/l glucose and feeding with 400 g/l lactose at a feeding rate of 0.6 ml/min at between 28 and 38 h increased the production amount and the rate further (187 U/ml, 5.9 U/ml/h). Continuous fermentation also enhanced the production rate (7.5 U/ml/h). Moreover, the biofilm reactor provided operation at the maximum level at a higher dilution rate (0.055 h^{-1} dilution rate) compared to a suspended-cell reactor (0.04 h^{-1} dilution rate) because of the immobilized cells in the biofilm reactor (Chapter 5).

Adsorption and desorption conditions were studied using silicic acid resin for the simultaneous fermentation and online recovery of human lysozyme (Chapter 6). At 25°C, pH 4, and 25% silicic acid ratio adsorption was 95.6%, while desorption at 25°C, pH 6.2 with 5% sodium dodecyl sulfate, 1 M NaCl and 20% ethanol as the eluent resulted in 42% desorption. Desorption up to 98% was achieved by four consecutive desorption processes. Using these selected recovery conditions, a simultaneous fermentation and online recovery system with tangential flow microfilter module generated 280.4 U/ml of human lysozyme.

The stability of lysozyme was evaluated during the storage of produced lysozyme at the room temperatures or below before downstream processing. A three factor Box-Behnken design was used to document the effects of the combination of pH, temperature, and time (Chapter 7). Results indicated that the lysozyme activity in the cell free fermentation medium was stable at the temperatures evaluated (4, 10, and 25°C) during 30 days of storage. Also, the human lysozyme activity was highest at 25°C, but decreased at 62.5^{o}C and 100°C at all pH levels.

Finally, primary models were built to describe the batch fermentation of human lysozyme production by *K. lactis* K7 in a biofilm reactor (Chapter 8). The model that best described

the biomass production was the modified Logistic model with 0.99 R^2 and 0.046 RMSE (Root Mean Square Error). Both human lysozyme production and lactose consumption were described well with the modified Gompertz model (0.99 R^2 and 7.1 RMSE for human lysozyme production and 0.99 R^2 and 3.4 RMSE for lactose consumption).

In conclusion, these studies demonstrated that the biofilm reactor enhanced the production of human lysozyme by *K. lactis* K7. However, purification and scale-up studies are required for industrial production.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

TECHNICAL ACKNOWLEDGEMENTS

CHAPTER 1

INTRODUCTION AND JUSTIFICATION

Lysozyme is an enzyme that catalyzes the hydrolysis of β (1→4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in a peptidoglycan. Peptidoglycans are the major components of the cell walls of both Gram-positive and Gram-negative bacteria (Schleifer and Otto, 1972). Because of its bactericidal activity, lysozyme has been of interest in medicine, cosmetics, and the food industry.

The most important application of lysozyme is as a food preservative. Food safety is an increasingly important public health issue in the world. According to the U.S. Centers for Disease Control and Prevention (CDC, 2011), each year roughly 1 in 6 Americans (or 48 million people) get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases. In the United States, the most common cause of foodborne illness is viral or bacterial contamination (Pigott, 2008). Effective control of many foodborne infections ultimately depends on designing foods and processing systems for greater safety. Today, there is a trend towards the consumption of foods with higher levels of nutrients and nutraceutical compounds without chemical preservatives. The use of antimicrobial compounds for food preservation minimizes the risk of foodborne illnesses and the loss of nutrients. Lysozyme is one of the natural antimicrobial compounds used as a preservative in many foods, such as cheese, fish, meat, fruit, vegetables, and wine (Hughey and Johnson, 1987; Gilby, 2001). Moreover, the antibacterial property of lysozyme has been exploited in a number of other applications, such as eye drops and wound healing creams (Naudi, 2000). Lysozyme has also been used in the treatment of gastrointestinal infections, post-radiation therapy, periodontosis, and dry-mouth (Jolles and Jolles, 1984).

For these purposes, egg-white lysozyme has been commonly used, but it poses immunological problems when consumed by human beings, because individuals can be sensitive to chicken egg have been shown to be allergic to lysozyme produced from

chicken egg (Pichler and Campi, 1992). Therefore, human lysozyme is preferred for food products that will be used by humans, and also it has four times greater activity than egg-white lysozyme (Choi et al., 2004). Commercical production human lysozyme is urgently needed to meet its current demand (Islam et al., 2006).

Human milk is an important source for human lysozyme, but human breast milk is a fairly poor source for commercial production of this enzyme (Yu et al., 2006). Therefore, several approaches for the expression of recombinant human lysozyme have been reported (Muraki et al., 1985; Yoshimura et al., 1987; Choi et al., 2004). Human lysozyme was successfully produced by genetically modified *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, which have been transformed by the insertion of human lysozyme gene (Choi at al., 2004; Iwata et al., 2004). *K. lactis* presents several advantageous properties in comparison to *S. cerevisiae*. These include *K. lactis*'s impressive secretory capacities, crabtree-negative effect in fully oxidative conditions, and food grade status (Swinkels et al., 1993; Gonzales-Siso et al., 2000).

Various fermentation modes, including batch, fed-batch, continuous, etc., should be evaluated to find the best strategy to increase the productivity (Schuler and Kargi; 2002). Some investigators have attempted to increase fermentation productivity using techniques such as immobilized cell reactors, cell-recycle reactors, and hollow fiber reactors. A biofilm reactor is one type of immobilized cell reactors (Demirci et al., 2007). Biofilm formation is a natural process wherein microbial cells attach to a support platform without use of chemicals and form thick layers of cells known as "biofilms" (Qureshi et al., 2005). Biofilm reactors show several advantages over suspended-cell reactors. Biofilm reactors are able to retain 5 to 10-times more biomass per unit volume of reactor, which can increase the production rate. Furthermore, biofilm reactors reduce the risk of washing out cells when operating at high dilution rates during continuous fermentation and eliminate the need for re-inoculation during repeated-batch fermentation. They also assist microorganisms resist the extreme conditions, and supply an easily recovered products from the reactors (Norwood and Gilmour, 2000; Demirci et al., 2007).

To date, human lysozyme production studies using *K. lactis* K7 have been performed by Maullu et al. (1999) and Huang and Demirci (2009). Maullu et al. (1999) conducted shake-flask studies and measured the yield of human lysozyme using cheese whey as its growth media. Huang and Demirci (2009) investigated cultivation conditions to enhance the production of human lysozyme by *K. lactis* K7 using shake flasks and suspended-cell bioreactors. However, flask studies are not scalable and suspended-cell bioreactors may not be the best due to their limited cell population. . Therefore, there is still a need to optimize human lysozyme production using novel approaches and make it cost effective for industrial production. This study was undertaken to enhance the production of human lysozyme by *K. lactis* K7 in biofilm reactors using a solid substrate (plastic composite support), optimum production conditions, and different fermentation modes and strategies in biofilm reactor were investigated. Because biofilm reactors and *K. lactis* K7 are suitable for large scale production, the results of this study can be used for the development of larger scale production of human lysozyme. As a result, industries such as the food, pharmaceutical, and cosmetic industries can use human lysozyme instead of egg-white lysozyme.

CHAPTER 2

LITERATURE REVIEW

The literature review will provide a background about food safety, conventional and novel food preservation methods including the use of natural antimicrobial compounds that minimize the risk of food-borne illnesses. Since the focus of this study is microbial production of lysozyme as a natural antimicrobial compound, information about lysozyme, the sources of lysozyme, and applications of lysozyme will be discussed. Finally, this literature review will provide information about the current methods and limitations of lysozyme production, bioreactors, and biofilm reactors.

2.1 Food Safety

Food safety constitutes a growing public health problem in the world. Because unsafe food causes many acute and life-long diseases, ranging from diarrhoeal diseases to various forms of cancer, foodborne illnesses create an enormous social and economic strain on societies. In 2011, the Centers for Disease Control and Prevention (CDC) estimates that each year roughly 1 in 6 Americans (or 48 million people) get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases (CDC, 2011). The CDC also reported that most of the foodborne disease in the U.S. is caused by *Salmonella* nontyphodial, Norovirus, *Clostridium perfringens*, *Campylobacter* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Toxoplasma gondii*, *E. coli* O157 (CDC, 2011).

It has been estimated that in the U.S. in 1997, diseases caused by food borne illnesses cost up to US $35 billion annually in medical costs and lost productivity (WHO, 2007). Due to the increasing number of food safety problems and rising consumer concerns, governments all over the world have increased the emphasis on improving food safety. It has been reported that 1.8 million people died from diarrhoeal diseases in 2005 alone (WHO, 2007). A great proportion of these cases can be attributed to the contamination of food and drinking water (WHO, 2007). In the United States, the most common cause of

foodborne illness is viral or bacterial contamination of foods (Pigott, 2008). Foodborne illnesses caused by microorganisms are a large and growing public health problem. Most countries, which have systems for reporting cases of foodborne illness, have reported significant increases over the past few decades in the incidence of foodborne illnesses caused by pathogens such as *Salmonella*, *Campylobacter jejuni* and enterohaemorrhagic *Escherichia coli*, and parasites such as *Cryptosporidium*, *Cryptospora*, and *Trematodes* (WHO, 2007).

2.2 Food Preservation Techniques

Providing a safe food product is a complex process requiring proper design of production and control within the food consumption chain. Major food preservation methods for each category are given in Figure 2.1.

Food Preservation Methods		
Inhibition	**Inactivation**	**Avoid Recontamination**
Low temperature storage	Sterilization	Packaging
Reduction of water activity	Pasteurization	Hygienic processing
Decrease of oxygen	Radiation	Hygienic storage
Increase of carbon dioxide	Ohmic heating	Aseptic processing
Acidification	Pressure treatment	
Fermentation	Cooking	
Adding preservatives	Frying	
Freezing		

Figure 2.1 Major food preservation methods (Rahman, 1999).

Major food preservation methods are categorized as inhibition of chemical deterioration and microbial growth; direct inactivation of microorganisms and enzymes; and prevention of recontamination before and after processing (Rahman, 1999). Conventional food preservation methods usually rely on thermal treatment to inactivate microbiological contaminants. However, thermal processing causes physical and chemical changes in the food. The usages of chemical preservatives and naturally occurring antimicrobial compounds are also common food preservation methods (Ramaswamy et al., 2007). Over the last two decades, a number of alternative novel non-thermal intervention processes, such as high hydrostatic pressure processing, pulsed electric field, irradiation and ultraviolet have evolved; specifically to address microbial contamination and satisfy consumer demand for "fresh", minimally-processed foods. Although their performance has some limitations, the lethality of some of the non-thermal methods can be increased by applying them in combination with natural antimicrobials (Ramaswamy et al., 2007).

2.3 Natural Antimicrobial Compounds

The natural compounds may exhibit antimicrobial activity in food as natural ingredients or may be used as additives to other food. Natural antimicrobials can be categorized in six groups including: lacto-antimicrobials, ovo-antimicrobials, phyto-antimicrobials, bacto-antimicrobials, acid-antimicrobials, and milieu-antimicrobials (Naudi, 2000).

Lacto-antimicrobials contain lactoferrin, lactoperoxidase, lactoglobulins, and lactolipids and are found in milk to protect the newborn mammal. Ovo-antimicrobials have been isolated from the egg and their main function in the egg is keeping microorganisms away from the yolk, the nutrient reservoir of the egg. Ovo-antimicrobials are lysozyme, ovotransferrin, ovoglobulin lgY, and avidin (Naudi, 2000). Phyto-antimicrobials are of plant origin. The antimicrobials in plants, such as phyto-phenols, saponins, essential oils, etc., function in the resistance or defense systems against microbial diseases or pests. Microorganisms produce a wide range of components called bacto-antimicrobials that influence the other microorganisms present in the environment. Bacto-antimicrobials contain probiotics, nisin, pediocin, reuterins, sakacins, etc. (Rahman, 1999; Naudi, 2000).

Acids inhibit microbial growth by lowering the pH, affecting the proton gradient across biological membrane, acidifying the cytoplasm, and interfering with chemical transport across cell membrane. Organic acids including acetic acid, lactic acid, sorbic acid, and citric acid, etc., can be formed in food by microbial fermentation or are found naturally in a variety of fruits. Milieu-antimicrobials are traditional antimicrobials and include sodium chloride, sugar, and smoke, all of which are abundant in the milieu (Naudi, 2000).

All natural antimicrobial compounds, which will be used as food preservatives, should be non-toxic, non-allergenic. Moreover, these antimicrobial compounds should be metabolized and excreted, and should not lead to residue build-up in the human body (Naudi, 2000).

2.4 Lysozyme and Its Antimicrobial Activity

Lysozyme is a natural antimicrobial compound, which was discovered in 1922 by Alexander Fleming (Naudi, 2000). Lysozyme is an enzyme, which is a type of murein hydrolases. Murein hydrolases are the common group that digests the peptidoglycan is found in bacteria. Based on their bond specificity, murein hydrolases are classified into three groups as: *i*) glycosidases, which split polysaccharide chains (lysozymes or muramidases and glucosaminidases), *ii*) endopeptidases, which split polypeptide chains, and *iii*) amidases, which cleave the junction between polysaccharides and peptides. While lysozyme splits the acetylmuramic and acetylglucosamine, the glucosaminidases split the link between acetylglucosamine and acetylmuramic acid in the cell wall (Osserman et al., 1974).

Lysozyme consists of 129 amino acids cross-linked by 4 disulfide bridges (Gill and Holley, 2000). Lysozyme can also be called N-acetylmuramideglycanohydrolases or muramidase (Naudi, 2000). The structure of lysozyme is given in Figure 2.2. Lysozyme has two major domains. In Figure 2.2, the alpha domain of the molecule is constituted primarily of alpha helices (magenta), while the beta domain, which is in the color gold,

contains beta sheets and helices (Muraki et al., 1996; Merolla and Fromer, 2008). The active site is within the cleft between the two domains.
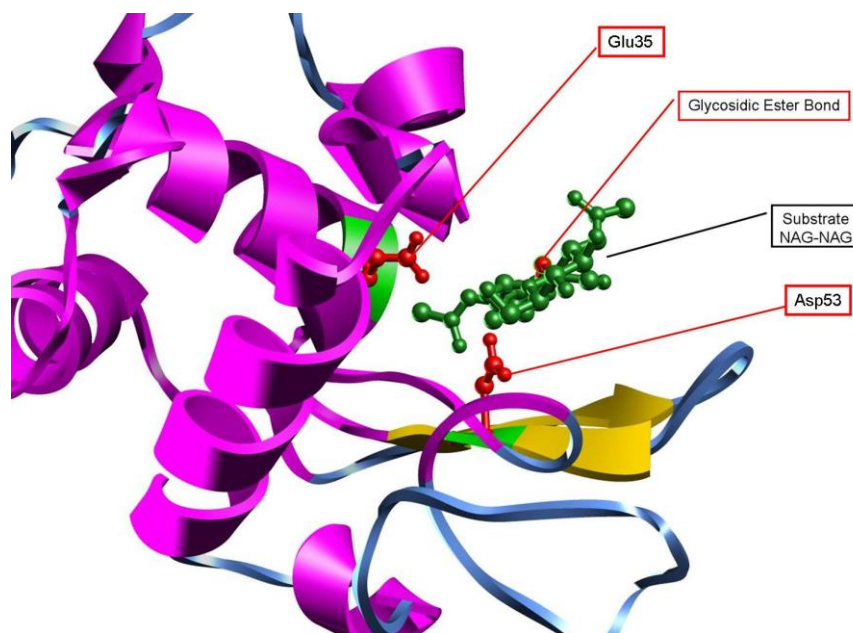
Figure 2.2 The structure of lysozyme (Merolla and Fromer, 2008).

The natural substrate of lysozyme is murein (peptidoglycan), which is a gigantic polymer of $(GlcNAc-MurNAc)_n$ polysaccharide strands cross-linked through short peptide bridges at lactyl groups of the muramic acid residues. Lysozyme catalyzes the hydrolysis (the insertion of a water molecule) of the β (1→4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in murein. This hydrolysis breaks the chain at that point. The high molecular weight murein is hydrolyzed to low molecular weight muropeptides by lysozyme. This causes a punctured cell wall and leads to lysis of the bacterial cell membrane and consequently cell death or growth inhibition (Meyer, 2003; Salazar and Asenjo, 2007). The β (1→4) glycosidic bonds between N-acetylglucosamine sugar (GlcNAc) and N-acetylmuramic acid sugar (MurNAc) to be hydrolyzed during the lysozyme reaction is shown in Figure 2.3.
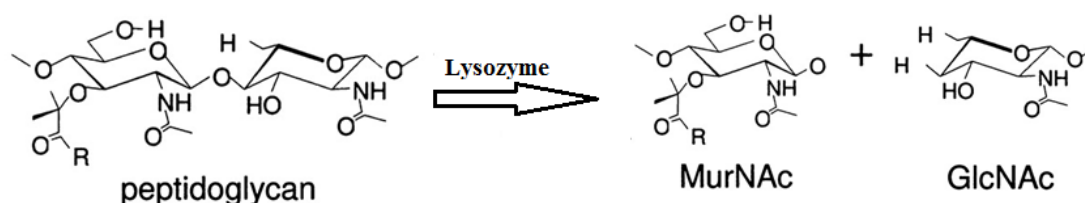
Figure 2.3 Lysozyme substrate description (adapted from the study of Pfeffer et al., 2006).

The amino acid side-chains glutamic acid 35 (Glu35) and aspartate 52 (Asp52) of lysozyme play multiple roles for enzymatic activity. Glu35 protonates the glycosidic oxygen by donating a proton to the glycosidic bond, cleaving the C-O bond in the substrate. Asp52 acts as a nucleophile to generate a glycosyl enzyme intermediate, so it can only react with a water molecule, to give the product of hydrolysis and leaving the enzyme unchanged (Vocadlo et al., 2001).

Lysozyme is more effective against Gram-positive bacteria than Gram-negative bacteria. Gram-positive cells have a thick cell wall composed of several layers of peptidoglycan (responsible for thick rigid structure), which are associated by a small group of amino acids and amino acid derivatives, forming the glycan-tetrapeptide. Towards the outside of the wall, peptidoglycan is connected to teichoic acids and polysaccharides. The reason of the differencein the degree of susceptibility to lysozyme of Gram-positive bacteria may be detailed composition of peptidoglycan in terms of the amount of teichoic acids, which may vary among the species. Teichoic acid is negatively charged which may bind the lysozyme and prevent its motion and diminish its hydrolytic action on the peptidoglycan (Meyer, 2003).

On the other hand, Gram-negative bacteria have a complex cell wall composition containing an outer membrane and a middle membrane. The outer membrane is composed of lipopolysaccharides, lipoprotein, and phospholipids, which covers the inner,

rigid peptidoglycan layer. The outer membrane has limited transport and barrier functions. Beneath the outer membrane is the middle membrane, composed of a thin layer of peptidoglycan or mucopeptide embedded in the periplasmic materials that contain several types of proteins. The resistance of Gram-negative bacteria to many enzymes including lysozyme, hydrophobic molecules (SDS and bile salts), and antibiotics (penicillin) is due to the barrier property of the outer membrane. Lipopolysaccharide molecules also have antigenic properties (Ray, 2004; Salazar and Asenjo, 2007). The spectrum activity of lysozyme can be broadened to Gram-negative bacteria when lysozyme is used in combination with other compounds. Gram-negative bacteria become susceptible to lysozyme after outer membrane of bacteria has been disrupted by compounds such as EDTA (cation chelating agent), aprotinin, organic acids, detergent (e.g. Triton X-100) or when lysozyme is conjugated to carbohydrates or when lysozyme is chemically modified by linking with a saturated fatty acid or hydrophobic peptide (Naudi, 2000; Salazar and Asenjo, 2007). Moreover, cell permeabilization by high hydrostatic pressure makes the Gram-negative bacteria susceptible to lysozyme (Salazar and Asenjo, 2007).

Lysozyme is not an effective on yeast by enzymatic activity, because yeast wall is composed mostly of mannoprotein and fibrous β (1→3) glucans with some branches of β (1→6) glucans. Therefore, enzyme systems for yeast cell lysis should include mixtures of β (1→3) glucanase, β (1→6) glucanase, and additionally protease, mannanase and chitinase, which act synergistically for lysing the cell wall (Salazar and Asenjo, 2007).

The activity of lysozyme on viruses is associated with the positive charge of lysozyme (Naudi, 2000). Lysozyme acts as a cationic protein that induces cell lysis via puncturing of the cell membrane through a protein phospholipid interaction mechanism. The lysozyme protein may also activate autolysin enzymes in the bacterial cell (Meyer, 2003). Ibrahim et al. (2001) also reported that while heat denaturation of lysozyme progressively inactivates the enzyme, its antimicrobial action on Gram-negative bacteria increases. Moreover, they have also reported that hen egg white lysozyme exhibits a broad spectrum

of antimicrobial activity after digestion with clostripain. This lysozyme peptide was found to be active against both Gram-positive and Gram-negative bacteria and the fungus *Candida albicans* (Ibrahim et al., 2001; Ibrahim et al., 1996). Düring et al. (1999) showed that native and heat-treated enzymatically inactive lysozyme caused similar in-activation levels on a particular *E. coli* strain. The antibacterial properties of denatured lysozyme may be related to the cationic nature of the peptide in combination with conformational changes leading to increased hydrophobicity. These characteristics increased the antimicrobial properties of several peptides. Specific peptides with cationic properties and without enzymatic activity derived from hen egg white and T4 lysozyme were also found to have antimicrobial activity (Düring et al., 1999; Pellegrini et al., 1997).

2.5 Sources of Lysozyme

Lysozymes are ubiquitous in both animal and plant kingdoms, and play important roles in natural defense mechanism (Naudi, 2000). Lysozyme has been found in bacteria, bacteriophage, insect, plant, and animals (Jolles, 1996). Although lysozymes from various sources exist, they differ in molecular characteristic, amino acid sequence, active center location, and enzymatic activity (Mulvey et al., 1974).

In the infection of bacterial cells by phages, they use bacteriophage lysozymesto favor the release of virions by lysis of the host cells or to facilitate the infection. Lysozyme has been described in bacteria, such as; *Arthrobacter crystalopoites*, *Bacillus subtilis*, *Bacillus thuringensis*, *Enterococcus hirae*, and *Streptomyces griseus*. In bacteria, the physiological function of lysozyme includes the autolysis of the cell, role during enlargement and division of the cell wall by the controlled splitting of bonds in the murein sacculus. Lysozyme has also been found in plants, such as; papaya, turnip, rubber tree, and barley. However, plant lysozymes seem to act more as a chitinase than as a lysozyme. Fungal cell walls contain chitin and chitinases are used by plants to combat fungal pathogens. Moreover, it was reported that lysozyme activities in plants have a sharp optimum at pH 4.5, while the chitinase activities have a broad optimum at pH 4.5-6.5 (Jolles, 1996). Jacobsen et al. (1990) found that lysozyme activity in barley is 10^{-4}

times that of chicken egg-white lysozyme. Insect lysozyme has a role in immune defense against bacteria and has been found in bees, dragon flies, cockroaches, true bugs, etc. (Jolles, 1996).

Naudi (2000) reported that one chicken egg contains about 0.3-0.4 g of lysozyme. Body fluids such as human tears and saliva contain 2.6 mg/ml, and 0.13 mg/ml of lysozyme, respectively (Naudi, 2000). Maga et al. (1998) reported that lysozyme is present at 400 μg/ml in human milk, which is higher than other mammalian milk such as cow milk (0.13 μg/ml), goat milk (0.25 μg/ml), and sheep milk (0.10 μg/ml). The lytic activities of several of lysozyme origins are given in Table 2.1.

Egg-white lysozyme is currently the major commercial source of lysozyme. However, human lysozyme has advantages over egg-white lysozyme. Human lysozyme is filtered through the kidney, reabsorbed by the proximal tubular cells, and catabolized in the lysosomes because of that it is not stored in the body and, therefore, does not cause any toxic effect (Naudi, 2000). However, among those with allergies to eggs the egg white lysozyme causes development of specific IgE antibody titers in the human (Naudi, 2000). American College of Allergy, Asthma & Immunology (2010) reported that 0.2% of population in the U.S. (more than 600,000 people) are allergic to egg. Sequence analysis shows that lysozyme from chicken egg white is only 60% identical with human lysozyme. The sequence differences generate functional differences in human and chicken egg-white lysozyme (Mulvey et al., 1974). These differences indicate that the antibodies against human and chicken egg-white lysozyme are not the same (Faure and Jollès, 1970). Therefore, it has been reported that human lysozyme is safer and less antigenic than egg-white lysozyme (Morita et al., 1995).

Furthermore, through a broad interval of pH, human lysozyme is more active in hydrolyzing the cell wall of bacteria than is egg-white lysozyme. It was reported that the activity of human lysozyme is about four times as great as that of egg-white lysozyme (Mulvey et al., 1974). Human lysozyme also has more arginine residues than egg-white

lysozyme (Naudi, 2000). Substrate binding mechanism and the physical properties of enzymes are dependant on the arginine residues (Masuda et al., 2005; Reid et al., 2006).

Table 2.1 Activities of lysozyme of various origins*.

Lysozyme	Lytic activity (%)
Hen	100
Japanese Pheasant	123
Turkey	176
Rabbit	204
Pig	245
Rat	255
Human	396

* Jolles, 1996.

2.6 The Factors Affecting Lysozyme Activity and Activity Measurement Techniques

2.6.1 The factors affecting lysozyme activity

Lysozyme is a heat stable enzyme because of four disulfide bonds in its structure (Naudi, 2000). Hughey and Johnson (1987) also reported that lysozyme is heat resistant at low pHs. Yang et al. (2011) reported that human lysozyme activity is not affected, when the temperature varied from 25 to 60°C, but the activity was slightly higher at around 40°C. Lysozyme is more stable against heat in solutions containing other solutes such as sugar and polyols, which serve as a protector for lysozyme against heat (Naudi, 2000).

Salton et al. (1958) stated that lytic activity is at its maximum level, when the ionic strength is around 0.1 and in the presence of potassium salts (Salton, 1958). While lysozyme is inactive in distilled water, it is activated by low concentrations of salt and its activity becomes stable in 20% alcohol (Naudi, 2000). Polysaccharides with carboxylic acid and sulphonic acid groups are inhibitory to lysozyme activity, but these inhibitory effects can be minimized by addition of 1-0.6% salt (Cunningham et al., 1991).

Moreover, it has been reported that metal ions bind carboxyl groups of Glu 35-Asp 52 in active side of enzyme (Cunningham et al., 1991). Some bacteria can also produce lysozyme inhibitors. For example; a peri-plasmic protein (Ivy) is produced by *Escherichia coli* and protects *it* against lysozyme in the presence of an outer membrane permeabilizing compound like lactoferrin (Vanderkelen et al., 2011). It has been also reported that covalent attachment of caffeic acid and cinnamic acid to the lysozyme reduces lytic activity of enzyme (Meyer, 2003). While Ibrahim et al. (2001) reported enhancement of lysozyme-inorganic salt lysis by proteases, Wilkens et al. (1982) reported that the loss of insoluble cell wall peptidoglycan by lysozyme treatment was pH independent and proteases have no effect on activity.

The rate of lysozyme catalysis depends on pH of the evironment. The egg-white lysozyme activity is maximum at around pH 5 and the activity decreases when the pH is <3.8 and >6.7 (Naudi, 2000). Lysozyme activity is also affected by salt concentration and this affects the pH spectrum of activity. Yang et al. (2011) reported that a salt concentration of 0.05 M provided lysozyme greater activity over a broader range of pH values (pH 5-9) than a salt concentration of 0.1 M. Cunningham et al. (1991) reported that lysozyme is heat stable in acidic solutions (pH 5.29, 100°C, and 30 min). However, the stability rapidly decreases in alkaline solutions andis destroyed at pH 8 at 65°C in 15 min (Cunningham et al., 1991).

2.6.2 Lysozyme activity measurement methods

Lysozyme activity can be measured by several approaches such as viscometric, lysoplate, and turbimetric methods.

i) Viscometric assay

The viscometric assay measures the changes in the viscosity during depolymerization of peptidoglycan, which is subjected to test samples, including lysozyme. This method provides a non-linear measure of depolymerrization, so its correlation with a standard is difficult (Jolles, 1996).

ii) Lysoplate method

The lysoplate method measures the decrease in the turbidity of cell walls, after the treatment with lysozyme. This method includes heat killed *Microccocus lysodekticus* cells in a buffer mixed with 1% molten agar. After solidified, wells are created on the agar. The samples are inoculated into these wells and incubated for a certain time, and then the occurrence of clear zones is an indication of lysozyme activity. Although there is a linear relation between clear zones and lysozyme activity, there are disadvantages, such as the composition of agar may have an inhibitory effect on enzyme activity and there maybe diffusional problems of samples through the agar (Gupta et al., 1987).

iii) Turbimetric method

The turbimetric method uses absorbance to measure the speed of lysis of lyophilized *Micrococcus lysodeikticus* cells, and the speed corresponds to the units of the lysozyme (Richard et al., 1965). Each unit is defined as the amount of enzyme able to decrease 0.001 in the optical density of lyophilized *M. lysodeikticus* cell suspensions. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 $Absorbance_{540nm}$ change per minute. The slope of the linear regression line refers to the speed of lysis of *M. lysodeikticus* suspension, and the slope estimates the concentration of human lysozyme in unit per milliliter (Richard et al., 1965).

Since the catalytic activity of lysozyme is dependent on the pH, ionic strength, substrate concentration, and sodium and potassium concentrations of the reaction mixture, these factors should be considered in the analysis of lysozyme. Since each microorganism has different susceptibility to lysozyme as explained before, a standard microorganism should be used for activity analysis to indicate a comparable unit of lysozyme. *Micrococcus lysodeikticus* is reported to be highly sensitive to lysozyme (Salton, 1958). Moreover, the lysozyme assays may also be affected by the sample medium. For example, biological fluids, such as salvia, may include arginine, which increases lysozyme activity (Jenzano and Lundblad, 1988). The lysoplate method can also overestimate the activity based on the properties of the sample, which cause

diffusional difference. Therefore, turbidimetric method provides more standard results. For example, Osserman and Lawlor (1966) reported the human lysozyme activity is 3-4 times higher than egg lysozyme activity based on turbidimetric method, but it is 12 times higher based on lysoplate method. This difference may be due to differences in the diffusability of the two enzymes in the agar medium, possibly reflecting differences in polymer formation, or interactions with acidic groups in the agar matrix (Osserman and Lawlor, 1966).

The turbidimetric method hasadvantages, because the incubation time is shorter than the lysoplate method and there is no diffusional problem. The turbidimetric method allows detection of enzyme activity in the range of 1 to 100 ng/ml (Jolles, 1996). Mörsky (1983) reported that the absorbance change during turbimetric method is linear at wavelengths 500-600 nm. Mörsky (1983) also determined the optimum pH as 6.2 with 50-75 mM phosphate buffer. Therefore, in turbidimtric method, *M. lysodeikticus* cell suspension is prepared with potassium buffer. Change in phosphate buffer concentration affect the rate of the cell lysis. Jenzano et al. (1986) reported the effects of salt solution on turbidimetric and lysoplate method. Parry et al. (1965) stated that the clearance due to settling of cell suspension is negligible during 20 min of turbidimetric assay. They also reported that high concentration of lysozyme (more than 30 μg) shows a decrease in rate of lysis after 30 seconds. Therefore, when there is a decrease in linearity, the samples are needed to be further diluted. The linearity also can be affected by the concentration of *M. lysodeikticus* because as the time passes there will be still *M. lysodeikticus* with not enough enzyme to degrade it. Therefore, *M. lysodeikticus* concentration should also be standardized. The reproducibility of the assay may also be poor due to lack of homogeneity of cell suspension.

Moreover, all these methods can be applied to all autolytic enzymes. A specific lysozyme enzyme assay can be established by the determination of the release of reducing muramyl ends (Jolles, 1996).

2.7 Applications of Lysozyme

Because of its bactericidal, anti-inflammatory, and antiviral activities, lysozyme has been of interest in the food, pharmaceutical, and cosmetic industries. Lysozyme is an attractive natural antimicrobial compound, because it is harmless to humans and is already produced naturally in human body and many foods, such as; eggs, milk and turnips. The FAO/WHO and several countries including Austria, Belgium, Denmark, Finland, France, Germany, Italy, The United Kingdom, Spain, and Australia have acknowledged the non-toxicity of lysozyme (Naudi, 2000).

Lysozyme is used as a food preservative in foods such as poultry, shrimp, sausage, and sake. Cunningham et al. (1991) reported that lysozyme has bactericidal activity against bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Bacillus cereus*, and *Staphylococcus aureu*s; all of which are foodborne pathogens. Lysozyme also can be used in hurdle food preservation techniques, such as in heat treated products to reduce the thermal requirement, and in inactivation of spores by high hydrostatic pressure (Lopez-Pedemonte et al., 2003; Cunningham et al., 1991). The combinations of lysozyme-lactoferrin and lysozyme-EDTA are used to increase the susceptibility of Gram-negative bacteria to lysozyme. Lactoferrin and EDTA interfere with the lipopolysaccharide layer of Gram-negative bacteria by increasing its permeability, which allows greater accessibility of lysozyme into bacterial cells (Naudi, 2000).

In dairy products, lysozyme is used for quality aspects and the control of the growth of foodborne and food spoilage microorganisms. Butyric acid bacteria are commonly found in milk and if not properly eliminated, they metabolize lactic acid into butyric acid, acetic acid, carbon dioxide, and hydrogen that can cause cheese to crack and make cheese unpalatable. In the cheese making process, lysozyme is added into milk. At the pH of the milk, lysozyme is positively charged, and when the curd forms, lysozyme is electrostatistically attracted and adsorbed to the negatively charged casein molecules forming curds. After separation of whey from the curd, 90% of the lysozyme activity remains with the curd. While cheese is aging, the center of the cheese develops anaerobic

conditions and the contaminating spores of *Clostridium tyrobutyricum* begin to germinate. In the vegetative form, the organism is susceptible to the lytic action of lysozyme. Lysozyme at 21 mg/L can kill 99% of vegetative cells of *C. tyrobutyricum* within 24 h incubation at 25ºC (Naudi, 2000). Therefore, it was reported that lysozyme at 20 to 35 mg/L successfully replaced commonly used chemical preservatives, such as; formaldehyde, nitrate, and hydrogen peroxide. The advantage of usage of lysozyme in cheese production is that lysozyme inhibits spoilage organisms, while not interfering with the starter culture (Naudi, 2000). While the growth of coliform isolates was inhibited by 1000 units/ml of lysozyme, the activity of starter cultures, in Gouda cheese, was not inhibited by lysozyme at a concentration up to 2500 units/ml (Bester and Lombard, 1990). Therefore, lysozyme can be used in the production of Gouda cheese upto 2500 units/ml to control coliform bacteria growth. *Listeria monocytogenes* is a concern in soft cheese and milk, because *L. monocytogenes* is a food borne pathogen that can multiply at refrigeration temperatures (Naudi, 2000). Johnson (1994) reported that the growth of *L. monocytogenes* can be delayed by lysozyme at 20 to 200 mg/l (Naudi, 2000).

With the trend towards the consumption of foods with high levels of nutrients and nutraceutical compounds without chemical preservatives, lysozyme has been used to supplement sulfur dioxide (SO_2) in wine to prevent malolactic fermentation and spoilage (Gilby, 2001). Naudi (2000) also reviewed the potential application of lysozyme in beer. Lysozyme at 50 ppm delayed the growth of common spoilage bacteria (*Lactobacillus brevis* and *Pediococcus damnosus*) in unpasteurized beer containing 5% alcohol at pH 4.7 (Naudi, 2000). Lysozyme has also been used in the production of yeast autolysate. Intact yeast cells were treated with lysozyme, which increase the release of nitrogen and proteins during incubation (Cunningham et al., 1991). In the wine industry, yeast autolysis is applied in the production of sparkling sherry and white wines. In addition to the wine industry, yeast extract, which is the product of yeast autolysis, is also used as an additive in the production of meat paste, meat pie filling, soups, sauces, and snacks. Yeast autolysate is a good source of nutrients such as proteins, vitamins, fiber, and micronutrients (Dharmadhikari, 2011).

In fish and sea food products, lysozyme has been applied to prevent edwardsiellosis, which is a bacterial infection caused by *Edwardsiella tarda*. Fruits and vegetables are low acid foods and are very susceptible to spoilage by *Clostridium botilinum* and *Listeria monocytogenes* (Naudi, 2000).

Lysozyme has also been used in chewing gum formulations to prevent periodontitis-causing bacteria, and gingivitis associated inflammation and bleeding (Naudi, 2000). Moreover, lysozyme has been used in baby formula to aid the immune system (Hughey and Johnson, 1987). Human lysozyme can be used for these applications due to its immunological advantages.

The pharmological and therapeutic applications of lysozyme are based on its antiviral activity, its ability to control the growth of susceptible bacteria and to modulate host immunity against infections and depressions of immune responses (Naudi, 2000). The antiviral activity of lysozyme is associated with the positive charge of lysozyme (Naudi, 2000). The presence of lysozyme has important roles in the treatment of various bacterial or viral (zona, herpes zoster) infections, colitis, various pains, allergies, and inflammations (Jolles and Jolles, 1984). Therefore, during recent years, lysozyme has been used either alone or in addition to a series of other components such as various antibiotics (tetracyclin, bacitracin), enzymes (α-amylase, papain), and vitamins, etc. in the pharmaceutical industry. Lysozyme has analgesic properties and has been used as a potentiating agent in antibiotic therapy. Because of the antibacterial property of lysozyme, lysozyme has been used in eye drops and wound healing creams. Lysozyme also shows antibacterial activity against mouth bacteria and has been used in periodontosis and in the treatment of dry-mouth. Lysozyme has also been used in treatment of gastrointestinal infections, lung cavity tuberculosis, and skin infections. EDTA-tris-lysozyme solutions are effective in the treatment of coliform infections of the bladder in humans. Precipitation of viral particles has been achieved by lysozyme application and it is able to control influenza viruses in experimental systems and viral

infections in cattle, horses, and pigs (Jolles and Jolles, 1984). The anti-inflammatory action of lysozyme makes it effective agent in the reduction of cancer related inflammatory processes, recovery, and healing of ulcers in artheriopathy, post-radiation therapy. Lysozyme is used in the prophylaxis and therapy of leukopenia induced by antiblastic and ionizing radiation. Moreover, lysozyme has been shown to inhibit the growth of HIV-1 in vitro (Islam et al., 2006; Jolles and Jolles, 1984; Jolles, 1996).

2.8 Production of Lysozyme

Commercial source of lysozyme is currently chicken egg white. The classical method of lysozyme production involves the repeated salt crystallization of the enzyme in chicken egg white. This process requires a week for the enzyme to be obtained with high purity. This separation process recovers 60-80% of the lysozyme present in egg white (Naudi, 2000). Ion-exchange and affinity membrane chromatography and ultrafiltration are other methods for the purification of lysozyme from chicken egg white (Gemili et al., 2007). Nowadays, scientists have created transgenic microorganisms that produce lysozyme. High levels of hen egg lysozyme have been produced using genetically modified *Pichia pastoris* and *Aspergillus niger* (MacKenzei et al., 1994; Naudi, 2000).

Recombinant human lysozyme has been expressed in plants, mice, bacteria and yeast. In the study of transgenic mice, human lysozyme concentration was found as 1.4 mg/ml in mice milk (Yu et al., 2006). The transgenic yeast, *Acremonium chrysogenum*, was able to produce 40 mg recombinant human lysozyme /L fermentation medium. However, both human lysozyme secreted from mice and *A. chrysogenum* showed some non-uniformity of human lysozyme (Huang et al., 2002). A synthetic human lysozyme gene was expressed in *Escherichia coli* and *Bacillus subtilis* (Muraki et al., 1985; Yoshimura et al., 1987). However, the human lysozyme produced either in *E. coli* or *B. subtilis* was inactive due to the formation of incorrect disulfide bonds (Choi et al., 2004). Although human lysozyme has been successfully expressed in rice seeds, carrot, and tobacco leaves, microorganisms are more advantageous hosts than plants because of high growth rates (Porro et al., 2005; Wilken and Nikolov, 2006).

Choi et al. (2004) investigated lysozyme production by *Saccharomyces cerevisiae* and obtained 74.5 units/ml of human lysozyme. However, *S. cerevisiae* has various disadvantages for continuous production such as the retention of product within the cell, hyperglycosylation of secreted protein, and Crabtree-positive. Crabtree-positive yeasts form ethanol under aerobic conditions, thus reducing ATP and biomass yield compared to Crabtree-negative yeasts. Iwata et al. (2004) found that *Kluyveromyces lactis* is more suitable as a host cell for the recombinant production of human lysozyme than *S. cerevisiae*.

K. lactis K7 is genetically engineered yeast, which is a transformant, obtained by complementing the his3-mutation of *K. lactis* WM37 strain. *K. lactis* K7 includes *K. lactis* GAL 7 promoter, the cDNA sequence coding for human lysozyme fused in-frame to *K. lactis* killer toxin signal sequence. The lysozyme produced by *K. lactis* K7 is secreted through the cell wall into culture medium. The secretion sequence is endoplasmic reticulum- Golgi - vesicle-cell surface (Schekman, 1982).

K. lactis has attractive properties, including its GRAS (generally regarded as safe) status, molecular genetic accessibility using both integrative and highly stabilized episomal vector systems, and excellent fermentative characteristics allowing for large-scale industrial applications. Moreover, its capability of exporting the zymocin complex, which is a large heterotrimeric (αβγ) N-glycosylated protein complex, suggests that *K. lactis* is suitable for secretion of large heterologous proteins (Stark and Boyd, 1986; Schaffrath and Breunig, 2000).

One of the properties of *K. lactis* favorable for recombinant protein expression is its so-called Crabtree-negative phenotype (Verduyn, 1991). *K. lactis* has resistance against antimycin A on glucose (*Rag* phenotype). Rag negative mutants depend on oxidative phosphorylation and are unable to grow on glucose when respiration is blocked (Schaffrath and Breunig, 2000). Moreover, *K. lactis* maintains species-specific

phenomena, such as the DNA-killer system; analysis of which promises new insights into linear plasmid biology, mechanisms of microbial competition, and vector development. Supported by the developments of powerful molecular genetic tools, *K. lactis* has been successfully established as an alternative system for biomass-directed industrial applications and large-scale expression of biotechnically-relevant gene products (Schaffrath and Breunig, 2000). Up to now, *K. lactis* has been used as an effective host cell in the productions of interleukin-1β, hepatisis B surface antigen, human serum albumin, α-galactosidase, and glucoamylase (Merico et al., 2004). Merico et al. (2004) studied the effect of physiological and cultural conditions on heterologous protein production and as a result of batch experiments they pointed out the importance of maintaining pH at 6.0 for growth and for protein production: this condition results in the complete utilization of the carbon source and, as a consequence, in a higher biomass level. In a comparison of carbon sources, the final biomass produced was highest value on glucose; however lactose allowed greater heterologous protein production. Merico et al. (2004) also observed that enriched culture medium with casamino acids provided higher biomass production.

Rossolini et al. (1992) studied application of targeting exogenous DNA by the use of integrative vectors in *K. lactis* transformants and comparison of lysozyme activities of these transformants and medium effect on activity. They analyzed human lysozyme generated by three *Saccharomyces cerevisiae* and seven *K. lactis* transformants. The *Saccharomyces* strains secreted the heterologous protein in equal amounts and the amount of secreted protein was higher in the medium containing carbon source, peptone, and yeast extract. Different *K. lactis* transformants showed varying amounts of secreted lysozyme. Rossolini et al. (1992) concluded that *K. lactis* K7 has the highest lytic activity when compared to other *K. lactis* transformants and *S. cerevisiae*. They also reported that the medium containing galactose, yeast nitrogen base and amino acid is more effective for human lysozyme production by *K. lactis* K7 than the medium containing galactose, peptone, and yeast extract. Iwata et al. (2004) also studied the efficient secretion of human lysozyme from *K. lactis* and concluded that addition of 1% casamino acid to

medium enhanced the secretion of human lysozyme. Rossolini et al. (1992) reported that
K. lactis K7 was still able to produce the same amount of lysozyme after growth for over
25 generations. This finding shows the genetic stability of *K. lactis* K7 for large scale
production.

To date, human lysozyme production using *K. lactis* K7 has been studied by Maullu et al.
(1999) and Huang and Demirci (2009). Maullu et al. (1999) conducted shake-flask
studies using cheese whey (pH 4-8) as its growth media at 28ºC. Huang and Demirci
(2009) investigated cultivation conditions to enhance the production of human lysozyme
by *K. lactis* K7 using shake-flasks and a suspended cell bioreactor. Huang and Demirci
(2009) concluded that greater human lysozyme production can be achieved under oxygen
limitation and acidic environments at both the 250-ml shake-flask and the bench-top
bioreactor at 25ºC. These authors produced human lysozyme at a cost of$1.26/25,000
units, while egg-white lysozyme costs $0.02/25,000 units. Therefore, there is still a need
for improvement to increase the yield of human lysozyme.

2.9 Modes of Fermentation Methods

Bioreactors include mechanical vessels in which organisms are cultivated in a controlled
manner and substrates are converted or transformed via specific reactions into products.
The bioreactors can be operated as batch, fed-batch, and continuous modes (Williams,
2002).

2.9.1   Batch fermentation

Batch bioreactors are closed fermentations. Figure 2.4 shows the bioreactor system and
its control tools. Bioreactors have systems to control environmental conditions to provide
the optimum growth parameters for maximum production. These environmental
conditions include temperature, controlled by the water jacket; pH, controlled by acid or
base addition; dissolved oxgen; controlled by the gas inlet through the sparger. Each
microorganism has optimum growth parameters and medium requirements for maximum

production. Therefore, control of environmental conditions play a crucial role in the fermentation processes.

The fermentation starts with medium sterilization and inoculation of microorganisms. Then, the fermentation process continues with lag, log, and stationary phases. At the end of fermentation, the reactor is drained for the product recovery (Pometto and Demirci, 2006).

The advantages of batch fermentation include reduced risk of contamination or cell mutation, lower capital investment when compared to continuous processes for the same bioreactor volume, more flexibility with varying product/biological systems, and higher raw material conversion levels resulting from a controlled growth period.
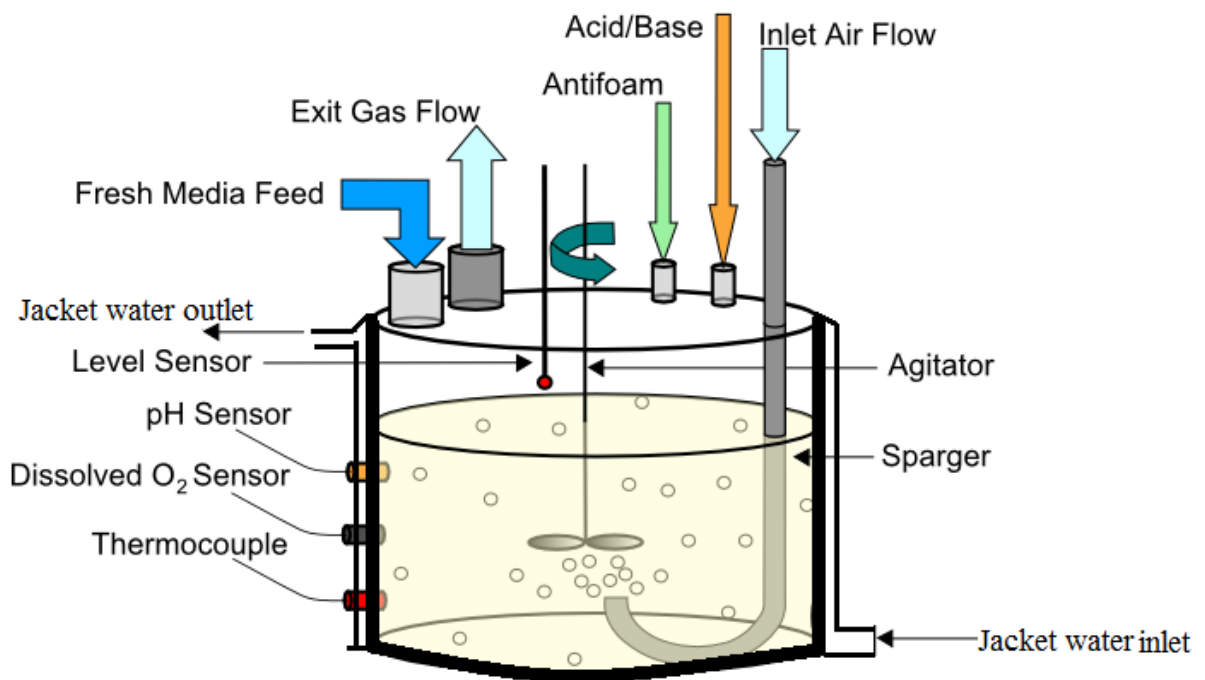
Figure 2.4 Batch and fed-batch bioreactor system (Adapted from Henson et al., 2013).

Archer et al. (1990) evaluated batch fermentation for hen-egg lysozyme production by *Aspergillus niger* with different fermentation mediums. As a result, maxiumum secreted level of lysozyme were achieved in the 50-100 mM range of added phosphate buffer although reduction in the mycelial yields was observed. MacKenzei et al. (1994) also produced secreted lysozyme at higher levels at 20-25°C than at 30-37°C and reported that soluble starch is a better carbon source for this fermentation than maltose, glucose, and xylose. Moreover, Choi et al. (2004) evaluated batch fermentation at different conditions for human lysozyme production by recombinant *Saccharomyces cerevisiae* and concluded that pH shift from 5.5 to 3 at stationary phase can incresea the production from 42.3 to 74.5 U/ml.

However, batch fermentations have many disadvantages, which include lower productivity levels due to the substrate limitations, time for filling, heating, sterilizing, cooling, emptying, and cleaning the reactor; increased focus on instrumentation due to frequent sterilization; greater expense incurred in preparing several subcultures for inoculation; higher costs for labor and/or process control for this non-stationary process. Batch fermentation can be applied for products that must be produced with minimal risk of contamination or organism mutation and operations in which only small amounts of product are produced (Williams, 2002).

2.9.2　Fed-batch fermentation

Fed-batch bioreactors are similar to the batch reactors except they have a portal to feed additional carbon to microorganisms in the bioreactor (The feeding portal to the reactor is shown with the blue arrow as fresh media in Figure 2.4) during the fermentation process (Pometto and Demirci, 2006). These systems provide a number of advantages. Extra substrate availability in fed-batch bioreactors enables higher production levels and yields. Fed-batch bioreactors also provide increased opportunity for optimizing environmental conditions of the microorganisms in regard to the phase of growth or production and age of the culture. Moreover, nearly stationary operation can be achieved in fed-batch bioreactors.

Park et al. (1992) evaluated fed-batch fermentation for recombinant protein production by *Bacillus subtilis* and and concluded that cell concentration, recombinant protein (β-galactosidase) level, and the specific enzyme expression level were increased from 19 to 184 g/L, 18.3 to 129 U/mL, and 3.2 to 5.7 U/mg protein, respectively, in fed-batch fermentation with controlled glucose concentration at 1 g/L as compared to conventional fed-batch culture. The authors utilized a concentrated glucose solution of 500 g/L simultaneously with alkali solution addition. Hardjito et al. (1993) also enhance the recombinant protein production by fed-batch fermentation. They stated that the time course of the glucose levels was the most significant factor on volumetric productivity. An average glucose feed rate of 1.31 g glucose/h provided both the maximum β-galactosidase production rate of 831-950 units ml /L/h and the maximum cell production rate of 0.520-0.524 mg/ml/h.

The disadvantages of fed-batch bioreactors are: lower productivity levels due to time-consuming procedures for sterile filling, media preparation, emptying and cleaning the reactor, and greater expenses in labor and/or dynamic process control for the process (Williams, 2002).

2.9.3 Continous fermentation

Continuous bioreactors involve addition of fresh medium continuously to the bioreactor, while spent culture medium, cells, and products are continually leaving the reactor (Pometto and Demirci, 2006). Figure 2.5 shows the continuous reactor with the blue arrows, which shows the inlet of the fresh medium and the outlet of the fermentation medium, which includes unconsumed nutrient sources and cells. The result is continuous productivity and output. The advantageous of continuous fermentations are: increased potential for high productivity; automation; reduced labor expense due to automation; less non-productive time expended in emptying, filling and sterilizing the reactor; consistent product quality due to invariable operating parameters; decreased toxicity risks

to staff due to automation; reduced stress on instruments due to repeated sterilization; and 10 to 100 times smaller bioreactor volume than the batch and fed-batch bioreactors.

Blondeau et al. (1994) studied the continuous production of heterologous human serum albumin by *Kluyveromyces lactis* and achieved the highest productivity (75 mg/l per h) and concentration (62 mg/l) with a dilution rate of 0.12 h^{-1} and with 38 g/l dry weight. Kiers et al. (1997) also studied continouos cultivation of *K. lactis* and reported that the growth of glucose-limited chemostat cultures (D = 0·05–0·40 h^{-1}) was entirely respiratory, without significant accumulation of ethanol or other metabolites in aerobic conditions. Mainwaring et al. (1999) evaluated continuous fermentation for hen egg white lysozyme production by *Aspergillus niger* strain (B1) in glucose-limited chemostat culture at a dilution rate of 0.07 h^{-1} and achieved maximum production (9.3 mg/g) at pH 4.5, which provided stable morphology of culture.
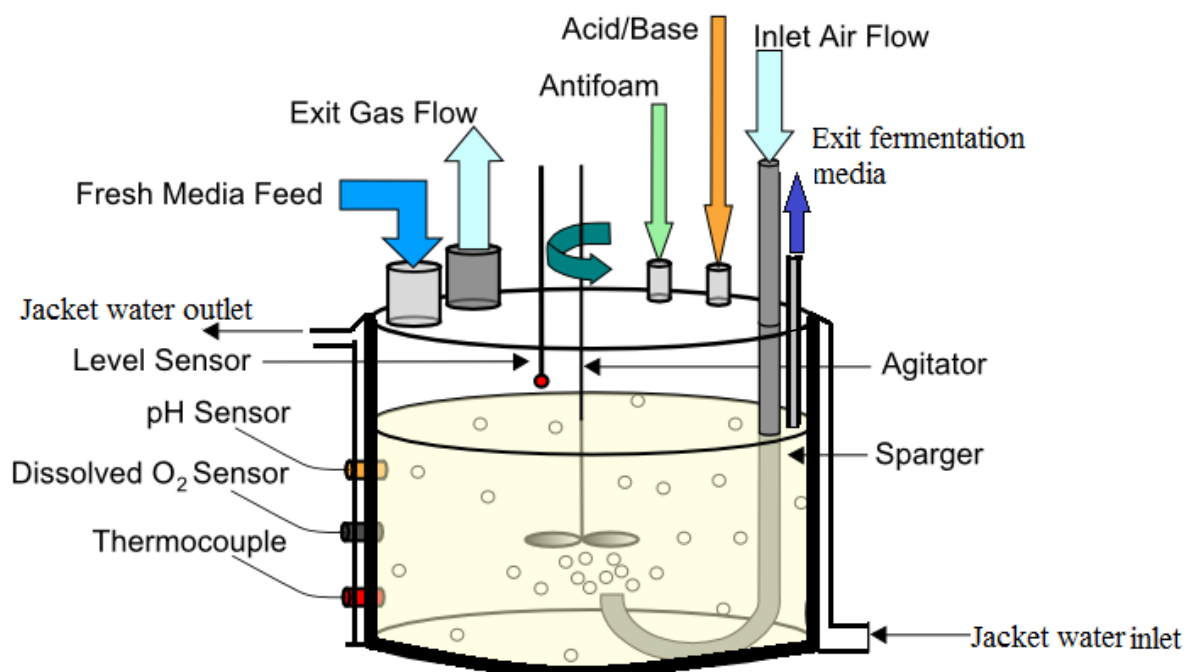
Figure 2.5 Continuous fermentation system (Adapted from Henson et al., 2013).

The disadvantages of continuous fermentations include: minimal flexibility (since only slight variations in the process are possible in throughput, medium composition, oxygen concentration and temperature); mandatory uniformity of raw material quality (necessary to ensure that the process remains continuous); higher investment costs in control and automation equipment; and increased expenses for continuous sterilization of the medium. Because of the advantages of continuous bioreactors, the industry is trying to increase the use continuous bioreactors (Pometto and Demirci, 2006; Williams, 2002). Regardless of the mode of fermentations, an increased biomass concentration in the reactor is required for high productivity. This can be achieved by cell-recycled, hollow-fiber, and immobilized-cell reactors.

2.10 Novel Bioreactors

High microbial population in bioreactors is essential to increase the productivity. In order to that, several approaches have been used used. Following are few of them:

2.10.1 Cell-recycled bioreactors

Bioreactors equipped with mechanical devices can be used to separate cells from fermentation broth, returning the cells to the reactor (Figure 2.6).

The filtration unit can be a membrane, or ultrafiltration device, etc. (Ohleyer et al., 1985; Qureshi and Ezeji, 2008). These bioreactors are called cell-recycled bioreactors and employ a filtration unit that allows for continuous collection of cell-free fermentation broth while keeping the biomass in the reactor ((Enzminger and Asenjo, 1986). These reactors allow continuous operation and increased productivity by eliminating batch downtime and allow high cell densities (Enzminger and Asenjo, 1986).
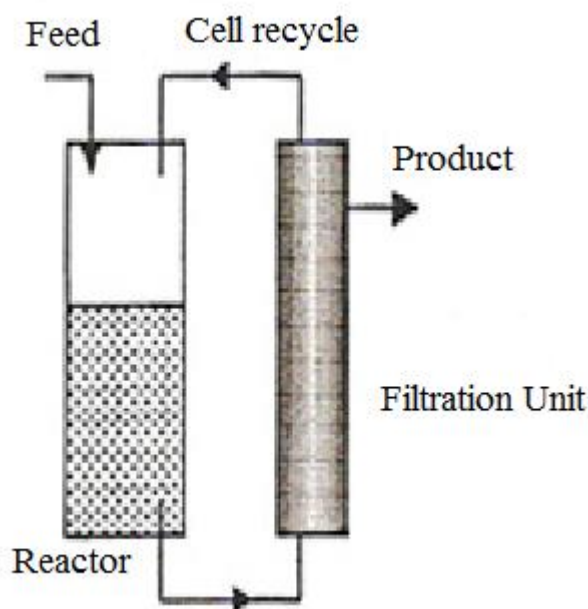
Figure 2.6 Membrane cell recycle reactor (Adapted from Qureshi and Ezeji, 2008).

Siegel and Brierley (1990) stated that cell recycle bioreactor can significantly reduce degradation-associated loss of a secreted protein product and found that maximal recombinant growth hormone releasing factor (GRF) concentration was increased from 5 mg/l to 30 mg/l by the use of a cell recycle reactor, and volumetric productivity was increased more than 10-fold to an average of 10 mg/l/h. Aeschlimann and Stockar (1991) conducted fermentation with cell recycle system for the production of lactic acid by *Lactobacillus helveticus* on a supplemented whey permeate medium and concluded that using recycled system provided about twice volumetric lactic acid productivity as much as with a nonrecycled system. Ohleyer et al. (1985) also applied cell-recycle reactor for lactic acid production by *Lactobacillus delbrueckii* and achieved a volumetric productivity of 150 g/l/h, while Leudeking and Piret (1959) obtained a productivity of 6.7 g/l/h without recycle system.

2.10.2 Hollow-fiber bioreactors

Hollow-fiber bioreactor is a another system in which the cells are separated from the medium using semi permeable membranes arranged in the form of hollow fibers (Jayaraman, 1992). Schematic diagrams of a hollow fiber reactor in longitudinal and cross-sectional views are given in Figure 2.7. The bioreactors typically consist of bundles of hollow fibers in a plastic cartridge and the culture media is continuously perfused in the intracapillary space through the plastic cartridge. The cells are grown in the extracapillary space that surrounds the fibers. The walls of the hollow fibers serve as semi-permeable ultrafiltration membranes. The membrane retains cells while permitting gas, nutrients, and metabolic waste products to diffuse freely across the membrane according to hydrostatic pressure differences and concentration gradients (Jackson et al., 1999).
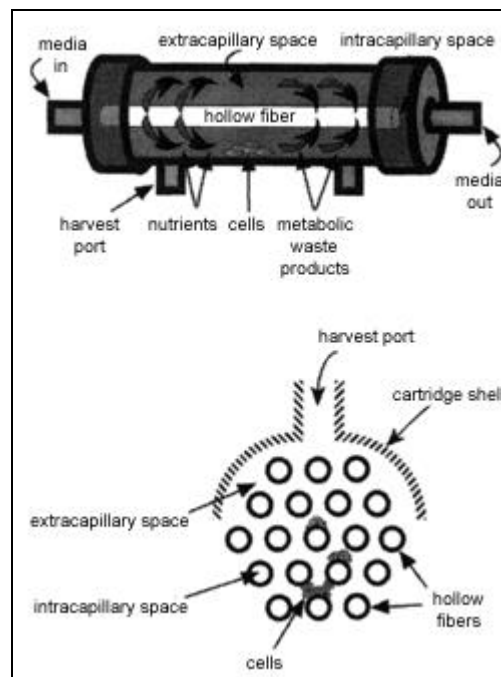
Figure 2.7 Hollow fiber bioreactor (Jackson et al., 1999).

Vick Roy et al. (1982) used hollow fiber fermentor for the production of lactic acid by *Lactobacillus delbrueckii* and 100 g/L/h of productivity was observed while the productivity was 6.7 g lactic acid/L/h in batch fermentation. Lloyd et al. (1997) studied the production of a recombinant toxoid by a marine *Vibrio* and achieved extended production phase with hollow-fibre bioreactors than batch culture, which provided maximal production phase for only 5 h of duration.

Both cell-recycled and hollow-fiber reactors provide increase of the productivity of fermentation by increase biomass in the reactors. However, hollow-fiber and cell-recycled reactors have their limitations in their high capital and operating costs. Moreover, filtration unit fouling is a problem in cell-recycled reactors and must be constantly monitored (Demirci et al., 2007). Similarly, membrane fouling is a problem in hollow fiber reactor during fermentation (Jayaraman, 1992).

2.10.3 Immobilized-cell bioreactors

Immobilization is the restriction of cell mobility in a fixed space. In an immobilized cell bioreactor, the cells are stuck to a sticky surface while nutrient flows over them. Therefore, the cells can be reused continuously. Immobilization provides cell reuse, eliminates the costly processes of cell recovery and cell recycle. Immobilization improves genetic stability and provides favorable microenvironmental conditions for cells. Immobilization eliminates cell wash out problems at high dilution rates. The combination of high cell concentrations and high flow rates allows high volumetric productivities (Schuler and Kargi, 2002).

Active immobilization and passive immobilization are the methods of immobilized-cell systems. Active immobilization is entrapment in polymer matrix (agar, alginate, polyacrylamide, chitosan, gelatin, and collagen) and covalent binding to surfaces using coupling agents (Demirci et al., 2007; Schuler and Kargi, 2002). In covalent binding, binding surfaces should be treated with coupling agents or reactive groups. Although covalent binding forces are strong and provide stable binding, the support materials with

desired functional groups are limited and the reactive groups may be toxic to the cells (Schuler and Kargi, 2002).

Shahbazi et al. (2004) used spiral-sheet bioreactor with active immobilized cells in sodium alginate beads for the production of lactic acid. They achieved a lactose conversion ratio of 79% and lactic acid yield of 0.84 g of lactic acid/g of lactose utilized during the first run with the immobilized *Lactobacillus helveticus* and a lactose conversion ratio of 69% and lactic acid yield of 0.51 g of lactic acid/g of lactose utilized with immobilized *Bifidus longum*.

Pham and Wright (2008) used immobilized *Saccharomyces cerevisiae* KAY446 cells in calcium alginate gel and increased the ethanol yield to 0.35 g of ethanol/g of glucose compared to suspended cell fermentation (0.30 g of ethanol/g of glucose). Kumaravel and Gopal (2010) also evaluated immobilization of *Bacillus amyloliquefaciens* MBL27 cells in calcium alginate beads and achieved the highest activity after the second cycle of use in repeated batch fermentation and the production was stable up to the fifth cycle.

In general, the disadvantages of active immobilization are toxicity of coupling agents and cross link agents on cell viability and activity, instability of polymer matrix, cell leakage from the gel matrix, limited mass transfer across the beads, poor operational stability, and high cost of the carrier (Demirci et al., 2007). On the other hand, passive immobization includes many advantages due to operational stability, low capital, operational cost, and it doesn't need any coupling agent, which might be toxic. Passive immobilization (Biofilms) occurs by natural adsorptions and multilayer growth of cells around or within the solid supports (Demirci et al., 2007; Schuler and Kargi, 2002).

2.11 Biofilm Reactors

2.11.1 Biofilm fundamentals
Biofilm is a microbial community, which is formed by the attachment of microbial community on biotic or abiotic surfaces and embedment in an extracellular matrix, which

is self-produced by microbial community (Burmølle et al., 2006; Xu et al., 2011). Biofilms are composed of approximately 15% cells and 85% matrix of extracellular substances by volume (Agle, 2007). The cells are placed in that matrix. Inside the matrix, there are water channels, which carry nutrients, dissolved oxygen (Agle, 2007).

Microbial community in biofilm structure has several advantages compared to planktonic cells. The bacteria gain more resistance to desiccation, grazing, and antimicrobial agents in the biofilm structure when compared to their planktonic cells (Burmølle et al., 2006). In biofilms, most of the bacteria species tend to exist as multispecies consortia and those biofilm structure triggers the syntrophic and other community interactions between microorganisms such as horizontal gene transfer and cometabolism, which is simultaneous degradation of substrates (Burmølle et al., 2006; Characklis and Marshall, 1990). Biofilm provides beneficial purposes in the natural environment as well as in the industry. It has been reported that biofilms play important role in the removal of dissolved and particulate contaminants from natural streams and in wastewater treatments plants. Moreover, biofilm has been used in biotechnology industry to improve the productivity and stability of the process. However, the biofilm may also have negative effects in the environment. For example; biofilm formation on the surfaces of food processing equipment causes decrease in the performance and lifetime of the equipment. Fouling of heat exchangers, pipelines, and ship hulls may occur due to the biofilm formation, which reduce heat transfer, increase fluid frictional resistance or increase corrosion. Moreover, biofilms has been focused by medicine and dentistry, because they can cause health problems (Characklis and Marshall, 1990). *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Escherichia coli* O157:H7 are examples of pathogens, which form biofilms on the surfaces (Kumar and Anand, 1998).

2.11.2 Principle of biofilm formation
Bacteria can change its life style from planktonic mode to biofilm or biofilm to planktonic mode. Transition from planktonic cells to biofilm is dependent on environmental and physiological factors, such as bacterial cell density, nutrient

availability, and cellular stress (Landini et al., 2010). Biofilm formation includes many steps. The main steps are a reversible adherence of planktonic cells to a solid surface, colonization, irreversible attachment of adhered cells, and biofilm dispersal (Xu et al., 2011). Figure 2.8 summarizes the biofilm formation schematically.

First, the substratum is conditioned by macromolecules in bulk liquid or by coated material. Then, the planktonic cells in the medium are transported to the surface by diffusion, convection, or self-motility. At this step, reversible adherence occurs. Reversible adhesion also contributes the long term attachment of whole biofilm to surfaces (Flemming and Wingender, 2010). Then, colonization starts to occur (Demirci et al., 2007). Colonization of bacterial cells enables bridging between cells. It is temporary immobilization of bacterial populations, which provides the development of high cell densities and cell-cell recognition (Flemming and Wingender, 2010). Then, biofilm matrix is formed and cause irreversible adherence (Demirci et al., 2007). The short range forces including dipole-dipole, hydrogen, ionic, and covalent bonds, and hydrophobic interactions forms the irreversible attachment. These short range forces mainly cause weak interaction with the surface. The matrix is mediating the strong interaction with the surface (Agle, 2007). The matrix, which surrounds the bacterial cells, includes proteins and extracellular polysaccharides and forms the scaffold for the three-dimensional architecture of the biofilm (Landini et al., 2010; Flemming and Wingender, 2010). The matrix is usually produced by the organisms themselves (Flemming and Wingender, 2010).

After irreversible adhesion takes place, secondary colonizers coadhere with organisms, which are already adhered on the surface. Moreover, coaggreagation can take place in bulk liquid and then the cells can adhere to the biofilm surface (Demirci et al., 2007). Then, detachment, erosion, and disruption occur simultaneously. These processes are dependent on fluid shear stress, weak internal cohesion, depletion of oxygen, or depletion of the nutrients in the biofilm. Erosion, which is resulted by liquid shear stress, causes the removal of small parts of biofilm. Rapid change or depletion of nutrients mainly causes

detachment of large parts of biofilm, which is called sloughing. Finally, when the growth is balance with the detachment, the system reach to pseudo-steady state and at this stage the thickness of the biofilm is considered as maximum (Demirci et al., 2007).
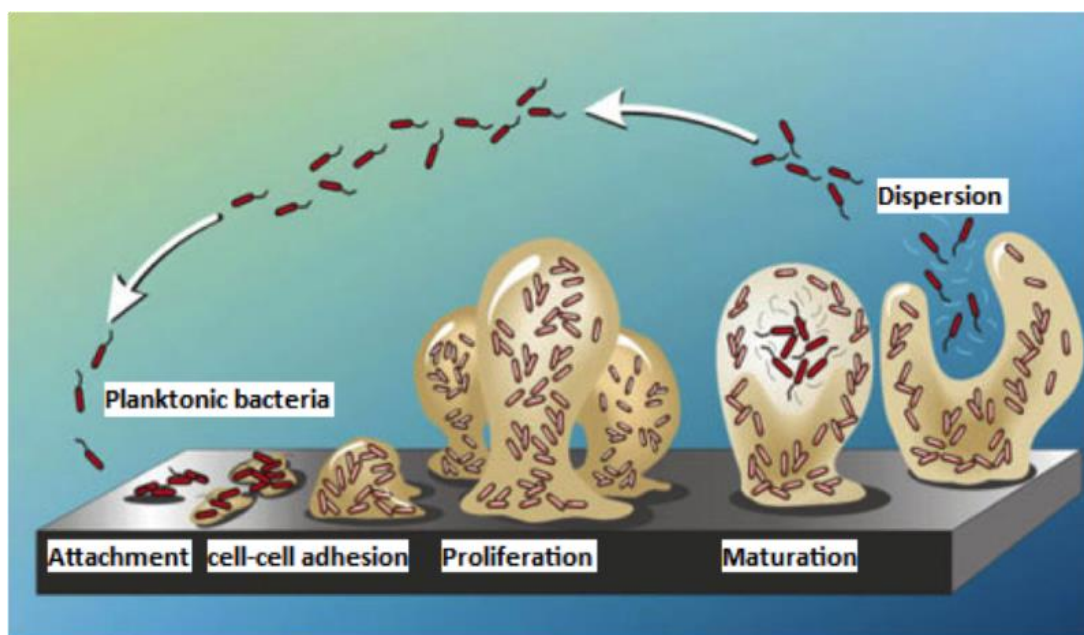
Figure 2.8 Biofilm formation (Parmar, 2012).

2.11.3 Properties of biofilm structure

Up to now, three different types have been suggested to describe for the structure of the biofilm, which are heterogeneous, pseudo-homogenous, and mushroom types (Wimpenny et al., 2000; Atkinson and Swilley, 1963; Nyvad and Fejerskov, 1997; Keevil and Walker, 1992; Stewart et al., 1995). It has been reported that different types of biofilm structure can be found in a well-mixed biofilm reactor (Wimpenny et al., 2000; Demirci et al., 2007). Heterogeneous type includes a dense, planar, homogenous biofilm, which is exposed to the flowing liquid (Atkinson and Swilley, 1963; Nyvad and Fejerskov, 1997). The biofilm that contains cells hold together by exo-polysaccharides, which are separated by water channels, are pseudo-homogeneous type (Atkinson and Swilley, 1963; Keevil and Walker, 1992; Stewart et al., 1995). In mushroom type, the

biofilm shape looks like mushroom and surrounded by water channels (Wimpenny et al., 2000).

The physical, chemical, and biological properties of biofilms are mainly dependent on the microbial species, their morphology, and the composition of the extracellular polymers. The extracellular polymers may change due to physiological stage of the microbial cells. Moreover, the properties of the biofilm vary with the environmental factors. Population distribution in the film, nutrient loading rate, and hydrodynamic shear stress are some of the examples for environmental factors. The biofilm properties affect the rate of transport of momentum, heat, and mass within or through the biofilm (Christensen and Characklis, 1990).

2.11.4 Factors affecting biofilm formation

The thickness of biofilm varies from a few microns to even a few centimeters depending on microbial species, biofilm age, nutrient availability, and environmental liquid shear stress. The attachment is greatly affected by the surface properties (i.e., roughness and charge) and the rate of microorganisms being transported to the surface (Teixeira and Oliveira, 1999; Demirci et al., 2007; Cheng et al., 2010). Many bacteria tend to attach to hydrophobic than hydrophilic surfaces (Christensen and Characklis, 1990). The colonization and growth are both affected by the factors including nutrient transport, diffusion, shear force of bulk flow, and growth rate of microorganisms (Cheng et al., 2010).

Different bacterial species may affect each other positively in many ways, including co-aggregation of cells, conjugation, and protection of one or several species from eradication against antimicrobial compounds. The reason of those protection systems might be enzyme complementation and organized spatial distribution of the cells in the biofilm. In addition to protective mechanisms, microorganisms can create a biofilm synergistically. However, some interactions between microbial communities in biofilms can affect the microbial growth negatively. These may be caused by the production of

bacteriotoxins and lowering of pH by production of acid by some microorganisms in the biofilm matrix (Burmølle et al., 2006).

Biofilm includes extracellular polymeric substances, which can vary greatly between biofilms, depending on the microorganisms present, the shear forces experienced, the temperature and the availability of nutrients. Biofilm strength can be increased in response to mechanical stresses by increasing extracellular polymeric substances (Flemming and Wingender, 2010). Moreover, the mechanical properties of the biofilm may be affected by the interaction of multivalent inorganic ions with extracellular polymeric substances. It has been reported that because Ca^{2+} mediate crosslinking of polyanionic alginate molecules, the mechanical stability of mucoid *P. aeruginosa* biofilms increase in the presence of Ca^{2+} (Körstgens et al., 2001). It has been reported that some of the microorganisms, such as *Streptococcus thermophilus* LY03 and related strains, has maximum extracellular polymeric substance yield at the optimal growth conditions (de Vuyst et al., 1998). In contrast, maximum production of extracellular polymeric substance occurs in mesophilic lactic acid bacteria at 20°C, which is at sub-optimal temperatures (Degeest and de Vuyst, 1999). The reason of the increase in the production of extracellular polymeric substances when cells are growing at lower temperatures may be related to an increase in the number of isoprenoid lipid carrier molecules available for the formation of extracellular polymeric substances (Sutherland, 1972). Moreover, it has been reported that extracellular polymeric substances synthesis increases when cells are exposed to harmful or sub-optimal environments (Ophir and Gutnick, 1994; Hutkins, 2006).

2.11. 5 Advantages of biofilm reactors

Biofilm reactors are more advantageous than the other novel bioreactors, such as cell-recycled reactors and hollow-fiber reactors. Biofilm reactors have lesser tendencies to develop membrane fouling and lower required capital costs. Moreover, biofilm reactors show many advantages over suspended cell reactors, especially in their higher biomass density and operation stability. Biofilm reactors are able to retain 5 or 10 times more

biomass per unit volume of reactor, thereby increasing production rates, reducing the risk of washing out when operating at high dilution rates during continuous fermentation, and eliminating the need for re-inoculation during repeated-batch fermentation. The biofilm matrix contributes to high resistance of microorganisms to extreme conditions of pH and temperature, contaminations, hydraulic shocks, antibiotics, and toxic substances (Demirci et al., 2007).

2.11.6 Type of biofilm reactors

As described in details so far, biofilm is formed naturally on solid surfaces, which can be applied to bioreactors in which microbial cells attach to the support structure without use of chemicals and form thick layers of cells known as "biofilm". As a result of biofilm formation, high cell concentrations can be achieved in the reactor. Therefore, biofilm reactor is considered as "passive immobilized cell reactor" (Demirci et al., 2007).

Batch, rotary continuous reactors, fixed-bed reactors including submerged beds, trickling filters, rotating disk reactors, packed bed, trickling bed, airlift reactors, and expanded bed reactors (i.e., fluidized bed and moving bed reactors) are examples of biofilm reactor systems (Demirci et al., 2007; Qureshi et al., 2005). Biofilms develop on static supports in fixed-bed reactors, which include packed bed, submerged beds, trickling filters, rotating disk reactors, and membrane biofilm reactors (Melo and Oliveira, 2001; Qureshi et al., 2005; Demirci et al., 2007).

i) Packed-bed reactor

Packed bed reactors are filled with support material and biofilm is formed by inoculation with the culture. Then the product is usually collected at the top of the reactor. Disadvantage of packed bed reactor is being blockade problem due to excessive cell growth (Qureshi et al., 2005).

ii) Submerged-bed and trickling filter reactors

The biofilm particles are completely immersed in the liquid in submerged beds reactors. The liquid flows downward through the biofilm bed, while the gas flows upward in trickling filters (Melo and Oliveira, 2001; Demirci et al., 2007).

iii) Rotating disk reactors

The biofilm develops on the surface of a vertical disk that is partially submerged and rotates within the liquid in rotating disk reactors (Melo and Oliveira, 2001; Demirci et al., 2007).

iv) Trickling bed reactors

Trickling bed reactors are fed at the top of the reactor and the product is collected at the bottom of the reactor (Qureshi et al., 2005).

v) Membrane aerated biofilm reactors

Porous gas-permeable membrane has been suggested as the support for biofilm reactors (Casey et al., 1999). A membrane aerated biofilm reactor includes a membrane lumen, which can be either open or closed. Air or oxygen is used to pressurize the lumen. This type of reactors have been used in volatile organic pollutant removal, and nitrification: denitrification processes. The oxidation of the pollutants occurs in the biofilm. The oxygen diffuses through the membrane into the biofilm and oxidation of the pollutants takes place inside the biofilm. Membranes can be in the form of tubular or flat. The material of the membrane can be polypropylene, which is hydrophobic porous, or silicone, which is a dense material, or a composite material, which includes a porous membrane coated with dense material (Casey et al., 1999).

vi) Fluidized bed reactors

In the fluidized bed reactors, the particles move up and down (Shieh and Keenan, 1986). The medium is fed upward through a bed of media such as silica sand. The velocities of medium feeding rate should be adjusted to induce the fluidization of the media. Recycle

of the effluent may also be applied to ensure uniform fluidization and adequate substrate loading rate. Fluidization provides a large surface area for biofilm formation and growth (Shieh and Keenan, 1986). In these reactors, cells grow around the adsorbent particles and form biofilm. The accumulation of sufficient active biofilm around the particles may take from two to four weeks. It is more advantageous than packed bed reactors and continuous stirred tank reactors with fibrous bed due to length of operation time. Excessive growth doesn't cause blockade in fluidized bed reactors (Qureshi et al., 2005). However, when the biofilms cover the media, the overall density of the biofilm coated media decrease and this cause a risk of washing out of the bioparticles from the reactor. This can be overcomed by controlling the expanded bed height at a given level and separating the wasted bioparticles as an excess sludge and returning the cleaned media to the reactor (Shieh and Keenan, 1986).

vii) Inversed fluized bed reactors

Inversed fluized bed reactor includes an airlift draft tube apparatus (Nikolov and Karamanev, 1987). The air is fed from the bottom of the inner tube and creates a recirculation of the medium inside the reactor. The downflow in the annulus forms an inverse fluidized bed due to expansion of the bed of particles whose density is less than that of the liquid. There is grid whose openings are greater than the light particles, in the internal tube, above the aerator. A 5-6 cm high bed of heavy inert particles such as quartz are placed on the grid supports and fluidized by the air-liquid stream. A liquid-biomass separator is assembled at the bottom of the reactor. A thermostating jacket is used to circulate the water for the temperature control inside the reactor. After inoculation, the cells are attached spontaneously to the light particles. Then, bioparticle density increase due to increase in the biofilm thickness and this causes expansion of the bed. After a certain time, the lower bed boundary reaches the lower draft tube opening and the heaviest particles enter the draft tube. Friction in the three phase fluidized bed causes removal of part of the biofilm. Then, the particles are returned to the top of the inverse fluidized bed by the gas-liquid stream and the removed biomass is separated by a separator (Nikolov and Karamanev, 1987).

viii) Expanded bed biofilm reactors

The expanded bed biofilm reactor circulates the medium throughout the moving bed; such as in airlift reactors and circulating-bed reactors. In both expanded biofilm bioreactors, all biofilm processes occur within continuously moving media maintained by high air or liquid velocity or by mechanical stirring (Demirci et al., 2007). Airlift reactors contain a riser tube (an inner tube) and a downcomer tube (an outer tube), which are placed in a concentric way. In these reactors, mixing is done by circulation. The circulation is achieved by the force applied by air at the bottom of inner tube. As a result, the liquid in the inner tube moves up and overflows downward. In some of the airlift reactors, an external loop is assembled circulate fermentation broth. If the air is replaced by an anaerobic gas, this reactor is called gaslift reactors (Qureshi et al., 2005). These configurations can provide some advantages and disadvantages depending on the microbial systems used.

2.11.7 Biofilm support materials

Support materials to form biofilm are essential part of the overall system. The following factors should be considered in the selection of solid supports for biofilm reactors. The support must favor microorganism adhesion; i) have a high mechanical resistance to liquid shear forces and particle collision; ii) be inexpensive; and iii) be widely available. Moreover, effect of some of the properties of solid support, such as surface charge, hydrophobicity, porosity, roughness, and density should be considered (Demirci et al., 2007; Melo and Oliveira, 2001; Capdeville and Rols, 1992). Stainless-steel mesh onto the outside of the draught-tube and solid hollow particles from woven mesh, polyster sponges, glass ceramic material, coal, sand, polymeric materials are some examples of supports for biofilm reactors (Atikson et al., 1979; Souza et al., 2008). Many studies have been conducted to investigate the biofilm formation on the surface and to improve the support materials by modifying the composition, increasing the surface area and strength. Martinova et al. (2010) reported that polyethylenevinylacetate material enhances the adhesion of biomass cells. This plastic material had average 1800 m^2/m^3 of specific area

and 70% bed porosity. They indicated that it is more advantageous than clay balls, ceramic pieces, and volcanic rocks due to its extended specific surface.

Terada et al. (2004) compared the adhesion rates of cells on to diethylamine fiber and polyethylene based fiber. The initial adhesion rates of cells on to diethylamine-fiber were 6-10 fold than the adhesion rate on to polyethylene based fiber. Moreover, the amount of cell attachment to the diethylamine-fiber was 3 fold than to polyethylene based fiber. Souza et al. (2008) reported that polymeric support made from PVC is an advantageous support in biodegradation process because of its high resistance and their densities being close to those of biofilms. They also reported that because it is non-porous, it prevents the adsorption of products and the medium and it provided homogeneous distribution inside the reactor. Chen et al. (2008) reported the importance of mechanical strength to withstand the high pressure in a packed-bed bioreactor. They used a sponge like a material as a support material, which has with pore size around 800 μm for bioethanol production in a packed bed bioreactor (Chen et al., 2008).

Some lignocellulosic material, such as sawdust, wood chips/shavings, rice husks, cotton towels, and straw, have been also used as solid support for biofilm formation (Zhang et al., 2009). The advantages of corn stalk as solid support was reported as readily available, inexpensive material, highly porous, and having a good water retention capacity and high resistance (Zhang et al., 2009). Moreover, agricultural by-products were used in the production of supports. Horiuchi et al. (2000) used charcoal pellets, which were produced from waste mushroom medium, as a solid support for biofilm formation. The production of charcoal pellets includes mixing of mushroom medium, consisting of a mixed powder of rice bran, corncob meal, and sawdust (70% conifer and 30% broadleaf tree); drying; pelletizing by an extrusion pelletizer; shaping into a cylindrical form; and thermally carbonization in a swing-type kiln at 600-800°C. The pellets are advantageous for the use in biofilm reactors because they are inert, porous and cheap to produce, having bacterial affinity (Horiuchi et al., 2000).

On the other hand, plastic composite support (PCS), developed at Iowa State University (U.S. patent number: 5,595,893), is an extrusion solid support made from polypropylene and several agriculture products at a rate of 11 rpm with a barrel temperature at 200°C and a die temperature of 167°C. The plastic composite supports have 3.5 mm of wall thickness and 10.5 mm of outer diameter (Ho et al., 1997a). The agricultural products provide essential nutrients to sustain cell growth on the PCS. Therefore, PCS provides both an ideal physical structure for biofilm formation and gradually releases nutrients for the microorganisms. Advantages of PCS are providing lower nitrogen requirement in the medium, longevity and durability for long-term fermentation due to PCS's strength and slow nutrient release characteristics (Ho et al., 1997b; Demirci et al., 2007).

2.11.8 Applications of biofilm reactors

2.11.8.1 Alcohol production

Biofilm reactors have been studied extensively for alcohol production. Zhang et al. (2009) used corn stalk as a solid support for the production of acetone, butanol, and ethanol by using *Clostridia beijerinckii*. They used a reactor, in which growing media was circulated for cell immobilization, and then media was fed and the product was discharged. While the productivity and yield in suspended cell bioreactor was reported as 0.22 g/L/h and 0.24 g/g, respectively. The productivity and yield was achieved in the reactor with the corn stalk support at the level of 0.6 g/L h and 0.32 g/g, respectively. Acetone, butanol, and ethonal production was also investigated in cell immobilized reactor with corn steep liquor as a medium ingredient, which is a byproduct of the corn wet-milling process. 6.29 g/L of total acetone-butanol-ethanol was produced during fermentation (Zhang et al., 2009).

Chen et al. (2008) used packed bed bioreactor with loofa spoange as a support for the production of bioethanol from uncooked raw starch. Regardless of circulation rate 100% cell immobilization was obtained within 120 min. The microorganisms used in the process were engineered yeast *Saccharomyces cerevisiae* codisplaying *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase on the cell surface. Although the

concentration of ethanol was obtained at the level of 42 g/L in 3 days, 55% drop of initial ethanol concentration was observed after five cycles due to decrease in cell mass and cell viability in the bioreactor. Therefore, they proposed addition of cells to the bioreactor, which cause 75% restoring of initial ethanol production.

A fluidized bed reactor was used for ethanol production by *Escherichia coli* KO11 (Dumsday et al., 1997). The reactor consisted of a vertical glass column. Macroporous glass beads, which were used as a solid support between stainless steel meshes at the top and bottom of the reaction zone, were fluidized by re-circulating the medium. The average ethanol yield and productivity were achieved as 0.40 g ethanol/g sugar added and 1.12 g/L/h respectively at a dilution rate of 0.06 h^{-1}.

Weuster-Botz et al. (1993) developed a continuous fluidized bed reactor operation system for ethanol production by *Zymomonas mobilis* using hydrolysed B-starch without sterilization. Two phases were applied in the operation system. Macroporous glass carriers, were filled up with monoculture of *Z. mobilis* in the first phase, were used as a solid support. In the second phase, a short residence time was applied for unsterile fermentation of hydrolysed B-starch to ethanol to prevent penetration of contaminant into the glass carriers. 99% of the unsterile hydrolysed B-starch was converted to glucose and 50 g/L ethanol was produced when the 120 g glucose/L was used in the substrate.

Ozmihci and Kargi (2008) evaluated ethanol production from cheese whey powder solution in a packed-column bioreactor. For biofilm formation, the reactor was fed with concentrated culture of *Klyveromyces marxianus* (DSMZ 7239) and sterile cheese whey powder solution and the medium was circulated due to 90% of the sugar was consumed. This process was repeated three times. As a result of the production, 22.5 g/L ethanol was produced when the feed sugar content was 100 g/L.

Biofilm reactors with plastic composite supports were also studied for ethanol production. Kunduru and Pometto (1996a, b) evaluated biofilm reactor with plastic

composite supoort chips for ethanol production. First, Kunduru and Pometto (1996a) tested different compositions for plastic composite support chips in packed-bed reactors. The supports consisted of polypropylene and up to 25% (w/w) of cellulose, soy hulls, soy flour, zein, oat hull, corn starch, corn hull. Moreover, they tested different cultures, which are pure and mixed cultures of Z*ymomonas mobilis* or *Saccharomyces cerevisiae* and mixed cultures of either of these ethanol-producing microorganisms and the biofilm-forming *Streptomyces viridosporus*. Maximum ethanol productivity was achieved at the level of 374 g/L/h (44% yield) on polypropylene composite-supports of soybean hull-zein-polypropylene by using *Z. mobilis.* Then continuous ethanol fermentations were evaluated for 60 days with *Z. mobilis* or *S. cerevisiae* in packed-bed reactors with plastic composite supports (Kunduru and Pometto, 1996b). The compositions of plastic composite-supports were polypropylene (75%) with ground soybean-hulls (20%) and zein (5%) for *Z. mobilis,* or ground soybean-hulls (20%) and soybean flour (5%) for *S. cerevisiae.* Maximum productivity was reported as 499 g/ L /h (37% yield) with *Z. mobilis* while the productivity without support was 124 g/L/h with *Z. mobilis.* Later, Demirci et al. (1997) also evaluated ethanol production in biofilm reactor with plastic composite support. As a result, 40% soybean hull, 5% soybean flour, 5% yeast extract-salt and 50% polypropylene was selected as the best composite composition. They also reported that *S. cerevisiae* produced 30 g/L ethanol on PCS with ammonium sulfate medium, which had lowered nitrogen content, in repeated batch fermentation. This production level was found two to ten times higher than on polypropylene-alone support.

2.11.8.2 Enzyme production

Increase in enzyme production by the application of biofilm reactors has been reported in many studies. Thermo-stable amylase production was increased five-fold by using immobilized recombinant *Escherichia coli* (EC147) on silicone foam support compared to suspended cell (Oriel, 1988).

Spouted bed reactor with 6 mm diameter, spherical, stainless steel biomass support particles was applied for continuous cellulase production by *Trichoderma viride* QM

9123. The broth was recycled, causing a jet at the base of a bed of particles was created to spout and circulate the particles. Steady state conditions were reported due to prevention of excess biomass as a result of high shear near the jet inlet. Overall, the amount of cellulase produced in biofilm reactor was more than 3 times that of production in suspended cell reactor (Webb et al., 1986). Cellulase production by using woven nylon pads biofilm reactor was reported by Hui et al. (2010). They reported similar cultural and nutritional requirement, except temperature, for free cell and immobilized cell. Temperature requirement was found 40°C for immobilized cell while the optimum temperature for free cell was reported as 30°C. The surface area of the support material affected the production of cellulolytic enzymes. Cellulase production in woven nylon pads biofilm reactor was achieved 4.5 times more than the production in suspended cell reactor for a much longer period. Kang et al. (1995) evaluated production of cellulase and xylanase by *Aspergillus niger* KKS, which was immobilized on Celite and polyurethane foams. The enzyme productivities by immobilized cell were twice as high as the productivities by shake flask culture.

Circulating bed reactor with biomass support particles was applied for the fed-batch and batch production of intracellular lipase by *Rhizopus chinensis* (Nakashiam et al., 1988). Cubic polyurethane foam with a porosity of around 0.97 and a pore size of 40 pores per linear inch, was used as a biomass solid support. Productivity in fed-batch operation was 1.3-1.5 times more than that obtained in batch cultivation.

Extracellular lignin peroxidase enzyme has been used to transform a wide range of hazardous compounds. Biofilm membrane stirred tank reactor was used for the improved production of lignin peroxidase and treatment of pentachlorophenol by *Phanerochaete chrysosporium*. They reported that lignin peroxidase production was affected by the surface area for biofilm growth and glucose consumption. As results, 10.5 mg/L/day of pentachlorophenol, which is five-fold more than the rate in flask-scale experiment, was disappeared in the biofilm reactor by the produced lignin peroxidase (Venkatadri et al., 1992). Moreover, stirred tank biofilm reactor with plastic composite support was used for

lignin peroxidase and manganese peroxidase production by *Phanerochaete chrysosporium* (Khiyami et al., 2006). Plastic composite support consisted of 50% (w/w) polypropylene, 40% ground dried soybean hull, 5% dried bovine albumin, 5% yeast extract, and mineral salts. Repeat batch fermentations were operated without reinoculation between batches. Aeration and alcohol addition were found effective on the yield. As a result, 50 U/L of lignin peroxidase and 63 U/L of manganese peroxidase were produced in biofilm reactor.

2.11.8.3 Organic acid production

Various organic acid productions have been studied in biofilm reactors. A multi-stage biofilm reactor was developed for acetic acid production by liquid surface culture of *Acetobacter aceti* M7. The biofilm reactor, which consisted of ten shallow flow horizontal reactors of laboratory scale, had total liquid surface area of 2,957 cm^2, total normal length of 18.9 m, working volume of 860 mL, liquid depth of 2.8 mm in average. The production temperature was held constant at 30°C and it was aerated by natural ventilation through the opening between the top edge of the composite horizontal reactors and the lids on them. A 4.3 g/L/ h production rate was achieved in the biofilm reactor with the application of step feeding of ethanol-rich medium (Park and Toda, 1992). Horiuchu et al. (2000) were also investigated the acetic acid production in biofilm reactor. They used a packed bed bioreactor for continuous acetic acid production. The pellets, which have high specific surface area (200 m^2/g) with numerous micropores (2-10 μm), were produced from waste mushroom medium by thermal carbonization. The production conditions occurred with no pH control, at constant temperature (30°C), and supplementation of oxygen-enriched air with a 40% O_2 content. The fermentation process took 120 day and 6.5 g/L/h productivity was obtained.

Andrews and Fonta (1989) used a fluidized bed biofilm reactor with activated carbon for lactic acid fermentation by *Streptococcus thermophilus*. Substrate inhibition was eliminated due to adsorption of substrate in the carbon. The productivity level was 12 g/L/h without pH control. Demirci et al. (1993) evaluated twelve different bacteria;

including species of *Bacillus, Pseudomonas, Streptomyces, Thermoactinomyces,* and *Thermomonospora*; and solid supports; including pea gravels, 3M-macrolite ceramic spheres, and polypropylene mixed with 25% of various agricultural materials (e.g. corn starch, oat hulls), which were extruded to form chips (polypropylene composite); for biofilm formation in continuous lactic acid production. The polypropylene composite was selected as the best support for biofilm formation with *P. fragi*, *S. viridosporus* T7A, and *Thermoactinomyces vulgaris*. Then, the biofilm producer strains were mixed with lactic acid producer strains. *S. viridosporus* T7A and *L. casei* on polypropylene-composite chips produced the highest amount of lactic acid (13 g/L lactic acid) among other combinations in the biofilm reactor without pH control. Demirci and Pometto (1995) were evaluated repeated batch fermentation for lactic production in biofilm reactor. The repeated batch operation included reactor medium change every 3 days for 24 batches. The pH was controlled at 5. *Streptomyces viridosporus* T7A for biofilm formation and *Lactobacillus casei* subsp. *rhamnosus* for L-lactic acid production were used in the system. The plastic-composite supports provided much more yield than polypropylene-alone supports. Pure culture in biofilm reactor produced more lactic acid than mixed culture and free cell fermentation. Ho et al. (1997b) investigated effects of different agricultural components on biofilm formation and lactic acid production. The highest biofilm population (2.3×10^9 CFU/g of support), cell density (absorbance of 1.8 at 620 nm), and lactic acid concentration (7.6 g/L) in minimal medium were obtained with the plastic composite supports containing soybean hulls, yeast extract, soybean flour, bovine albumin, and mineral salts. The leaching rate of nutrients inside the plastic composite support and lactic acid accumulation on the PCS were also studied (Ho et al., 1997a). The leaching rates in the PCS with dried bovine albumin, dried bovine erythrocytes, and/or soybean flour were lower than the rate in PCS blended with only yeast extract. No correlations were observed between lactic acid accumulation in the support and lactic acid production or biofilm formation.

Vasillev et al. (1993) evaluated the production of gluconic acid by cells of *Aspergillus niger* immobilized on polyurethane foam in repeated-batch shake-flask and bubble-

column fermentations. The polyurethane foam cubes were 1.0 -0.3 cm^3 and had an average pore size of 0.6-0.8 mm. They reported that immobilized cells were able to be maintained for 65-70 h with high productivity. About 143 g/L gluconic acid was produced with foam cubes in the bubble column.

Many studies have also been conducted for citric acid production in biofilm reactors. Sanroman et al. (1994) investigated adsorption and entrapment techniques in polyurethane foams for citric acid production by *Aspergillus niger* in fluidized bed reactor were then compared. The adsorption technique was provides more durable activity than the entrapment technique. The production of citric acid in biofilm dipping reactor was observed by Sakurai et al. (1999). The biofilm dipping reactor composed of horizontal glass cylinder, segment shaped stainless steel meshes. The temperature was maintained constant at 30°C and optimum dipping period was determined as in the range below 20s to hold the critical dissolved oxygen level in the range of 0.9-1.3 mg/L for citric acid production. Wang (2000) used a rotating disk reactor for citric acid production by *Aspergillus niger*. Plastic disks mounted on a horizontal shaft with polyurethane foam, which is a porous biomass support, were used as a solid support for biofilm formation. The disks were exposed to both air and the medium. The volumetric productivity was obtained (0.896 g/L/h) three times higher than that obtained with a stirred-tank fermentor (0.33 g/L/h). The immobilized biofilm provides a stable bioactivity for over 8-cycles of fermentation. Citric acid production was also studied with the immobilization on cellulose microfibrils (Sankpal et al., 2001). In their study, both continuous and batch production of citric acid from sucrose were investigated using *Aspergillus niger* NCIM 588. Mycelia of *A. niger* formed a uniform biofilm on cellulose microfibril under the conditions rich in oxygen. A recycle reactor was used in fed-batch mode and the volumetric productivity was improved to 1.85 g/L/h of citric acid, which is 15-fold over than the productivity in shake-flasks and 1.6-fold over than in a conventional aerated batch reactor. In continuous fermentation, 2.08 g/L/h of volumetric citric acid productivity for 26 days was achieved without loss of productivity. Pramod and Lingappa (2008) employed polyurethane foam for the immobilization of *A. niger* strains MTCC

281 and KLP20. As a carbon source carob pod extract were used. Immobilized *A. niger* strains MTCC 281 and KLP20 were able to produce 33 and 38 g/L citric acid while free cells produced 23 and 27 g/L citric acid, respectively.

Rotating-disk reactors with self-immobilized mycelia of *Rhizopus oryzae* on the plastic disks was used to produce fumaric acid (Cao et al., 1996; 1997). Rotary biofilm reactor included six plastic discs (125 cm^2/disc) as a solid support for biofilm formation. The discs were placed on a horizontal shaft and exposed to the fermentation medium and the air space with rotation at 22 rpm. The productivity was achieved at the level of 3.78 g/L/h within 24 h in the rotary biofilm reactor with the supplement of $CaCO_3$. The volumetric productivity level was reported as three folds higher than with a stirred-tank fermenter with $CaCO_3$. The duration of fermentation in rotary biofilm reactor became shorter than the stirred-tank system and the immobilized biofilm stayed active for more than 2 weeks (Cao et al., 1997). Cao et al. (1996) also developed an integrated system of simultaneous fermentation-adsorption for the production and recovery of fumaric acid from glucose by *Rhizopus oryzae*. Nitrogen rich medium was used for the self-immobilization *R. oryzae* on to the plastic discs of a rotary biofilm contactor. Coupled adsorption column was used to remove the produced fumaric acid continuously to eliminate the inhibition, moderate the pH decrease, enhance the fermentation rate and sustain cell viability. As a result, average yield of 85 g/L was obtained by repetitive fed-batch cycles, which took 20 h. The productivity level was achieved at the level of 4.25 g/L/h, while the productivity was reported as 0.9 g/L/h in stirred tank reactor.

Urbance et al. (2003 and 2004) investigated succinic acid production using *Actinobacillus succinogenes* in biofilm reactor with plastic composite support. A mixture of 50% (wt/wt) polypropylene, 35% dried ground soybean hulls, 5% dried bovine albumin, 2.5% soybean flour, 2.5% yeast extract, 2.5% dried red blood cells, and 2.5% peptone was selected as the best composition of plastic composite support for succinic acid production. The medium was modified by the substitution of industrial yeast extract for vitamins and fatty acid (Urbance et al., 2003). The yield of succinic acid production

increased to 70% in biofilm reactor from 64% in suspended cell fermentation. Then, continuous and repeat-batch fermentation modes were applied for succinic acid production in biofilm reactor with plastic composite support. In continuous fermentation, 71.6% yield was achieved at the dilution rate of 0.2 h^{-1} while the yield for fed-batch fermentation was reported as 86.7% with an initial glucose concentration of 40 g/L (Urbance et al., 2004).

Organic acids, such as butyric, acetic and propionic were produced concurrently with the production of hydrogen through photosynthesis and fermentation from wastewater as the raw material. Horizontal packed-bed bioreactor with expanded clay beads as a support material was used. Optimum production was obtained when operation was conducted without a buffer agent (Leitea et al., 2008).

2.11.8.4 Miscellaneous

In addition to organic acid, alcohol, enzyme production, biofilm reactors have been used for many other productions, such as antibiotics, polymers, starter cultures, etc. Three-phase fluidized-bed biofilm fermenter systems with complete-mixed and plug-flow patterns were analyzed for penicillin production. Complete-mixed contacting pattern was found to be superior to plug flow pattern due to decrease in the inhibitory effect of carbon source (Park and Wallis, 1984). Park et al. (1989) used a fluidized-bed bioreactor with bioparticles, which were biofilm of *Cephalosporium acremonium* around celite particles for the production of Cephalosporin, which is an antibiotic. A significant improved production of the antibiotic in biofilm reactor with bioparticles compared to free mycelial was reported. Srivastava and Kundu (1999) also investigated Cephalosporin production in biofilm reactor. Airlift bioreactor with pellets and Siran supported bioparticles were used to improve the production by *Cephalosporium acremonium*. The maximum specific growth rate of free cells, pellets and Siran carrier were observed to be 0.037, 0.033 and 0.045 h^{-1}, respectively.

Pongtharangkul and Demirci (2006a, b, c) investigated nisin production in biofilm reactor with plastic composite support, which composed of 50% polypropylene, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% dried bovine albumin, and 0.272% of sodium acetate, 0.0004% of $MgCl_2 \cdot 6H_2O$, and 0.002% of NaCl. It was reported that the high-biomass density of the biofilm reactor caused a significantly shorter lag time of nisin production relative to a suspended-cell reactor. Moreover, Pongtharangkul and Demirci (2007) applied online recovery system of nisin by silicic acid coupled with a micro-filter module to reduce detrimental effects caused by adsorption of nisin onto producer, enzymatic degradation by protease, and product inhibition during fermentation. Significant nisin production was achieved by the application of online recovery (7,445 IU/ml) when compared to the production without the online recovery (1,897 IU/ ml).

Bacterial cellulose production was also enhanced by the application of biofilm reactor with plastic composite support (Cheng et al., 2009a). Plastic composite support, which contains polypropylene, soy flour, yeast extract, riboflavin, and salts, was selected due to its nitrogen content, moderate leaching rate, and amount of biofilm attachment. The results indicated that cellulose production and mechanical properties of cellulose improved by the application of biofilm reactor. 2.5 fold higher cellulose yield (7.05 g/L) was achieved by the application of the PCS biofilm reactor than the control (2.82 g/L). Higher stress at break, Young's modulus, and strain at break were also observed for the PCS-grown bacterial cellulose than one produced from agitated culture.

The production of pullulan, which is also polysaccharide, was evaluated in biofilm reactor with plastic composite support, which has polypropylene, soybean hulls, defatted soy bean flour, yeast extract, dried bovine red blood cells, and mineral salts (Cheng et al., 2009b). Pullulan concentration was observed to be 32.9 g/L, which was 1.8 fold higher than the results from suspended cell culture (Cheng et al., 2009b). After medium optimization, the pullulan production increased to the level of 60.7 g/L with 95.0% purity (Cheng et al., 2010b). Mulchandani and Luong (1988) employed biofilm reactor for pullulan production by surface immobilized cells and polyurethane foam entrapped cells.

While, both type of cells showed similar fermentation characteristics, more shear resistant was observed in polyurethane foam entrapped cells, which is advantageous for repeated usage of cells.

The production of xantham gum in conventional stirred tank fermenter is energy-intensive and expensive due to high operation cost because of its high viscosity. Yang et al. (1996) used a centrifugal packed-bed reactor for xanthan gum production by *Xanthomonas campestris* cells, which were immobilized in a rotating fibrous matrix by natural attachment to the fiber surfaces. The volumetric xanthan productivity in biofilm reactor with rotating fibrous matrix was found to be 3 g/L/h, which was 6-fold higher than the productivity in suspended cell reactor.

2.11.9 Limitations of biofilm reactors

Although biofilm reactors are more advantageous for most of the fermentation process, there are some inherit limitations. Diffusion of oxygen and substrate into the cell or release of the extracellular product to the medium can be limited in biofilm reactors. The number of the cell layers in the biofilm may vary due to the type of strain, changes in environmental conditions, etc. Increase in the cell layer may cause increase in biofilm thickness, which may create diffusion resistance to the substrate and nutrients (Qureshi et al., 2005). Therefore, thin biofilm applications were proposed, but the biomass activity of the thin biofilm is a concern (Capdeville and Rols, 1992). Moreover, the reason of the diffusion resistance may also be dependent on the interaction among the compounds, such as inert mass, exopolysaccharides, and absorbed organic matters, which affects the mass transfer at the biofilm-liquid interface (Nicolle et al., 2000; Qureshi et al., 2005; Cheng et al., 2010). The penetration of the substrate is also dependent on the porosity of the biofilm (Nicolella et al., 2000). The product itself also may cause limitations in the biofilm reactor due to its toxicity (Qureshi et al., 2005). Another limitation factor for biofilm reactors is the long starting time of immobilization processes (Nicolella et al., 2000). Moreover, specific type of biofilm reactor design may cause some limitations (Boltz et al., 2012). For example; the operation time may be limited due to the reactor

design. Reactor blockage is a disadvantage for packed bed reactors due to cell growth rate, packing density of the support, and supply of nutrients (Qureshi et al., 2005). Particulate biofilm reactors have also problems with respect to clogging, uniform fluidization, control of biofilm thickness, and start up time (Nicolella et al., 2000). Moreover, scaling up of support materials is challenging.

2.12 Modeling of Microbial Growth and Production

The growth, production, and substrate consumption during the fermentation can be predicted by mathematical models and this can help to increase the possibility of optimization of the process (Volesky and Votruba, 1992). The growth needs to be measured experimentally in order to build models. Bacterial growth often starts with lag phase where the specific growth rate starts at a value of zero. Then, in the logarithmic phase, the growth rate increases and it reaches to a maximal value (μ_m) in a certain period of time. The x intercept of the tangent line on the growth curve, where the specific growth rate is at the maximum level, gives lag time (λ), which provides an average value. In the end, the growth rate decreases and reaches to zero, so that an asymptote (A) is reached. The growth curve in the logarithm of the number of organisms against time gives a sigmoidal curve (Figure 2.9) (Zwietering et al., 1990). Sigmodial functions, such as modified Gompertz (Eqn. 2.2) and Logistic (Eqn.2. 3) equations are the most used models because they can cover all phases including lag, log, and stationary. Mechanistic models derived based on the biological theory while empirical models are based on the observed data (van Boekel, 2008). In the logistic model (Eqn 2.1; $N_{(t)}$: existing population; N_{max} : maximum population) the growth rate is propotional to momentary of the existing population and the remaining resources available to the existing population. In the modified logistic model, the parameters, which include microbial characteristics, are obtained experimentally (van Boekel, 2008).

$$\frac{\mathrm{d}N(t)}{\mathrm{d}t} = k \cdot N(t)\left[1 - \frac{N(t)}{N_{max}}\right] \qquad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots \text{Eqn. 2.1}$$

ln(*N/No*)
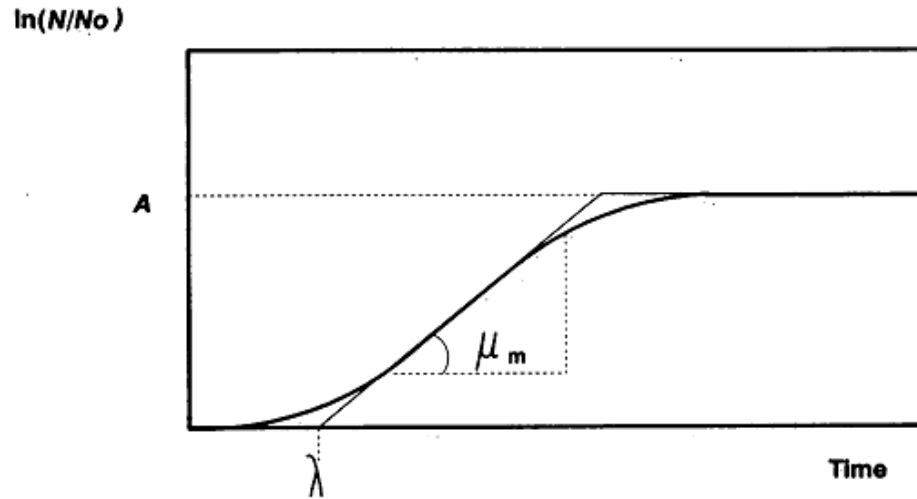
Figure 2.9. A growth curve and parameters to build model (Zwietering et al., 1990).

$$y = \frac{A}{\left\{1 + \exp\left[\frac{4\mu_m}{A}(\lambda - t) + 2\right]\right\}}$$ .... Eqn. 2.2

$$y = A\exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]\right\}$$ Eqn.2.3

The fitted models should be validated for the goodness-of-fit by mean square error (MSE) and R^2. Lower MSE means that the prediction will be more appropriate. After the observed values (*x* axis) versus predicted values (*y* axis) from both models are graphed, if the predictions are in perfect agreement with the observed values, the intercept should be 0, the slope should be 1, and R^2 should be 1. A slope of less than 1 indicates that the model under-predicts the observation (Zhao et al., 2001).

Arroyo et al. (2005) used the modified Gompertz model to describe the growth of *Pichia anomala*, a strain of yeast associated with olive fermentation to determine maximum specific growth rate, lag phase period, maximum specific growth rate at evaluated salt

concentration, temperature, and pH. Mu et al. (2006) described the kinetics of batch anaerobic hydrogen production by mixed anaerobic cultures by modified Gompertz equation. Chowdhury et al. (2007) also reported that both modified Gompertz and Logistic model equations was fitted well to the experimental data for the growth of *Pediococcus acidilactici* and the production of bacteriocin (pediocin). Moreover, Zajsek and Gorsek (2010) described the batch kefir fermentation for ethanol production by mixed natural microflora by Gompertz equations, which was able to give the growth of lactic acid bacteria, the lag time, the specific production rate and the maximum ethanol mass concentration and described the formation of ethanol as the fermentation proceeded.

2.13 Separation of Lysozyme from Fermentation Broth

Like any other fermentation products, it is essential to have an effient and economical separation and purification process. Up to now, the recovery of lysozyme from the fermentation broth has been studied by several reasearches (Maullu et al., 1999; Shin 2002; Cochran 2006). The Maullu et al. (1999) started the purification process by centrifuging the fermentation broth at 5,000 x g for 15 min at 4°C. The supernatant fluid was precipitated by ammonium sulphate subsequently at 80% of saturation. Then, the pellet was suspended in phosphate buffer and dialysed against the same buffer. Then, the concentration was achieved by Amicon cell with 3000 Da cut-off membrane. Then, the isolated protein was fractionated in Carboxy-methyl Saphadex C-50 column. Then, the fractions, which had lysozyme activity, were dialysed against 20 mmol/L phosphate buffer at pH 7 and concentrated in an Amicon cell. Shin (2002) proposed a method to extract lysozyme from an aqueous solution using surfactants and recover it as a solid. The process included direct addition of surfactant to the aqueous solution containing lysozyme. Then, the lysozyme forms an insoluble lysozyme-ligand complex by the contact with surfactant. The surfactant-free lysozyme was recovered as a solid by dissolving the insoluble compound in acetone. Overall, lysozyme was able to be recovered by 70% and retained the enzymatic activity. Then, Cochran (2006) used Expanded Bed Chromatography (EBC). The chromatography is a fast method for the recovery of proteins from crude process streams after dilution. The fermentation broth

had 200 mg/L lysozyme and CM-Hyper-Z was used a resin. As a result, the dynamic binding capacity for lysozyme was stated as 50 mg/mL in buffer, and 20 mg/mL in undiluted fermentation broth containing 500 g/L cells. The binding capacity was increased to 30 mg/mL by harvesting undiluted fermentation broth and circulating it through the EBC column. The combination of fermentation and recovery process didn't cause any dramatic effects on biomass accumulation or metabolic rate.

Parente and Ricciardi (1999) reported that fermentation processes for bacteriocin production with cell recycle or immobilized cells can improve the productivity over batch fermentations. Moreover, these systems can be integrated with recovery systems, based on adsorbing bacteriocin on resins or silica compounds and can reduce the inhibition effect of product on cell growth or the negative effects of by-products on product formation. Pongtharangkul and Demirci (2007) significantly enhanced the nisin production (7,445 IU/ml) by using an online recovery system with silicic acid as adsorbent when compared to the batch fermentation without the online recovery (1,897 IU/ ml). They suggested that the reason of increase in the production might be higher biomass density due to application of tangential flow microfilter module. Liu et al. (2010) also proposed an online recovery system with foam during fermentation of *Lactococcus lactis subsp. lactis* ATCC 11454 for nisin production and total nisin production (4,870 +/- 180 IU/ml) was increased by 30.3% with online foam separation. Moreover, online recovery systems have been applied applied for biobutanol production in a *Clostridium acetobutylicum* biofilm reactor. Macroporous resin, KA-I was used as an adsorbent and the solvent concentration and productivity increased by 4 to 6-fold and 3 to 5-fold, respectively, compared to traditional batch fermentation using planktonic culture without a recovery system (Liu et al., 2014).

2.14 The State of Art of "Enhanced Human Lysozyme Production by *Kluyveromyces lactis* K7 in Biofilm Reactor"

Lysozyme is an important component in the prevention of microbial growth in foods. Moreover, lysozyme has been of interest in industries of medicine and cosmetics. For these purposes, egg-white lysozyme has been commonly used. However, egg-white lysozyme poses immunological problems when consumed by certain people. Therefore, studies about human lysozyme production are needed. Human lysozyme gene expression in microorganisms and their advantages have been reported. *Kluyveromyces lactis* K7 is a genetically modified organism that expresses human lysozyme. *K. lactis* has effective secretory capacity and Crabtree-negative effect. These characteristics make *K. lactis* suitable host for large scale fermentation.

To date, human lysozyme production by *K. lactis* K7 was investigated in shake-flasks and suspended cell bioreactor in batch and fed-batch fermentation modes. Application of different bioreactor design can further enhance the production of human lysozyme by *K. lactis* K7. Biofilm reactors with plastic composite supports have been reported as advantageous fermentation system that provides a substitute of high-biomass density systems with lower capital cost. During the past decade, biofilm reactors have been successfully applied for production of many value-added products, such as enzymes, organic acids, bioenergy, etc. In addition to bioreactor designs, selection of fermentation modes also affects the productivity of fermentation. Fed-batch and continuous fermentation modes provide high productivity by the elimination of carbon source effect. By establishing and optimizing different fermentation modes, it is possible to improve productivity in a most effective way.

Therefore, the goal of this research was to enhance the human lysozyme production by using *K. lactis* K7 and biofilm reactor with plastic composite support with different fermentation modes and strategies. In order to order to achieve the goal, several objectives have been fulfilled. First, the optimal plastic composite support composition type for human lysozyme production in biofilm reactor has been selected (Chapter 3).

Then, the optimum growth conditions (temperature, pH, aeration) of *K. lactis* K7 for human lysozyme production in biofilm reactor with selected plastic composite support has been determined (Chapter 3). After evaluating the different nitrogen sources and effect of NaCl and NH$_4$Cl, the optimum fermentation medium has been determined for human lysozyme production in biofilm reactor with plastic composite support (Chapter 4). Different fed-batch fermentation strategies to eliminate the carbon source effect have been tested. Moreover, continuous fermentation mode has been applied and optimum dilution rate has been determined to increase the production rate (Chapter 5). Then, the recovery of the produced human lysozyme in biofilm reactor has been evaluated and an online recovery system, which included silicic acid as a resin and tangential flow filtration system, has been applied (Chapter 6). The human lysozyme stability in cell free fermentation medium has been evaluated at various temperatures and the effects of pH, time and temperature on human lysozyme activity have been studied (Chapter 7). Finally, mathematical models for the batch fermentation for human lysozyme production by *K. lactis* K7 in biofilm reactor with plastic composite support have been developed (Chapter 8).

CHAPTER 3

**PRODUCTION OF HUMAN LYSOZYME IN BIOFILM REACTOR
AND OPTIMIZATION OF GROWTH PARAMETERS OF
KLUYVEROMYCES LACTIS K7**

3.1 Abstract

Lysozyme (1,4-β-N-acetylmuramidase) is a lytic enzyme, which degrades the bacterial cell wall. Lysozyme has been of interest in medicine, cosmetics, and food industries because of its anti-bactericidal effect. *Kluyveromyces lactis* K7 is a genetically modified organism that expresses human lysozyme. There is a need to improve the human lysozyme production by *K. lactis* K7 to make the human lysozyme more affordable. Biofilm reactor provides high biomass by including a solid support, which microorganisms grow around and within. Therefore, the aim of this study was to produce the human lysozyme in biofilm reactor and optimize the growth conditions of *K. lactis* K7 for the human lysozyme production in biofilm reactor with plastic composite support (PCS). The PCS, which includes polypropylene, soybean hull, soybean flour, bovine albumin, and salts, was selected based on biofilm formation on PCS (CFU/g), human lysozyme production (Unit/ml), and absorption of lysozyme inside the support. To find the optimum combination of growth parameters, a three-factor Box–Behnken design of response surface method was used. The results suggested that the optimum conditions for biomass and lysozyme productions were different (27°C, pH 4, 1.33 vvm for biomass production; 25°C, pH 4, no aeration for lysozyme production). Then, different pH and aeration shift strategies were tested to increase the biomass at the first step and then secrete the lysozyme after the shift. As a result, the lysozyme production amount (141 U/ml) at 25°C without pH and aeration control was significantly higher than the lysozyme amount at evaluated pH and aeration shift conditions ($p<0.05$).

3.2 Introduction

Lysozyme is an enzyme that degrades the bacterial cell wall by catalyzing the hydrolysis of β (1→4) glycosidic linkages between N-acetylmuramic acid and N-acetyl glucosamine found in peptidoglycan layer, which is the major component of the cell wall of both Gram-positive and Gram-negative bacteria (Schleifer and Otto, 1972). However, it is more effective on Gram-positive bacteria due to its higher peptidoglycan content. Because of its bactericidal activity and thermal stability, lysozyme has been of interest in medicine, cosmetics, and the food industry. The most important application of lysozyme is usage as a food preservative, such as in cheese, fish, meat, fruit, vegetables, and wine. Other potential applications of lysozyme include use in heat treated products to reduce thermal requirement, use to prevent gas formation and cracking of the cheese wheels by *Clostridium tyrobutyricum*, added as a supplement with SO_2 in wine making to prevent malolactic fermentation and spoilage, and use in baby formula to aid the immune system development (Hughey and Johnson, 1987; Gilby, 2001). Moreover, the antibacterial property of lysozyme has been exploited in a number of other applications, such as eye drops and wound healing creams. Lysozyme has also been used in treatment of gastrointestinal infections, post-radiation therapy, periodontitis, and dry-mouth (Jolles and Jolles, 1984; Jolles, 1996; Islam et al., 2006). For all these purposes, egg-white lysozyme has been commonly used, but it poses immunological problems when applied to human beings. Individuals sensitive to chicken egg have been shown to be allergic to lysozyme produced from chicken egg (Pichler and Campi, 1992). Because of this, human lysozyme is a better anti-inflammation agent and preservative for food products that will be used by humans, and it has a four times greater specific activity than egg-white lysozyme (Choi et al., 2004). Therefore, there is an urgent need to increase the production of human lysozyme to meet its current and future demands.

Human breast milk is an important source for human lysozyme, but it is not a good source for commercial production of this enzyme (Yu et al., 2006). Therefore, several approaches for the expression of recombinant human lysozyme have been reported (Yoshimura et al., 1987; Choi et al., 2004). Human lysozyme was successfully produced

by genetically modified *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, which have been transformed by the insertion of a human lysozyme gene (Choi at al., 2004; Iwata et al., 2004). *K. lactis* presents several advantageous properties in comparison to *S. cerevisiae*. These include *K. lactis*'s secretory capacities, crabtree-negative effect in fully oxidative conditions, and food grade status (Swinkels et al., 1993; Gonzales-Siso et al., 2000).

To date, human lysozyme production studies using *K. lactis* K7 have been performed by Maullu et al. (1999) and Huang and Demirci (2009). Maullu et al. (1999) conducted flask studies and measured the yield of human lysozyme using cheese whey as its growth media. Huang and Demirci (2009) investigated the production of human lysozyme by *K. lactis* K7 using shake flasks and suspended-cell bioreactor. Lactose as carbon source at the level of 9% (*w/v*) at 25°C was concluded as optimum conditions for human lysozyme production in suspended cell bioreactor. Fed-batch fermentation was also tested and they increased the production level from 110.3 U/ml (in batch fermentation) to 123.6 U/ml. However, flask studies cannot be scaled-up and suspended cell bioreactor may not be the best reactor system due its limited cell population. Therefore, there is a need to study human lysozyme production by using novel approaches and make it more economical for the industry.

Some studies attempted to increase the productivities during fermentation by using techniques such as immobilized cell reactors, cell-recycle reactors, and hollow fiber reactors (Mehaia and Cheryan, 1984; Najafpour et al., 2004; Choudhury and Swaminathan, 2006). However, each one has their own limitations (Jayaraman, 1992; Schuler and Kargi, 2002; Demirci et al., 2007). Biofilm reactor is a natural form of immobilized cell reactors (Demirci et al., 2007). Biofilm formation occurs naturally by the attachment of microbial cells to the support without use of any chemicals agent in biofilm reactors (Qureshi et al., 2005). Biofilm reactors are more advantageous than the other novel bioreactors, such as cell-recycled reactors and hollow-fiber reactors. Biofilm reactors have lesser tendencies to develop membrane fouling and lower required capital

costs (Demirci et al., 2007). Moreover, biofilm reactors show many advantages over suspended cell reactors, especially in their higher biomass densities and operation stability. Therefore, biofilm reactors enhance production rates, reducing the risk of washing out when operating at high dilution rates during continuous fermentation, and eliminating the need for re-inoculation during repeated-batch fermentation (Demirci et al., 2007). Moreover, the biofilm matrix contributes to high resistance of microorganisms to extreme conditions of pH and temperature, contaminations, hydraulic shocks, antibiotics, and toxic substances (Demirci et al., 2007). Support material is an important key factor in biofilm reactor due to its capacity of microbial adhesion, mechanical resistance, affordability, availability, etc. (Capdeville and Rols, 1992; Melo and Oliveira, 2001; Demirci et al., 2007). Plastic composite support (PCS), developed at Iowa State University (U.S. patent number: 5,595,893), is an extrusion of agricultural by-products and polypropylene. It has 3.5 mm of wall thickness and 10.5 mm of outer diameter (Ho et al., 1997b). While polypropylene acts as solid matrix, agricultural by-products sustain the cell growth. Advantages of PCS include providing lower nitrogen requirement of the medium, longevity and durability for long-term fermentation due to PCS's strength and slow nutrient release characteristics (Cheng et al., 2010a). Many studies showed that using PCS biofilm reactors can enhance production of ethanol, organic acids, and bacteriocins. For example; two to ten times higher ethanol productivity was achieved in PCS ring biofilm reactors (Demirci et al., 1997). Higher lactic acid productivity was also obtained by pure culture PCS biofilm reactors (0.81 g/l/h) in repeated-batch fermentation when compared with suspension (0.65 g/l/h) (Demirci and Pometto, 1995).

Therefore, this study was undertaken to select the suitable PCS for biofilm reactor and optimize the growth parameters of *K. lactis* K7 (temperature, pH, aeration) to achieve maximum human lysozyme production in biofilm reactor with the selected PCS.

3.3 Materials and Methods

3.3.1 Microorganism and growth medium

Kluyveromyces lactis K7 (ATCC-MYA-413) was obtained from American Type Culture Collection (Manassas, VA). The culture was grown in medium containing 2% (*w/v*) glucose, 1% (*w/v*) yeast extract, and 2% (*w/v*) peptone for 20-24 h at 25ºC. The working culture was maintained on the agar slant with 20 g/l of glucose, 10 g/l of yeast extract, 20 g/l of peptone, and 20 g/l of agar and transferred to a fresh sterile agar slant every 2 weeks.

3.3.2 Plastic composite support (PCS) tubes

The PCS tubes were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw corotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Pometto et al. (1997). The compositions of PCS tubes, which were evaluated in this study, were given in Table 1. Polypropylene (PP) 50% (*w/w*) and soybean hulls (SH), soybean flour (SF), and salts (S) are the common ingredients for all PCS types evaluated in this study. In addition to PP, SH, and SF, the PCS tubes yeast extract (YE), dried bovine red blood cell (RBC), and dried bovine albumin (BA) are the ingredients for some of the PCS tubes evaluated in this study.

3.3.3 Test tube fermentation for PCS selection

Four types of PCS with different compositions (Table 3.1) were evaluated for both biofilm formation and human lysozyme production using test tube fermentation with three replicates. The PCS tubes provided the opportunity to evaluate the effects of additional ingredients into the PCS on human lysozyme production in biofilm reactor because while the first PCS had the base ingredients, the other PCS tubes had one more additional ingredient (Table 3.1). The PCS tubes were cut into disks with 0.3 mm of thickness. For each replicate, 0.3 g of PCS disks were sterilized dry in capped 50-ml culture tubes for 1 h at 121°C. Fifteen-ml of sterilized medium was added aseptically to

the sterile PCS disks and each test tube was inoculated with 1% (*v/v*) of 24-h grown *K. lactis* K7. Five repeated-batch fermentations for 10 days (each batch fermentation took 2 days) at 25°C were performed for biomass formation. The fermentation medium was composed of 0.67% (*w/v*) yeast nitrogen base, 9% (*w/v*) lactose, and 1% (*w/v*) casamino acid, which were determined as optimum medium compositions for human lysozyme in suspended cell bioreactor by Huang and Demirci (2009). Control fermentation without PCS was also performed simultaneously. After five repeated batch fermentation, while the medium was analyzed to determine the lysozyme production amount (U/ml), the PCS disks were analyzed to determine the biofilm cell population by stripping sand method (Ho et al., 1997a).

Table 3.1. The nutrient composition of plastic composite supports.

Support Types	Ingredients of extrusion mixture (%*w/w*)						
	PP[a]	SH[b]	SF[c]	YE[d]	BA[e]	RBC[f]	S[g]
SH-SF-S	50	40	10				+
SH-SF-YE-S	50	40	5	5			+
SH-SF-YE-BA-S	50	35	5	5	5		+
SH-SF-YE-BR-S	50	35	5	5		5	+

[a] PP, polypropylene resins (Quantum USI Division, Cincinnati, Ohio).

[b] SH, ground (20 mesh), vacuum dried (48 h at 110°C; 30 in. of mercury) soybean hulls (Cargill Soy Processing Plant, Iowa Falls, Iowa).

[c] SF, defatted soybean flour (Archer Daniels midland, Decature, III.).

[d] YE, yeast extract; Ardemine Z (Champlain Industries Inc.).

[e] BA, dried bovine albumin (American Protein Corp., Ames, Iowa).

[f] RBC, dried bovine red blood cells (American Protein Corp., Ames, Iowa).

[g] S, 2 g/kg of sodium acetate, 1.2 g/kg of $MgSO_4.7H_2O$, and 0.06 g/kg of $MnSO_4.7H_2O$.

The PCS types were also evaluated for lysozyme absorption by PCS disks. Screw-cap culture tubes (in replicates of three) with 0.3 g of PCS disks and the control tube without PCS disks were sterilized dry at 121°C for 1 h. Each culture tube was then filled with 15 ml of sterilized deionized water and incubated at 25°C for 24 h to hydrate the PCS disks inside the culture tubes and to solubilize any readily leachable material from the PCS (Ho et al., 1997b). Lysozyme solution was prepared with standard lysozyme in fermentation medium, and dispensed into screw-cap culture tubes (15 ml for each tube). The culture tubes were incubated at 25°C for 2 days. After incubation, the solution in each culture tube was decanted and the activity of the final residual lysozyme not absorbed by the materials was measured. The difference between residual lysozyme not absorbed by the material and the initial lysozyme activity is lysozyme accumulation in the PCS. A control experiment with lysozyme solution without PCS was also performed simultaneously to determine if the lysozyme is degraded or absorbed. The results were given as the percentage reduction of lysozyme activity after 2 days (Table 3.2).

3.3.4 Batch fermentation in biofilm reactor

After determination of the best plastic composite support in test tubes, a Sartorious Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel was used to construct biofilm reactor, which had 12 PCS tubes (6.5 cm long) bound to the agitator shaft in a grid-like fashion, with six rows of two parallel tubes (Figure 3.1). The reactor vessel with PCS was autoclaved with water at 121°C for 90 min. Then, 1.5 l of sterilized fermentation medium was added aseptically to the reactor vessel with PCS. For biofilm formation, a 24-h grown culture of *K. lactis* K7 (1% *v/v*) was inoculated. Then, five repeated-batch fermentations were performed at the (no pH control, 25ºC, 100 rpm, and no aeration) conditions in suspended cell bioreactor as suggested by Huang and Demirci (2009). After forming biofilm, 72-h batch fermentations were conducted for each condition determined by Box-Behnken design of surface response methodology in terms of temperature (20-40°C), pH (4-7) and aeration levels (0-1.5 vvm) (Table 3.3). Two replicates were performed for each level. The response variables were biomass and lysozyme production.
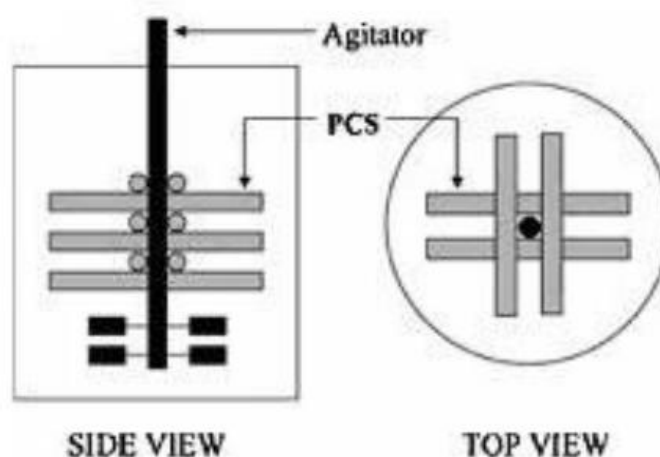
Figure 3.1 Diagram of the PCS biofilm reactor.

3.3.5 Analysis

3.3.5.1 Biomass

The suspended biomass was determined by measuring optical density (OD) using a spectrophotometer (Beckman Coulter, Fullerton, CA) by obtaining absorbance at 600 nm, which then converted to biomass concentration by using equation: *Biomass* (g/l) = $0.564 \times OD_{600}$ as suggested by Huang and Demirci (2009).

3.3.5.2 Biofilm cell population

The stripping sand method was used to determine the relative biofilm population on the supports (Ho et al., 1997a). The PCS discs in the 50-ml test tubes were washed in 100 ml of sterile 0.1% (w/v) peptone water by turning the tubes upside-down 10 times. Then, PCS discs were aseptically transferred into a 50-ml test tube, which includes 2 g of sterile sand and 9 ml of sterile peptone water. After vortexing the tube three times in 30-s intervals, the samples were serially diluted, and spiral plated onto agar plates by using Autoplate 4000 (Spiral Biotech, Norwood, MA). The medium composition of agar plates was 2% (*w/v*) glucose, 1% (*w/v*) yeast extract, and 2% (*w/v*) peptone, 2% (*w/v*) agar.

Then the agar plates were incubated at 25°C for 48 hr. The results were expressed in CFU/g PCS.

3.3.5.3 Lactose concentration

Lactose concentration determinations were conducted using a Waters's high pressure liquid chromatography (HPLC) equipped with a refractive index detector (Waters, Milford, MA). Lactose was separated using Bio-Rad Aminex HPX-87H column (300×7.8 mm; Bio-Rad, Richmond, CA) with 0.8 ml/min of 0.012 N sulfuric acid as mobile phase. The detector temperature and column temperature were maintained at 35ºC and 65ºC, respectively. Prior to the analysis, the fermentation samples were centrifuged for 2 min at 5200 g to separate the cells from the medium. Then, the samples were diluted 3-fold. Finally, the samples and the standard solutions were filtered with 0.2 μm PTFE membrane filters and injected to HPLC. The injection volume was 20 μl. Identification was done with comparison of retention times of compounds and external standard. For the quantification, a calibration curve was constructed by plotting peak areas versus concentrations of lactose standards (5, 10, 15, 20, 30 g/l).

3.3.5.4 Lysozyme activity

Lysozyme activity was carried out using a method adapted from Richard et al. (1965). The procedure for lysozyme assay is as follows: A 0.5 mg/ml of *Micrococcus lysodeikticus* cell suspension (Sigma-Aldrich, St. Louis, MO) was prepared using potassium phosphate buffer at pH 6.2. The assay solution consists of 600 μl of 0.5 mg/ml *M. lysodeikticus* cell suspension, 200 μl of 300 mM sodium chloride solution, and 400 μl of sample. The absorbances were measured every 10 s for 1 min at 540 nm. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 A540 change per minute. The slope of the linear regression line refers to the speed of lysis of *M. lysodeikticus* suspension, and the slope estimates the concentration of human lysozyme in unit per milliliter.

3.3.6 Statistical analysis

The statistical analyses were performed by using MINITAB Statistical Software package (Version 15, Minitab Inc., State College, PA). Analysis of variance (ANOVA) was performed for investigating statistically significant differences between production amounts at different PCSs and growth parameters. A p-value of <0.05 was considered to be significant.

3.4 Results

3.4.1 PCS selection

The results of PCS selection for human lysozyme production in biofilm reactor were summarized in Figure 3.2. Test tube fermentation runs were conducted to select the best PCS type for human lysozyme production and the samples were analyzed for biomass formation on PCS, human lysozyme production. Moreover, an experiment, which mimicked the conditions of test tube fermentation without microorganism with standard human lysozyme, was also performed to see the human lysozyme absorption by PCS.
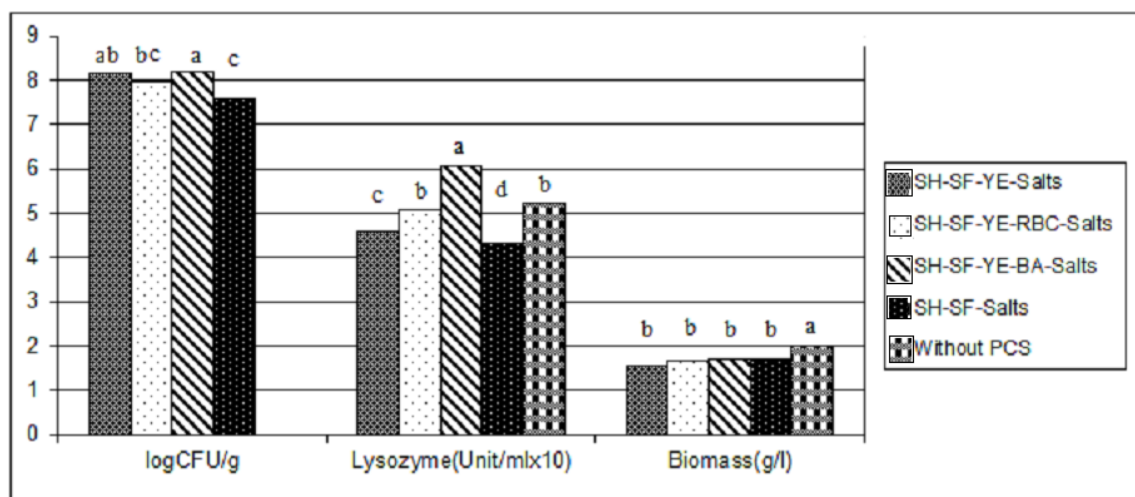
Figure 3.2 Effects of different PCS blends on the number of viable attached cells on the PCS, lysozyme production in test tubes (n =3) (Different letters shows the significant difference between treatments based on ANOVA test ($p<0.05$)).

The biomass formation on PCS was ranged from 8.14 to 7.57 log CFU/g. Biomass formation on SH-SF-YE-BA-S was higher than the other PCS types (Figure 3.2). The biomass production in the medium was ranged from 1.69 to 1.53 g/l in the PCS test tubes, but the differences were not significantly different ($p \geq 0.05$). However, the biomass production of suspension cells in the test tube without PCS was 1.98 g/l, which was significantly higher than that of test tubes with PCSs ($p < 0.05$). The lysozyme activity (60.50 U/ml) was found to be significantly higher for SH-SF-YE-BA-S than the other PCSs and the control (test tube without PCS) ($p < 0.05$).

Table 3.2 Lysozyme absorption by plastic composite supports.

PCS type	% absorption (1st batch)	% absorption (2nd batch)	% absorption (3rd batch)	% absorption (4th batch)	% absorption (5th batch)
SH-SF-S	7.12^{bA}	6.43^{bB}	5.95^{bC}	5.74^{bD}	5.70^{bD}
SH-SF-YE-S	7.26^{bA}	6.55^{bB}	6.00^{bC}	5.85^{bD}	5.82^{bD}
SH-SF-YE-BA-S	4.05^{aA}	3.75^{aB}	3.68^{aC}	3.65^{aC}	3.63^{aC}
SH-SF-YE-RBC-S	14.85^{cA}	9.02^{cB}	8.00^{cC}	7.95^{cD}	7.90^{cD}

Capital letters (A, B, C, D) show the significant difference between values within the same row. Different lower case letters (a, b, c) show the significant difference between values within the same column based on ANOVA test, tukey post-test ($p < 0.05$).

As results of absorption test, significant decrease in the lysozyme absorption amount was observed until the fourth repeated batch run ($p < 0.05$) for all PCS types except SH-SF-YE-BA-S (Table 3.2). The absorption amount in SH-SF-YE-BA-S didn't change significantly after third repeated batch run. At the end of fifth batch run, while the lysozyme absorption amount in SH-SF-YE-RBC-S was the lowest, the lysozyme absorption amount by PCS, which included SH-SF-YE-BA-S, was found to be the lowest among the evaluated PCS types ($p < 0.05$). There were no significant difference in the absorption amount by PCSs, which were consisted of SH-SF-S and SH-SF-YE-S ($p \geq 0.05$). Moreover, a control experiment without PCS was performed and no significant

changes in the amount of lysozyme activity were observed during five repeated batch runs ($p \geq 0.05$).

3.4.2 Batch fermentation in biofilm reactor

3.4.2.1 Optimization of growth parameters by response surface method

Different pH (4-7), temperature (20-40ºC), aeration (0-1.5 vvm) were tested to determine optimum conditions for biomass and lysozyme production in biofilm reactor with selected PCS. Table 3.3 shows the Box-Behnken design and experimental for lysozyme and biomass production amounts as a result of batch fermentation. Figures 3.3 and 3.4, which are response surface plots, show the effects of combinations of different pH, aeration, and temperature on biomass and lysozyme productions, respectively. The lysozyme production was varied from 19 to 73 U/ml. The biomass production was ranged from 0.55 to 6.66 g/l. Both lysozyme and biomass production decreased when the temperature was 40ºC compared to 20ºC. For example; while at 40°C, pH 4, and 0.75 vvm, the biomass and lysozyme productions were 3.49 g/l and 22 U/ml, respectively, at 20°C, pH 4, and 0.75 vvm, the biomass and lysozyme productions were 5.15 g/l and 51 U/ml, respectively.

Moreover, both pH and aeration decrease caused increase in lysozyme production. In contrast to lysozyme production conditions, biomass production increased when the aeration level and pH increased. For example; when the conditions were 20°C, pH 5.5, and 1.50 vvm, the biomass and lysozyme productions were 6.32 g/l and 20 U/ml, respectively. When the aeration was turned off at the same temperature and pH, the lysozyme production increased to 61.50 U/ml and biomass production decreased to 1.48 g/l. Moreover, at 20°C, pH 4, and 0.75 vvm, the production amounts of biomass and lysozyme were 5.15 g/l and 51 U/ml, respectively. When the pH increased to 7 at the same temperature and aeration conditions, the biomass production increased to 5.35 g/l and lysozyme production decreased to 18 U/ml. As seen from the given examples above,

the aeration affected the biomass production much more, while the pH affected biomass production less than the other conditions (Figure 3.3).

Table 3.3 Effect of different growth parameter combinations on biomass and lysozyme production in biofilm reactor with PCS.

Temperature (^{o}C)	pH	Aeration (vvm)	Experimental		Predicted	
			Biomass (g/l)	Lysozyme (Unit/ml)	Biomass (g/l)	Lysozyme (Unit/ml)
30	7.0	0.00	1.93	43.50	2.08	41.25
30	5.5	0.75	5.82	38.75	5.85	38.00
30	5.5	0.75	5.89	37.50	5.85	38.00
20	5.5	1.50	6.32	20.00	6.47	19.19
20	7.0	0.75	5.35	18.00	5.37	17.94
30	4.0	1.50	6.40	24.00	6.26	26.25
30	7.0	1.50	6.66	19.00	6.49	19.88
20	5.5	0.00	1.48	61.50	1.32	63.81
40	4.0	0.75	3.49	22.00	3.47	22.06
40	7.0	0.75	3.61	15.00	3.62	16.44
20	4.0	0.75	5.15	51.00	5.14	49.56
40	5.5	1.50	3.81	18.00	3.97	15.69
40	5.5	0.00	0.55	37.50	0.40	38.31
30	4.0	0.00	1.77	73.00	1.94	72.13
30	5.5	0.75	5.75	38.95	5.85	38.00
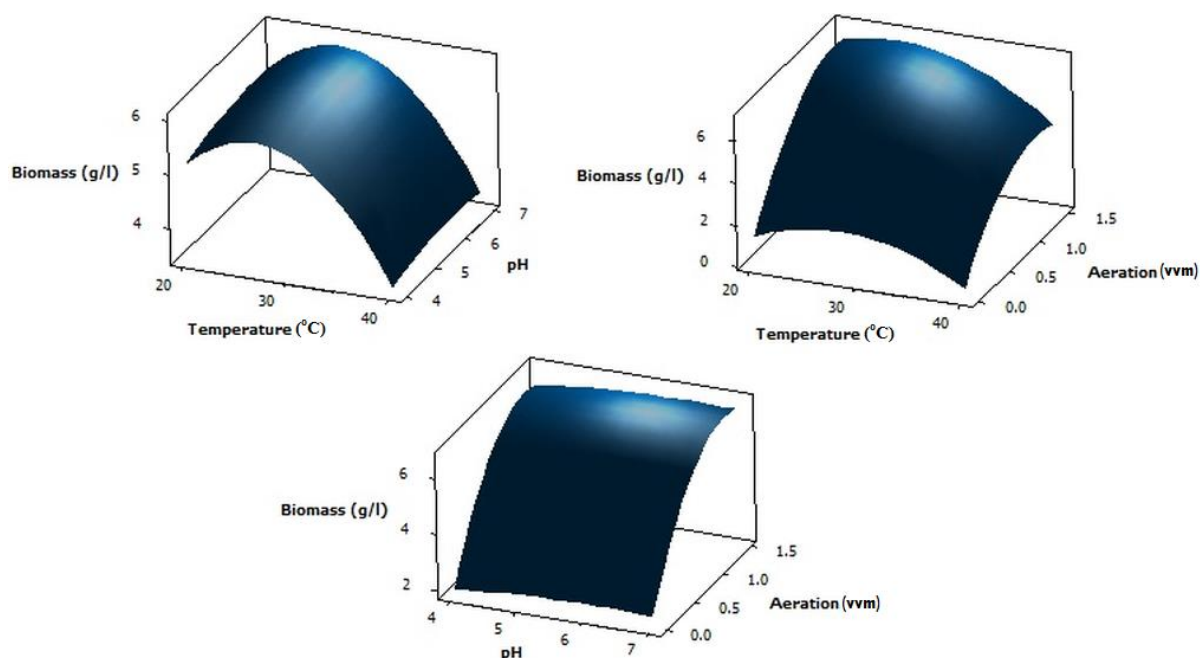
Figure 3.3 Response surface plots for biomass production.

By the application of multiple regression analysis on the experimentally determined values, the following second order polynomial equations (Eqn.s 3.1 and 3.2) were obtained for lysozyme and biomass productions, respectively. The sample variations of 99.6% of biomass production and 99.3% of human lysozyme production were attributed to the factors stated in the models for biomass and human lysozyme production in biofilm reactor with PCS. The predicted R^2 values were 94.5 and 89.2% for biomass and lysozyme models, respectively. The predicted R^2 explains the predictive capability of the model (Anderson and Whitcomb, 2000). The adjusted R^2 values, which are related to correlation strength between response and predicted value, were 98.9% and 98.04% for lysozyme and biomass models, respectively. Table 3.3 shows the predicted values for biomass and lysozyme production amounts as result of the application of regression models for different combinations of growth conditions. Table 3.4 and 3.5 show the analysis of variance results for the models. For both models, the model was significant and the lack of fit was insignificant at the 95% confidence interval.
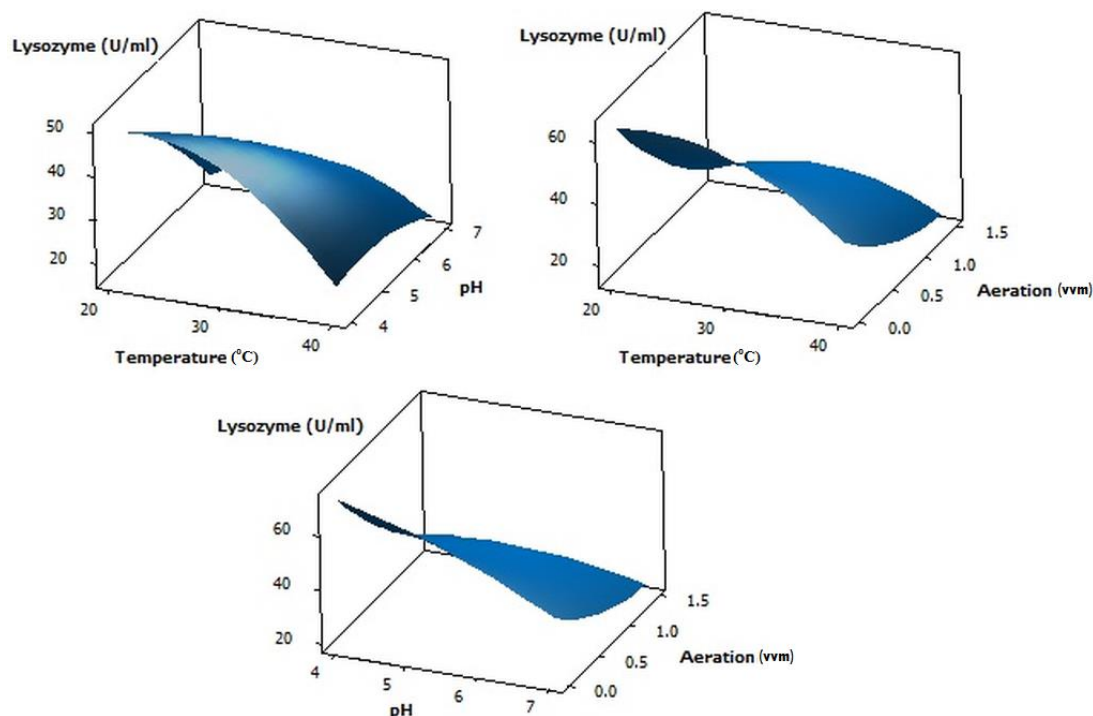
Figure 3.4 Response surface plots for lysozyme production.

As a result of performing optimization by optimizer module in Minitab, the maximum lysozyme production was estimated as 80 U/ml when the aeration level, pH, and temperature were 0 vvm, 4, and 25ºC, respectively. On the other hand the optimum biomass production was estimated as 6.89 g/l when the temperature, aeration level, and the pH were 27ºC, 1.33 vvm, and 6, respectively.

Lysozyme (U/ml)= 109.42 + 1.59×Temperature – 7.95×pH – 87.19 ×Aeration – 0.08×Temperature ×Temperature + 5.4×Aeration ×Aeration + 0.43×Temperature ×pH + 0.73×Temperature ×Aeration + 5.4×pH×Aeration   (R^2 = 0.993) **…………..….** Eqn. 3.1

Biomass (g/l)= – 10.65 + 0.74×Temperature + 0.74×pH + 8.39×Aeration – 0.01×Temperature ×Temperature – 2.68×Aeration×Aeration – 0.05×Temperature×Aeration   (R^2= 0.996) **…………………………..…………** Eqn.3.2

Table 3.4 Analysis of variance for lysozyme response surface quadratic model obtained from experimental design.

Source	Degree of freedom	Sum of squares	F value	p value
Regression	9	4256.78	78.88	0.000
Linear	3	3410.93	38.08	0.001
Quadratic	3	410.12	22.80	0.002
	3	435.72	24.22	0.002
Interaction				
Residual	5	29.98		
Lack of fit	3	28.74	15.52	0.061
Pure error	2	1.24		
Total	14	4286.76		

Table 3.5 Analysis of variance for biomass response surface quadratic model obtained from experimental design.

Source	Degree of freedom	Sum of squares	F value	p value
Regression	9	58.00	153.65	0.000
Linear	3	44.02	71.55	0.000
Quadratic	3	13.35	106.10	0.000
	3	0.62	4.99	0.058
Interaction				
Residual	5	0.20		
Lack of fit	3	0.19	13.60	0.069
Pure error	2	0.01		
Total	14	58.21		

For the validation of the model, batch fermentation was conducted at optimum condition levels for biomass and lysozyme production in duplicate. The results were in agreement with the predicted values. At the optimum conditions, the experimentally determined biomass and lysozyme productions were 6.53±0.25 g/l and 83.75±0.77 U/ml, respectively. Figure 3.5 shows the fermentation at the optimum conditions for human lysozyme production.
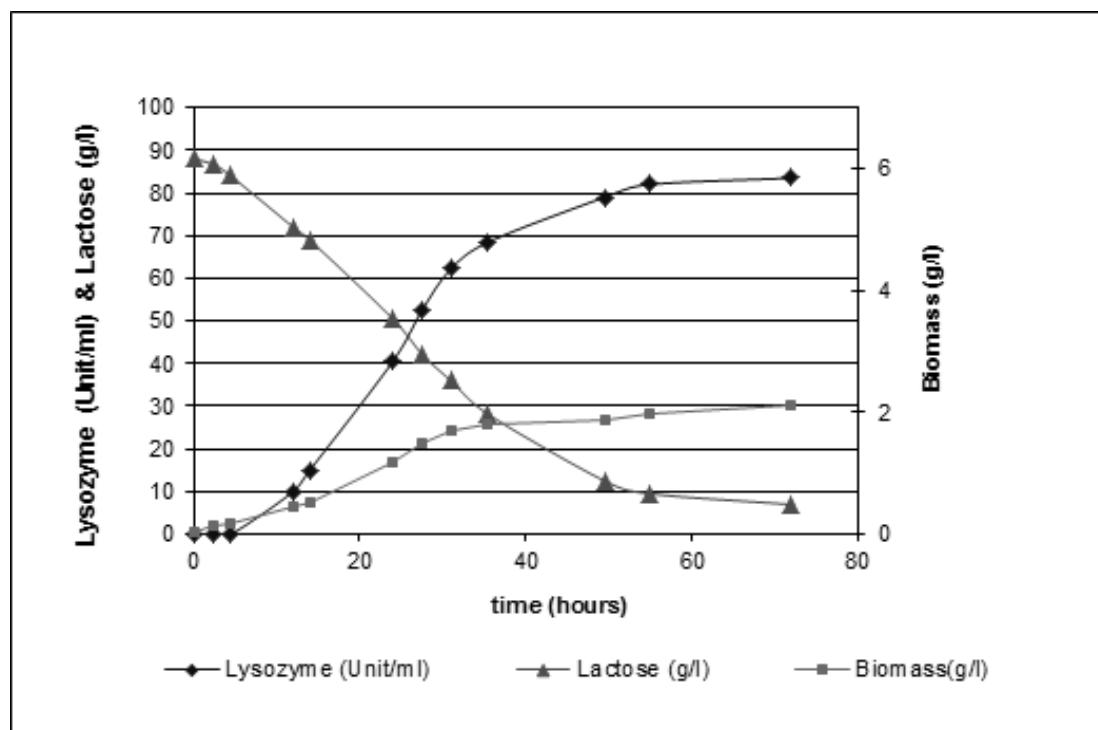
Figure 3.5 Fermentation in biofilm reactor at 25°C, pH 4, and no aeration.

3.4.2.2 pH and aeration shift trials

Different pH and aeration shift trials were conducted to see the effect of pH and aeration changes during fermentation, since different optimum conditions for lysozyme and biomass productions were determined. The results of pH and aeration shift trials were summarized in Table 3.6. As seen in Figure 3.5, 24th hour is in the middle of log phase and 48th hour is at the beginning of stationary phase. Therefore, the shifts were tested at the 24th and 48th hours to see the effect of different phases of fermentation on pH and aeration shift trials.

Table 3.6 pH and aeration shift strategies in batch fermentation.

pH and aeration shift strategies	Initial pH	Final pH	Lysozyme (U/ml)	Biomass (g/l)
pH shift (6 to 4) and aeration shift (1.33 vvm-0 vvm) at 48 h	6.00	4.00	77^e	6.83^{ab}
pH shift (6 to 4) and aeration shift (1.33 vvm-0 vvm) at 24 h	6.00	4.00	127^c	4.39^{cd}
pH shift (6 to no control) with 1.33 vvm constant aeration at 24 h	6.00	5.30	55^f	6.15^b
pH shift (6 to no control) and aeration shift (1.33 vvm-0 vvm) at 24 h	6.00	4.54	92^d	4.33^d
pH shift (6 to no control) without aeration at 24 h	6.00	3.98	135^{bc}	2.98^e
no pH and no aeration control	5.85	3.95	141^{ab}	1.92^f

Different letters within the same column shows the significant difference between treatments based on ANOVA test ($p<0.05$).

The fermentation is started at pH 6 and aeration at 1.33 vvm until 24th and 48th hour, and then the pH and aeration were shifted to 4 and 0, respectively. While biomass production was higher with the shift at 24th hour than 48th hour, higher lysozyme production amount was obtained at the fermentation with the shift at 24th hour than 48th hour. Then, the pH was shifted from 6 to no control with different aeration strategies, including shifts from 1.33 vvm to no-aeration, constant aeration at 1.33 vvm, and no aeration. As a result, fermentation without aeration with pH shift from 6 to no control at 24th hour provided more lysozyme production (135 U/ml) than the production at the optimum conditions, which was determined by response surface method. The pH changes during fermentation were also recorded. Since pH decrease to around 4 were observed at the fermentation run, which included a pH shift from 6 to no pH control, a fermentation was also run without pH and aeration control to see the effect of gradual pH decrease during fermentation on the human lysozyme production. As a result, pH decreased gradually to 3.95 and the lysozyme production amount increased up to 141 U/ml (Figure 3.6).
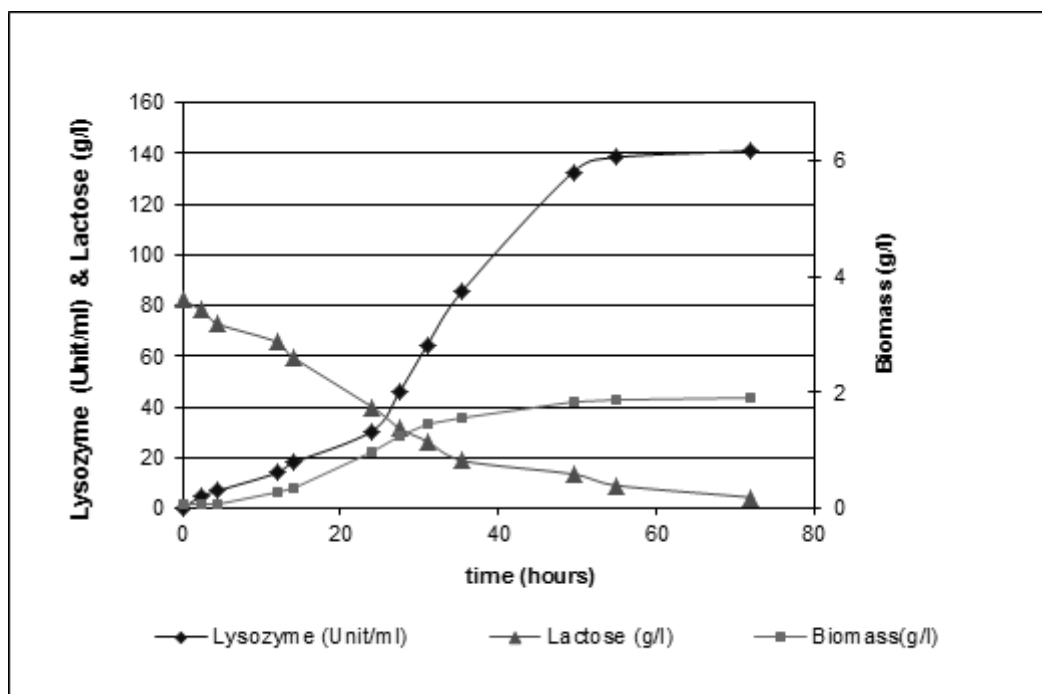
Figure 3.6 Fermentation in biofilm reactor at 25°C without pH, and aeration control.

3.5 Discussion

3.5.1 PCS selection

The best PCS type was selected out of four different types of PCS based on biofilm population, lysozyme production, and lysozyme absorption inside the PCS tubes. Higher production was achieved in the test tube with SH-SF-YE-BA-S than the other PCSs and the control ($p<0.05$). Although there were no differences in biomass amount in the medium of test tubes with PCSs, the reason of high production amount may be related to the cell population increase inside the test tube due to biofilm formation on PCSs because highest amount of biofilm formation was observed on PCS, which was consisted of SH-SF-YE-BA-S, among evaluated PCS types. Moreover, Cheng et al. (2010b) reported that the nitrogen content per g PCS may cause differences in the productions in test tubes. Similarly, the lysozyme productions in test tubes, which include PCS types with nitrogen sources (bovine albumin, yeast extract, and red blood cells), were higher than the production amount in test tubes with PCSs without nitrogen source (SH-SF-S) ($p<0.05$).

In addition to effects of biofilm formation and nitrogen sources on production, absorption of product by PCS may also affect the production amount in the test tubes. Therefore, the PCS types were tested to determine lysozyme absorption amount inside the PCS (Table 3.2). The absorption of lysozyme by PCSs decreased during repeated batch runs. This may be related to the saturation levels of PCS tubes. The PCS type with lowest absorption amount was found as PCS, which was consisted of SH-SF-YE-BA-S. A control experiment without PCS was also performed and no degradation during time was observed. Ho et al. (1997b) reported that porosity is correlated with interstitial volume, which is influenced by the ingredients of PCS. They also reported that soybean hull is the most significant ingredients that affect the porosity followed by dried bovine red blood cell, and bovine albumin. As given in Table 3.1, the concentrations of soybean hull in SH-SF-YE-S and SH-SF-S were higher than SH-SF-YE-BA-S and SH-SF-YE-RBC-S. Moreover, SH-SF-YE-RBC-S had dried bovine red blood cells, which affect the porosity more than dried bovine albumin. Therefore, since the lysozyme absorption in SH-SF-YE-BA-S were less than the other PCS types that were evaluated, the results were in agreement with the study of Ho et al. (1997b). The reason of the difference between lysozyme production in test tubes including SH-SF-YE-BA-S and SH-SF-YE-RBC-S may be the difference in absorption amounts of lysozyme inside the each PCS types because although both dried bovine albumin and dried red blood cell provide additional nitrogen source, lysozyme production was found higher in test tube including SH-SF-YE-BA-S than SH-SF-YE-RBC-S. Therefore, PCS with SH-SF-YE-BA-S was selected for biofilm reactor to be used for human lysozyme production.

3.5.2 Batch fermentation in biofilm reactor

Because the biofilm formation affects the rate of transport of heat and mass (Christensen and Characklis, 1990), the growth parameters in biofilm reactor may become different than parameters in suspended cell bioreactor. Therefore, the growth parameters of *K. lactis* K7 in biofilm reactor were optimized in this study.

As a result of optimization, the required temperature condition for higher lysozyme production was found to be lower than the required temperature for higher biomass production. MacKenzei et al. (1994) reported that enhancement of protein folding and efficiency of secretion pathway may be favored at temperatures lower than the optimum temperature for biomass production. In addition to temperature, different optimum pH and aeration values were observed for biomass (pH 6, 1.33 vvm) and lysozyme (pH 4, 0 vvm) production. Choi et al. (2004) reported that pH can facilitate the efficiency of the secretion of the extracellular product through the cell due its effect on the cellular membrane, structure of the lipid bi-layers and membrane proteins. Moreover, aeration was a significant effect on both biomass and lysozyme production. While the biomass production was higher at higher aeration, the lysozyme production was higher without aeration. Garciagaribay et al. (1987) studied the production of β-galactosidase by *Kluyveromyces marxianus* at different oxygen transfer rates and concluded that production of the enzyme is higher at lower oxygen rates while the biomass production was lower at the same conditions. Similarly, Cruz-Guerrero et al. (1999) reported that while *Kluyveromyces marxianus* requires high oxygen level for high rates and yields of biomass, the pectinolytic enzymes production was higher under anaerobic conditions. It has been reported that although oxygen limitation can cause plasmid instability, gene expression might increase in anaerobic conditions. For example; Li et al. (1992) observed the effect of the levels of dissolved oxygen on the expression of recombinant proteins in four recombinant *Escherichia coli* strains and concluded higher dissolved oxygen levels provided higher plasmid content and similar recombinant protein yields was detected. Moreover, high oxygen might also limit the protein production amount due to oxidative damage to proteins (Palomares et al., 2004).

The regression models also show the effects of temperature, pH, and aeration on human lysozyme and biomass productions. The Eqn. 3.1 shows that aeration, pH, temperature, and also their interactions have significant effects on model for human lysozyme production ($p<0.05$). Similarly, the Eqn.3.2 shows that aeration, pH, temperature, and

also their interactions except interactions of pH with temperature and aeration have significant effects on the model for biomass production ($p<0.05$).

Since different optimum parameters were obtained for maximum biomass and lysozyme production, aeration and pH shift strategies were evaluated to increase the biomass and then secrete the lysozyme from the microorganism to enhance the production. Therefore, optimum conditions determined by response surface methodology were used in these trails. In the beginning of fermentation, production was carried out at the optimum conditions for biomass production and then the conditions were shifted to the levels, which were optimum for lysozyme production. Aeration and pH shift trials provided higher lysozyme production when the shifts were done in the middle of logarithmic phase (24th hour) rather than in the late logarithmic phase (48th hour). A pH and aeration at 48th hour of fermentation cause higher biomass production than the shift at 24th hour because production was conducted at the conditions, which were optimum for biomass for longer time than the shift at 24th hour. As a result of different pH and aeration shift trials, pH shift from 6 to no control without aeration at 24th hour provided more lysozyme production (135 U/ml) than the production (83.75 U/ml) without pH shift at the determined optimum conditions for lysozyme by response surface methodology. Then, a slightly higher lysozyme production (141 U/ml) was obtained by a gradual decrease in pH without pH and aeration control at 25°C than the production with a pH shift from 6 to no control at 24th hour.

Overall, higher lysozyme production amount (141 U/ml) was achieved in biofilm reactor when compared to lysozyme production (110.3 U/ml) in suspended cell batch-fermentation as reported by Huang and Demirci (2009). Figure 3.6 shows the biomass and human lysozyme production and lactose consumption amounts during the fermentation at 25°C without pH and aeration control. Based on these results, maximum human lysozyme production rate was 4 U/ml/h in biofilm reactor with PCS, while the maximum production rate was reported as 2 U/ml/h in suspended cell bioreactor by Huang and Demirci (2009). The reason of the increase in production rate may be increase

in cell population in the reactor and the presence of biofilm population that were already in the log phase. Similarly, Pongtharangkul and Demirci (2006a, b, c) reported that the high biomass density of the biofilm reactor caused a significantly shorter lag time of nisin production relative to a suspended cell reactor.

3.6 Conclusion

In summary, PCS containing polypropylene, soybean hulls, soybean flour, yeast extract, bovine albumin, and salt, (SH-SF-YE-BA-S) was selected based on human lysozyme and biofilm production and minimum lysozyme absorption by PCS. Temperature at 25°C, pH at 4 and no aeration were determined as optimum growth parameters of *K. lactis* K7 for human lysozyme production. As a result of pH and aeration shift strategies, the pH decrease without any control provided the highest lysozyme production level without aeration. Moreover, higher production level and production rate were observed in biofilm reactor than suspended cell reactor. Therefore, this study suggested that biofilm reactors with PCS can be utilized to enhance human lysozyme production.

References

Anderson, M. J. and P. J. Whitcomb. 2000. *DOE Simplified: Practical Tools for Effective Experimentation*. Portland, OR: Productivity Press.

Capdeville, B. and J. L. Rols. 1992. Introduction to biofilms in water and wastewater treatment.In *Biofilms – Science and Technology*, 13-20. L. F. Melo, T. R. Bott, M. Fletcher,and B. Capdeville, ed. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Cheng, K., A. Demirci, and J. M. Catchmark. 2010a. Advances in biofilm reactors for production of value-added products. *Applied Microbiology and Biotechnolgy* 87: 445–456.

Cheng, K., A. Demirci, and J. M. Catchmark. 2010b. Enhanced pullulan production in a biofilm reactor by using response surface methodology. *Journal of Industrial Microbiology and Biotechnolgy* 37: 587–594.

Choi, S., H. Paik, S. Lee, T. Nihira, and Y. Hwang. 2004. Enhanced productivity of human lysozyme by pH-controlled batch fermentation of recombinant *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering* 98(2): 132–135.

Choudhury, B. and T. Swaminathan T. 2006. Lactic acid fermentation in cell-recycle membrane bioreactor. *Applied Biochemistry and Biotechnology* 128:171-84.

Christensen, B. E. and W. G. Characklis. 1990. Physical and chemical properties of biofilms. In *Biofilms*, 93-131. W. G. Characklis and K. C. Marshall, ed. New York, New York: John Wiley& Sons, Inc.

Cruz-Guerrero, A., E. Barzana, M. Garcia-Garibay, and L. Gómez-Ruiza. 1999. Dissolved oxygen threshold for the repression of endo-polygalacturonase production by *Kluyveromyces marxianus. Process Biochemistry* 34: 621-624.

Demirci, A. and A. L. III Pometto. 1995. Repeated-batch fermentation in biofilm reactors with plastic-composite supports for lactic acid production. *Applied Microbiology Biotechnology* 44: 585–589.

Demirci, A., A. L. III Pometto, and K-L.C. Ho. 1997. Ethanol production by Saccharomyces cerevisiae in biofilm reactors. *Journal of Industrial Microbiology and Biotechnology* 19: 299–304.

Demirci, A., T. Pongtharangkul, and A. L. Pometto III. 2007. Applications of biofilm reactors for production of value-added products by microbial fermentation. In *Biofilms in the Food Environment,* 167-189. H. P. Blaschek, H. H. Wang, and M. E. Agle ed. Iowa, USA: Blackwell Publishing and The Institute of Food Technologists.

Garcia-Garibay, M., J. Torres, A. Lopez-Munguia-Canales, and L. T. Casas. 1987. Influence of oxygen transfer rate on β-galactosidase production *Kluyveromyces marxianus*. *Biotechnology Letters* 9(6): 417-.420.

Gilby, C. 2001. Lysozyme – a natural way to manage fermentation risks?. Available at; http://www.cix.co.uk/~cgilby/lysozyme1.pdf. Accessed 20 October 2012.

Gonzalez-Siso, M. I., M. A. Feire-Picos, E. Ramil, M. Gonzalez-Dominguez, A. R. Torres, and M. E. Cerdan. 2000. Respirofermentative metabolism in

Kluyveromyces lactis: insights and perspectives. *Enzyme Microbial Technology* 26: 699-705.

Ho, G. K. L., A. L. Pometto, P. N. Hinz, and A. Demirci. 1997a. Nutrient leaching and end product accumulation in plastic composite support for L-(+)-lactic aicd biofilm fermentation. *Applied Environmental Microbiology* 63: 2524–2532.

Ho, G. K. L., A. L. Pometto, P. N. Hinz, J. S. Dickson, and A. Demirci. 1997b. Ingredient selection for plastic composite supports used for l-(+)-lactic acid biofilm fermentation by *Lactobacillus casei* subsp *rhamnosus*. *Applied and Environmental Microbiology* 63: 2516–2523.

Huang, E. L., and A. Demirci. 2009. Enhanced human lysozyme production by *Kluyveromyces lactis. Food and Bioprocess Technology* 2: 222–228.

Hughey, V. L., and E. A. Johnson. 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Applied and Environmental Microbiology* 53(9): 2165-2170.

Islam, R., J. Kite, A. S. Baker, A. Ching, and M. R. Islam. 2006. Affinity purification of hen egg lysozyme using sephadex G75. *African Journal of Biotechnology* 5(20): 1902-1908.

Iwata, T., R. Tanaka, M. Suetsugu, M. Ishibashi, H. Tokunaga, M. Kikuchi, and M. Tokunaga. 2004. Efficient secretion of human lysozyme from the yeast, Kluyveromyces lactis. *Biotechnology Letters* 26: 1803-1808.

Jayaraman, V. K. 1992. The solution of hollow-fiber bioreactor design equations. *Biotechnology Progress* 8 (5): 462–464.

Jolles, P. and J. Jolles. 1984. What's new in lysozyme research? *Molecular and Cellular Biochemistry* 63: 165 189.

Jolles, P. 1996. *Lysozymes: Model Enzymes in Biotechnology and Biology*. Basel, Boston, Berlin: Birkhauser.

Li, X. L., J. W. Robbins, and K. B. Taylor. 1992. Effects of the levels of dissolved oxygen on the expression of the recombinant proteins in 4 recombinant Escherichia coli strains. Journal of Industrial Microbiology 9(1): 1-9.

MacKenzei, D. A., L. C. G. Gendron, D. J. Jeenes, and D. B. Archer. 1994. Physiological optimization of secreted protein production by *Aspergillus niger*. *Enzyme and Microbial Technology* 16(4): 276:280.

Maullu, C., G. Lampis, T. Basile, A. Ingiani, C. M. Rossolini, and R. Pompei. 1999. Production enriched lysozyme biomass from cheese industry by products. *Journal of Applied Microbiology* 86: 182-186.

Mehaia, M. A. and M. Cheryan. 1984. Hollow fibre bioreactor for ethanol production: Application to the conversion of lactose by *Kluyveromyces fragilis*. *Enzyme and Microbial Technology* 6: 117-120.

Melo, L. F. and R. Oliveira. 2001. Biofilm Reactors. In *Multiphase Bioreactor Design*, 271-309. J. M. S. Cabral, M. Mota, and J. Tramper, ed. New York: NY: Taylor & Francis.

Najafpour, G., H. Younesi, and K.Syaahidah Ku Ismail. 2004. Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. *Bioresource Technology* 92: 251-260.

Palomares, L. A., S. Estrada-Mondaca, and O. T. Ramírez. Production of Recombinant Proteins. In *Methods in Molecular Biology*, vol. 267: *Recombinant Gene Expression: Reviews and Protocols*, 15-51. P. Balbás and A. Lorence, ed. Totowa, NJ: Humana Press Inc.

Pichler, W. J. and P. Campi. 1992. Allergy to lysozyme/egg white-containing vaginal suppositories. *Ann Allergy* 69: 521-525.

Pometto, A. L. III, A.Demirci, and K. E. Johnson. 1997. Immobilization of microorganisms on a support made of synthetic polymer and plant material. U.S. patent 5,595,893.

Pongtharangkul, T. and A. Demirci. 2006a. Evaluation of culture medium for nisin production in repeated-batch biofilm reactor. *Biotechnology Progress* 22: 217–224.

Pongtharangkul, T. and A. Demirci. 2006b. Effects on pH profiles on nisin production in biofilm reactor. *Applied Microbiology and Biotechnology* 71: 804–811.

Pongtharangkul, T. and A. Demirci. 2006c. Effects of fed-batch fermentation and pH profiles on nisin production in suspended-cell and biofilm reactors. *Applied Microbiology Biotechnology* 73: 73–79.

Qureshi, N., B. A. Annous, T. C. Ezeji, P. Karcher, and I. S. Maddox. 2005. Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. *Microbial Cell Factories* 4(24): 1-21.

Richard, M. P., C. C. Ramesh, and M. S. Khem. 1965. A rapid and sensitive assay of muramidase. *Proceedings of The Society for Experimental Biology and Medicine* 119: 384–386.

Schleifer, K. H. and K. Otto. 1972. Peptidoglycan .Types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews* 36(4): 407-477.

Shuler, M. L. and F. Kargi. 2002. Engineering Principles for Bioprocesses. In *Bioprocess Engineering Basic Concepts*, 245-285. Prentice Hall Inc., Upper Saddle River, NJ.

Swinkles, B. W., A. J. J. van Ooyen, and F. J. Bonekamp. 1993. The yeast *Kluyveromyces lactis* as an efficient host for heterologous gene expression. *Antonie van Leeuwenhoek* 64: 187-201.

Yoshimura, K., A. Toibana, K. Kikuchi, M. Kobayashi, T. Hayakawa, K. Nakahama, M. Kikuchi, and M. Ikehara. 1987. Differences between *Saccharomyces cerevisiae* and *Bacillus subtilis* in secretion of human lysozyme. *Biochemical and Biophysical Research Communications* 145(2): 712-718.

Yu, Z., Q. Meng, H. Yu, B. Fan, S. Yu, J. Fei, L. Wang, Y. Dai, and N. Li. 2006. Expression and bioactivity of recombinant human lysozyme in the milk of transgenic mice. *Journal of Dairy Sci*ence 89: 2911–2918.

CHAPTER 4

ENHANCED HUMAN LYSOZYME PRODUCTION IN BIOFILM REACTOR BY MEDIUM OPTIMIZATION

4.1 Abstract

Lysozyme is an antimicrobial compound, which has been used in pharmaceutical and food industries. Chicken egg is the commercial source of lysozyme. However, human lysozyme is more effective and safer than egg-white lysozyme. Human milk is an important source for human lysozyme, but it is not feasible to provide the needed lysozyme commercially. Biofilm reactors provide passive immobilization of cells onto the solid support, which may lead to higher productivity. The aim was to evaluate the fermentation medium composition for enhanced human lysozyme production by *Kluyveromyces lactis* K7 in biofilm reactor with plastic composite supports. Yeast nitrogen base was selected as the best nitrogen source, when compared to the yeast extract and corn steep liquor. Moreover, inhibition effect of NaCl and NH$_4$Cl at the concentrations of 25 mM and 50 mM, respectively, was observed. Three factors Box–Behnken Response Surface design was conducted and the results suggested 16.3% lactose, 1.2% casamino acid, and 0.8% yeast nitrogen base as optimum medium composition for maximum human lysozyme production. Overall, the human lysozyme production by *K. lactis* K7 was increased to 173 U/ml, which is about 23% improvement in biofilm reactor and 57% improvement compared to the suspended-cell fermentation.

4.2 Introduction

Unfortunately several foodborne illness outbreaks are still being reported every year in all over the world. There are many ways to prevent foodborne illnesses including development of more effective processing methods and preventing cross-contamination during production and transportation, and handling phases of foods. One of the methods

is using antimicrobial food additives if the other methods fail. Lysozyme is one of the food additives, which serves as a natural antimicrobial agent (Cunningham et al., 1991).

The natural substrate of lysozyme is murein (peptidoglycan), which is a common cell wall polymer in Gram-negative and Gram-positive bacteria (Madigan et al., 2006). It is a gigantic polymer of $(GlcNAc-MurNAc)_n$ polysaccharide strands cross-linked through short peptide bridges at lactyl groups of the muramic acid residues. Lysozyme catalyzes the hydrolysis (the insertion of a water molecule) of the β (1→4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in murein. The high molecular weight murein is hydrolyzed to low molecular weight muropeptides by lysozyme. This causes a punctured cell wall and leads to lysis of the bacterial cell membrane and consequently cell death or growth inhibition (Meyer, 2003; Salazar and Asenjo, 2007).

The most common source of lysozyme for commercial production is egg-white of chicken. However, egg-white lysozyme may cause immunological problems when used by humans who have an allergy to eggs. Therefore, use of human lysozyme in industry is safer than the use of egg-white lysozyme (Pichler and Campi, 1992). Moreover, human lysozyme has greater antimicrobial activity than egg-white lysozyme. However, it is not feasible to provide needed lysozyme commercially. Therefore, several approaches for the production of human lysozyme have been reported (Porro et al., 2005; Wilken and Nikolov, 2006; Choi et al., 2004; Muraki et al., 1985; Yoshimura et al., 1987). *Kluyveromyces lactis* K7 is a genetically modified microorganism that has effective secretory capacity and crab-tree negative effect, which provides stability of metabolism during environmental changes, in fully oxidative conditions (Verduyn, 1991). These characteristics make *K. lactis* a suitable host cell for the production of human lysozyme on a commercial scale. *K. lactis* K7 is a transformant obtained by complementing the his3-mutation of *K. lactis* WM37 strain. *K. lactis* K7 includes the *K. lactis* GAL 7 promoter, the cDNA sequence coding for human lysozyme fused in-frame to the *K. lactis* killer toxin signal sequence (Schekman, 1982). The lysozyme produced by *K. lactis* K7 is

secreted through the cell wall into culture medium. The secretion sequence is endoplasmic reticulum- Golgi - vesicle-cell surface (Schekman, 1982). Rossolini et al. (1992) reported that *K. lactis* K7 was still able to produce the same amount of lysozyme after growth for over 25 generations. This finding shows the genetic stability of *K. lactis* K7 for large scale production.

To date, human lysozyme production using *K. lactis* K7 has been studied by only few researchers. Maullu et al. (1999) conducted flask studies using cheese whey (pH 4-8) as its growth media at 28ºC. Huang and Demirci (2009) also investigated cultivation conditions to enhance the production of human lysozyme by *K. lactis K7* using shake flasks and a suspended cell bioreactor. Huang and Demirci (2009) achieved to produce human lysozyme (110 U/ml) in suspended cell bioreactor, which costs $1.26/25,000 units, while 25,000 units of chicken egg white lysozyme cost $0.02. Therefore, there is still need to improve the human lysozyme production by *K. lactis* K7 to make the human lysozyme more affordable. Increased cell concentration in the reactor is required for high productivity. For this purpose, biofilm reactors can be used to enhance the fermentation process. Biofilm reactor is a passive immobilized cell reactor (Demirci et al., 2007). Biofilm formation is a natural process whereby microbial cells attach to the support structure without use of chemicals and form thick layers of cells known as "biofilms" (Qureshi et. al, 2005). Many studies showed that using plastic composite supports (PCS) in biofilm reactors can enhance production of ethanol, organic acid, enzymes, and bacteriocin. For example; two to ten times higher ethanol productivity was achieved in PCS ring biofilm reactors (Demirci et al., 1997). The PCS biofilm reactor was also evaluated for lactic acid production of repeated-batch fermentation (Demirci and Pometto, 1995). Higher concentrations of lactic acid were produced by pure- and mixed-culture PCS biofilm reactors (60 and 55 g/L, respectively) when compared with suspension. Up to now, the growth parameters of *K. lactis* K7 were optimized for human lysozyme production in biofilm reactor (Chapter 3). As a result, the production of human lysozyme was increased to 141 U/ml compared to 110 U/ml, which was the highest production level in the literature for suspended cell bioreactor. Also, it is well known that

fermentation medium composition is very essential to meet the need for possible maximum production. Many fermentation medium optimization researches have been done to enhance the production (Tabbene et al., 2009; Pongtharangkul and Demirci, 2006; Tang et al., 2004). Therefore, this study was undertaken to enhance further the human lysozyme production by using *K. lactis* K7 and biofilm reactor with plastic composite support by optimizing the fermentation medium concentration.

4.3 Materials and Methods

4.3.1   Microorganism and medium

Kluyveromyces lactis K7 (ATCC-MYA-413) was obtained from American Type Culture Collection (Manassas, VA). The culture was grown in medium containing 2% (*w/v*) glucose, 1% yeast extract, and 2% peptone for 20-24 h at 25ºC. The working culture was maintained on the agar slant with 20 g/l of glucose, 10 g/l of yeast extract, 20 g/l of peptone, and 20 g/l of agar and transferred to a sterile agar slant biweekly.
Base fermentation medium included 0.67% (w/v) yeast nitrogen base, 9 % lactose, and 1% casamino acid (Huang and Demirci, 2009).

4.3.2 Plastic composite support (PCS) tubes

The PCS tubes were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Pometto et al.(1997). The composition of PCS tube used in this study was determined in our earlier study as polypropylene (50% (*w/w*) and soybean hulls, soybean flour (SF), salts, yeast extract, and dried bovine albumin (Chapter 3).

4.3.3   Batch fermentations in biofilm reactor

4.3.3.1 Biofilm formation in the reactor

Sartorious Biostat B Plus bioreactor (Sartorious, Allentown, PA) equipped with a 2-L vessel was used to construct biofilm reactor, which had 12 PCS tubes (6.5 cm long) bound to the agitator shaft in a grid-like fashion, with six rows of two parallel tubes (Chapter 3). The Figure 4.1 shows the design of the biofilm reactor with PCS. The reactor vessel with PCS was autoclaved with water at 121°C for 90 min. Then, 1.5 l of sterilized fermentation medium was replaced with water aseptically in the reactor vessel with PCS. For biofilm formation, a 24-h grown culture of *K. lactis* K7 (1% *v/v*) was inoculated. Then, to have a stable biofilm on the supports, five repeated-batch fermentations were performed at the (no pH control, 25ºC, 100 rpm, and no aeration) as suggested by our earlier study (Chapter 3).

4.3.3.2. Batch fermentation in biofilm reactor

Batch fermentations were conducted to select the best medium composition in the biofilm reactor, which had already stable biofilm in it. The conditions were 25ºC, no pH control, 150 rpm, and no aeration as suggested by our earlier study (Chapter 3). Each batch fermentation took 80 h. Samples were taken during fermentation periodically for about 80 hours during fermentation. The maximum activities obtained at each condition were used to compare the effects of different treatments on human lysozyme fermentation. After each batch, a fresh sterile medium was prepared at the composition that was going to be tested. The spent fermentation medium in the reactor was pumped aseptically out of the reactor and the fresh sterile medium was pumped in to the biofilm reactor and another batch has been started.
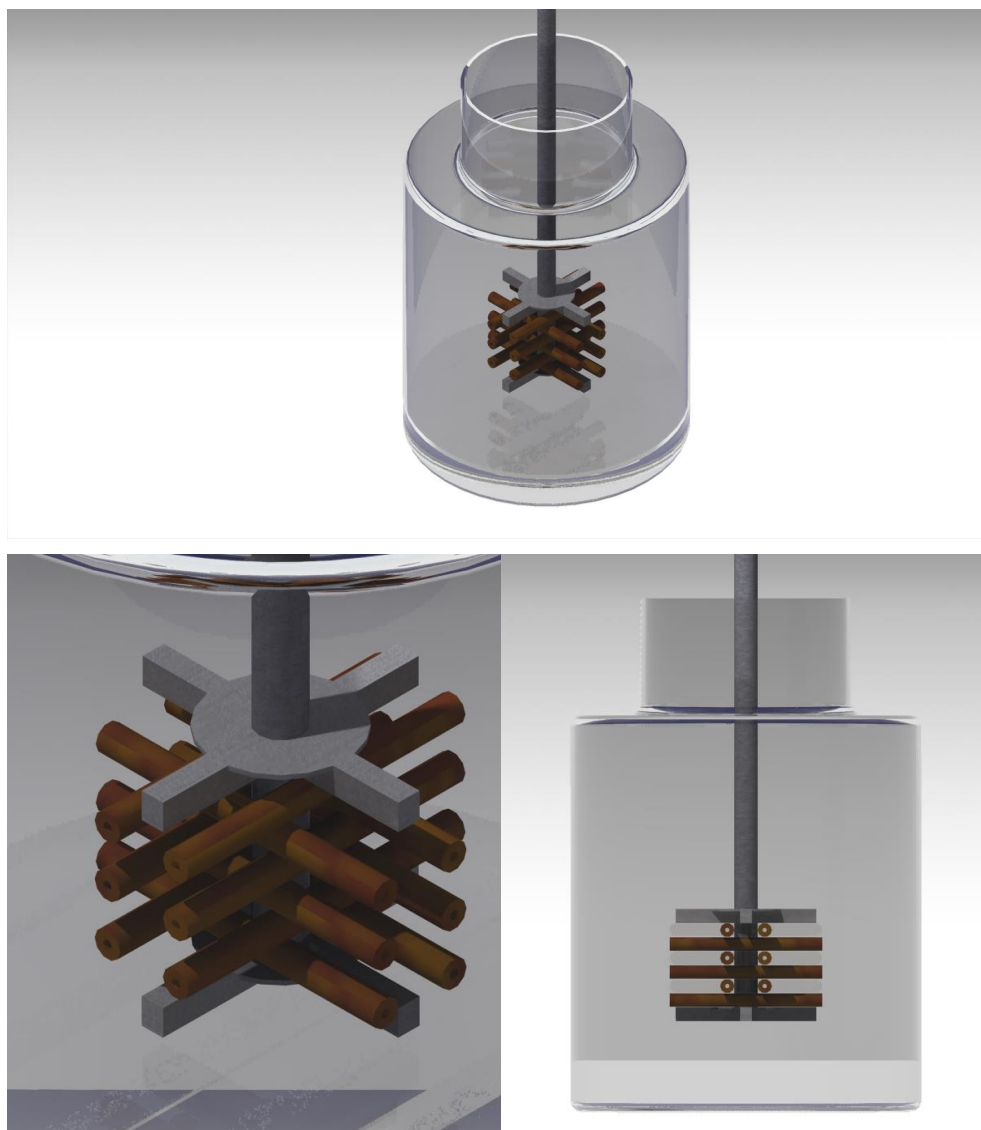
Figure 4.1 Biofilm reactor with PCS set up.

4.3.4 Determination of effects of various nitrogen sources and salts on human lysozyme fermentation

After forming biofilm, 72-h batch fermentations were conducted to evaluate effects of nitrogen sources and salts on lysozyme production with one factor at a time approach.

i)   Yeast extract, yeast nitrogen base, corn syrup liquor were tested at 0.75% (w/v) concentrations to see the effects of different nitrogen sources on production. In

addition to the different nitrogen sources listed above, the fermentation medium consisted of lactose (%9 (w/v)) and casamino acid (1.2 % (w/v)) in nitrogen source evaluations

ii)   Salt addition to the medium was also tested to see the effect of ionic strength on secretion of lysozyme. Therefore, lysozyme fermentations were performed with NH_4Cl and NaCl at 0, 25, and 50 mM. The fermentation medium also included 9% lactose (%9 (w/v)), 0.75% yeast nitrogen source, and 1.2 % casamino acid.

4.3.5 Optimization of fermentation medium

After determination the effects of different nitrogen sources and salts on the lysozyme production, 72-h batch fermentations were conducted for each condition determined by Box-Behnken design of surface response methodology in terms of lactose (5-25 g/l), yeast nitrogen base (0.5-1.5 g/l), and casamino acid (0.5-1.5 g/l) (Table 3). Two replicates were performed for each level. The response variables were biomass and lysozyme production. After the optimum conditions were determined, the validation fermentation runs were duplicated.

4.3.6   Analysis

4.3.6.1 Biomass

Biomass was determined by measuring optical density (OD) using a spectrophotometer (Beckman Coulter, Fullerton, CA) by obtaining absorbance at 600 nm, which will then converted to biomass concentration by using equation: *Biomass* (g/l) $= 0.564{\times}OD_{600}$ as suggested by Huang and Demirci (2009).

4.3.6.2 Lactose concentration

Lactose concentration determinations were conducted using a Waters's high pressure liquid chromatography (HPLC) equipped with a refractive index detector (Waters, Milford, MA). Lactose was separated using Bio-Rad Aminex HPX-87H column (300×7.8

mm; Bio-Rad, Richmond, CA) with 0.8 ml/min of 0.012 N sulfuric acid as mobile phase. The detector temperature and column temperature were maintained at 35 and 65ºC, respectively. Prior to the analysis, the fermentation samples were centrifuged for 2 min at 5,200 × g to separate the cells from the medium. Then, the samples were diluted 3-fold. Finally, the samples and the standard solutions were filtered with 0.2 μm PTFE membrane filters and injected to HPLC. The injection volume was 20 μl. For the quantification, a calibration curve was constructed by plotting peak areas versus concentrations of lactose standards (5, 10, 15, 20, 30 g/l).

4.3.6.3 Lysozyme activity

Lysozyme activity was carried out using a method adapted from Richard et al. (1965). The procedure for lysozyme assay is as follows: A 0.5 mg/ml of *Micrococcus lysodeikticus* cell suspension (Sigma-Aldrich, St. Louis, MO) was prepared using potassium phosphate buffer at pH 6.2. The assay solution consists of 600 μl of 0.5 mg/ml *M. lysodeikticus* cell suspension, 200 μl of 300 mM sodium chloride solution, and 400 μl of sample. The absorbances were measured every 10 s for 1 min at 540 nm. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 A540 change per minute. The slope of the linear regression line refers to the speed of lysis of *M. lysodeikticus* suspension, and the slope estimates the concentration of human lysozyme in unit per milliliter.

4.3.7 Statistical analysis

The statistical analyses were performed by using MINITAB Statistical Software package (Version 15, Minitab Inc., State College, PA). Analysis of variance (ANOVA) and Tukey test were performed for investigating statistically significant differences between production amounts at different conditions. A p-value of <0.05 was considered to be significant.

4.4 Results

4.4.1 Salt and nitrogen source effect

The results of nitrogen source evaluations for human lysozyme production in biofilm reactor were given in Table 4.1. The suspended biomass production increased by the addition of yeast extract and corn syrup liquor (2.85 and 2.26 g/l, respectively) compared to yeast nitrogen base (2.02 g/l) ($p<0.05$). However, lower lysozyme productions were observed by the addition of yeast extract (77.5 U/ml) and corn syrup liquor (72.5 U/ml) compared to yeast nitrogen base (145 U/ml) ($p<0.05$).

Table 4.1 Effects of different nitrogen sources on lysozyme and biomass production.

Nitrogen source	Lysozyme (U/ml)	Biomass (g/l)
Yeast extract	77.5^b	2.85^a
Corn Syrup liquor	72.5^c	2.26^b
Yeast nitrogen base	145.0^a	2.02^c

Different letters within the same column shows the significant difference between treatments based on ANOVA test ($p<0.05$).

Then, batch fermentations with the medium, which includes the best nitrogen source, were conducted with and without NH_4Cl and $NaCl$ (Table 4.2). The salts were tested at two different concentrations (25 and 50 mM). Addition of salts at both low and high concentrations caused decrease in lysozyme and biomass production compared to the one without any added salt ($p<0.05$). The productions with different salts ($NaCl$ and NH_4Cl) were not significantly different ($p\geq0.05$). Increase in the concentrations of salts in the fermentation medium decreased the biomass and lysozyme production significantly ($p<0.05$). For example, while 1.53 g/l of biomass and 67 U/ml of lysozyme were produced with 50 mM $NaCl$ addition, the productions increased to 2.02 g/l of biomass and 145 U/ml of lysozyme when there was no salt in the fermentation medium.

Table 4.2 Effects of salt addition on lysozyme and biomass production.

Salt	Lysozyme (U/ml)	Biomass (g/l)
No Salts	145[a]	2.02[a]
25 mM NaCl	103[b]	1.87[ba]
50 mM NaCl	67[c]	1.53[c]
25 mM NH$_4$Cl	109[b]	1.89[ba]
50 mM NH$_4$Cl	60[c]	1.57[c]

Different letters within the same column shows the significant difference between treatments based on ANOVA test ($p<0.05$).

4.4.2 Optimization of fermentation medium by response surface method

Different lactose (5-25 %), yeast nitrogen base (0.5-1.5 %), casamino acid (5-25 %) were tested to determine optimum medium for lysozyme production in biofilm reactor with plastic composite support. Table 4.3 shows the Box-Behnken design and experimental results for lysozyme and biomass productions as a result of 81-hr batch fermentation. The lysozyme production was varied from 70 to 175 U/ml. The biomass production was ranged from 1.50 g/l to 2.46 g/l. Both lysozyme and biomass production decreased when the lactose concentration was 5 % compared to 25 %. For example; at 25 % lactose, 1 % casamino acid and 1.5 % yeast nitrogen base, the biomass and lysozyme productions were 2.37 g/l and 110 U/ml, respectively, while at 5 % lactose, 1 % casamino acid and 1.5 % yeast nitrogen base, the biomass and lysozyme productions were 1.55 g/l and 75.5 U/ml, respectively. Although the effect of lactose on lysozyme production was higher than the effects of casamino acid and yeast nitrogen base, both casamino acid and yeast nitrogen base concentration decrease caused decrease in lysozyme production ($p<0.05$). For instance; when the lactose and yeast nitrogen base concentrations in the fermentation medium is constant at 5 % and 1 %, respectively, increase in casamino acid from 0.5 % to 1 % resulted in increase in the lysozyme (70 to 77 U/ml) and biomass (1.50 to 1.56 g/l) productions. Similarly, at constant 5 % lactose and 1 % casamino acid when the yeast

nitrogen base were increased from 0.5 to 1.5 %, both lysozyme (69 to 75.5 U/ml) and biomass (1.46 to 1.55 g/l) productions increased.

Table 4.3 Effect of different medium composition combinations on biomass and lysozyme production in biofilm reactor with PCS.

Dependent variables			Experimental		Predicted	
Lactose (%)	Casamino acid (%)	Yeast nitrogen base (%)	Lysozyme (U/ml)	Biomass (g/l)	Lysozyme (U/ml)	Biomass (g/l)
5	0.5	1.0	70.0	1.50	69.6	1.51
5	1.5	1.0	77.5	1.56	76.2	1.57
25	1.0	1.5	110.0	2.37	108.8	2.39
15	1.5	1.5	170.0	2.25	170.8	2.24
25	0.5	1.0	110.0	2.40	111.3	2.38
25	1.5	1.0	115.0	2.46	115.4	2.45
15	1.5	0.5	172.0	2.12	172.1	2.12
15	1.0	1.0	175.0	2.36	174.0	2.36
5	1.0	0.5	69.0	1.46	70.2	1.44
15	0.5	1.5	165.0	2.12	164.9	2.12
5	1.0	1.5	75.5	1.55	76.1	1.54
15	1.0	1.0	173.0	2.34	174.0	2.36
25	1.0	0.5	119.0	2.35	118.4	2.36
15	0.5	0.5	168.0	2.10	167.3	2.11
15	1.0	1.0	174.0	2.38	174.0	2.36

By the application of multiple regression analysis on the experimentally determined values, the following second order polynomial equations were obtained for lysozyme (Eqn. 4.1) and biomass (Eqn. 4.2) productions.

Lysozyme (U/ml)= -69.1563+ 26.36×Lactose + 28.25×Casamino acid + 28.75×Yeast nitrogen base – 0.78×Lactose^2 – 11×Casamino acid^2 – 10×Yeast nitrogen base^2 – 0.78×Lactose×Yeast nitrogen base **..** Eqn. 4.1

Biomass (g/l)= 0.12 + 0.14×Lactose + 0.63×Casamino acid + 1.03×Yeast nitrogen base – 0.003×Lactose^2 – 0.34×Casamino acid^2 – 0.51×Yeast nitrogen base^2 + 0.11×Casamino acid×Yeast nitrogen base **…..……………………………………………..** Eqn. 4.2

The sample variations of 99.96% of lysozyme and 99.87% of biomass were attributed to the factors stated in the models for biomass and human lysozyme production in biofilm reactor with PCS. The predicted R^2 values (predictive capability of the model) were 94.5% and 89.2% for biomass and lysozyme models, respectively. The adjusted R^2 values were 98.9% and 98.04% for lysozyme and biomass models, respectively. Higher adjusted R^2 means higher correlation strength between response and predicted value. Table 4.3 shows the predicted values for biomass and lysozyme production amounts as results of the application of regression models for different combinations of lactose, casamino acid, and yeast nitrogen base concentrations. Tables 4.4 and 4.5 show the analysis of variance results for the models. For both models, the model was significant and the lack of fit was insignificant at the 95% confidence interval.

As a result of performing optimization by optimizer module in Minitab, the maximum lysozyme production was estimated as 176 U/ml when the lactose, yeast nitrogen base, and casamino acid concentrations were 16.3, 0.8, and 1%, respectively. At these conditions, the biomass was estimated as 2.4 g/l. On the other hand the maximum biomass production was estimated as 2.5 g/l when the lactose, yeast nitrogen base, and casamino acid concentrations were 22, 1.1, and 1%, respectively.

Table 4.4 Analysis of variance for lysozyme response surface quadratic model obtained from experimental design.

Source	Degree of freedom	Sum of squares	F value	p value
Regression	9	26022.8	1385.11	0.000
Linear	3	3345.3	2519.84	0.000
Quadratic	3	22615.6	3611.27	0.000
Interaction	3	60.1	28.77	0.003
Residual	5	10.4		
Lack of fit	3	8.4	2.81	0.273
Pure error	2	2.0		
Total	14	26033.1		

Table 4.5 Analysis of variance for biomass response surface quadratic model obtained from experimental design.

Source	Degree of freedom	Sum of squares	F value	p value
Regression	9	1.941	507.53	0.000
Linear	3	1.557	327.84	0.000
Quadratic	3	0.379	297.64	0.000
Interaction	3	0.004	7.12	0.044
Residual	5	0.002		
Lack of fit	3	0.001	1.10	0.508
Pure error	2	0.001		
Total	14	1.943		

For the validation of the model, batch fermentation was conducted at optimum condition levels for biomass and lysozyme production in duplicate. The results were in agreement with the predicted values. At the optimum conditions, the experimentally determined biomass and lysozyme productions were 2.3±0.25 g/l and 173.06±0.89U/ml, respectively. Figure 4.2 shows the lysozyme fermentation at the optimum conditions for human lysozyme production.
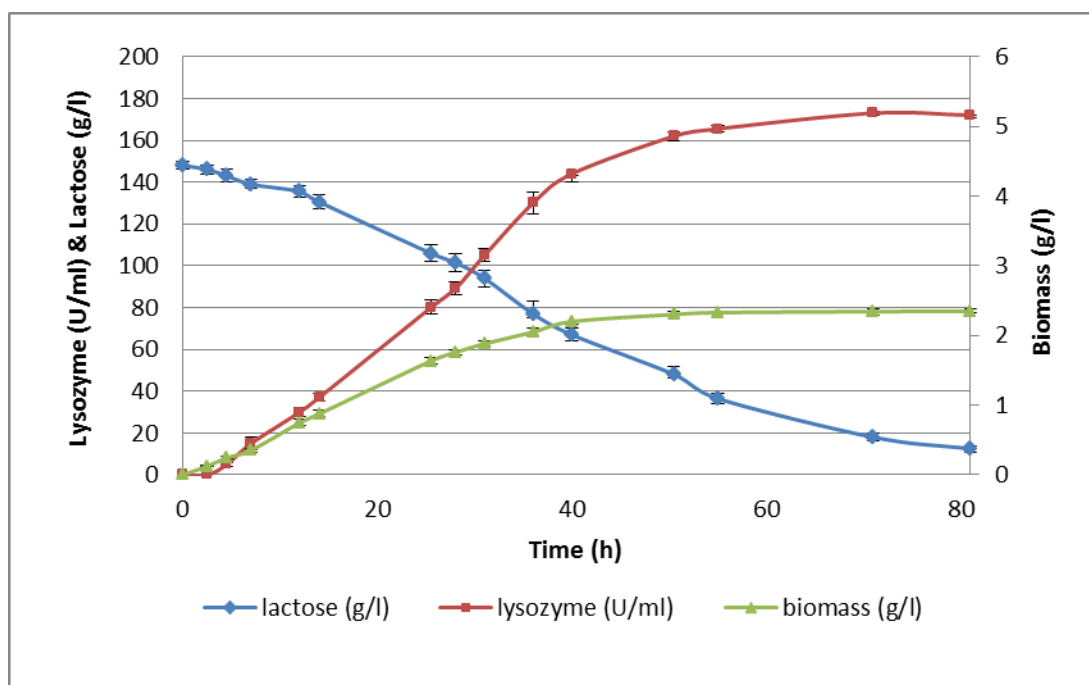
Figure 4.2 Fermentation in biofilm reactor at optimum conditions.

4.5 Discussion

Every microorganism has specific requirements for carbon, nitrogen, and minor nutrient sources. Both growth and production of by-products can be optimized by determination of nutrient requirements of microorganisms. Since immobilization of cells may cause different mass transfer in the reactor, the nutrient requirements of the suspended cell and immobilized cell may become different. The fermentation with immobilized cells can cause a decrease in the substrate inhibition (Galaction et al., 2010).

In this study, the nutrient requirements of *K. lactis* K7 in biofilm reactor were optimized to obtain maximum lysozyme production. First, different nitrogen sources were tested for human lysozyme production by *K. lactis* K7. Although, the biomass production was higher with the medium includes yeast extract, higher lysozyme production was achieved by the addition of yeast nitrogen base ($p<0.05$). Yeast nitrogen base is a complex medium component, which provides salts, vitamins, amino acids to the cells. Iwata et al. (2004) proposed that use of amino acids in the medium affects the extracellular production of human lysozyme by *K. lactis*. In the study of Iwata et al. (2004), addition of 1% casamino acid to medium enhanced the secretion of human lysozyme. Therefore, since casamino acid is a mixture of amino acids except tryptophan and small peptides, it was also included into medium composition in this study. Since *K. lactis* K7 has GAL7 promoter (Rossolini et al., 1992), galactose is needed for the production of human lysozyme. Therefore, lactose was used as carbon source, which provides galactose to induce the production of lysozyme and glucose to increase the biomass level. *K.lactis* K7 is able to transport lactose across the cell membrane by inducible lactose permease system and then by the action of β-galactosidase lactose is hydrolyzed in to glucose and galactose. Then, they are utilized in glycolytic pathways of the cells (Stewart and Russel, 1987).

Then, salts were tested to see whether they facilitate the human lysozyme secretion or not. The medium was supplemented with NH_4Cl and $NaCl$ at concentrations 25-50 mM. In the study of Archer et al. (1990), 50 mM NH_4Cl and $NaCl$ increased the secretion of chicken egg-white lysozyme from *Aspergillus niger.* Malcolm et al. (1989) reported that maintenance of high ionic strength may increase the yield of chicken egg-white lysozyme by restricting its association with fungal cell wall. However, addition of extra salts to the medium inhibited growth of *K. lactis K7* since lower biomass concentrations were observed when compared to the fermentation with the medium without salts ($p\geq0.05$). Since the biomass concentration decreased, this caused decrease in the lysozyme production. Therefore, salts were not included into medium in the optimization of medium by response surface methodology.

Finally, different lactose (5-25%), yeast nitrogen base (0.5-1.5%), casamino acid (0.5-1.5%) were tested to determine optimum medium composition for lysozyme production in biofilm reactor with plastic composite support by Box-Benkhen Design. Figure 4.3, which includes the response surface plots, shows the effects of combinations of different concentrations of lactose, casamino acid, and yeast nitrogen base on human lysozyme production.
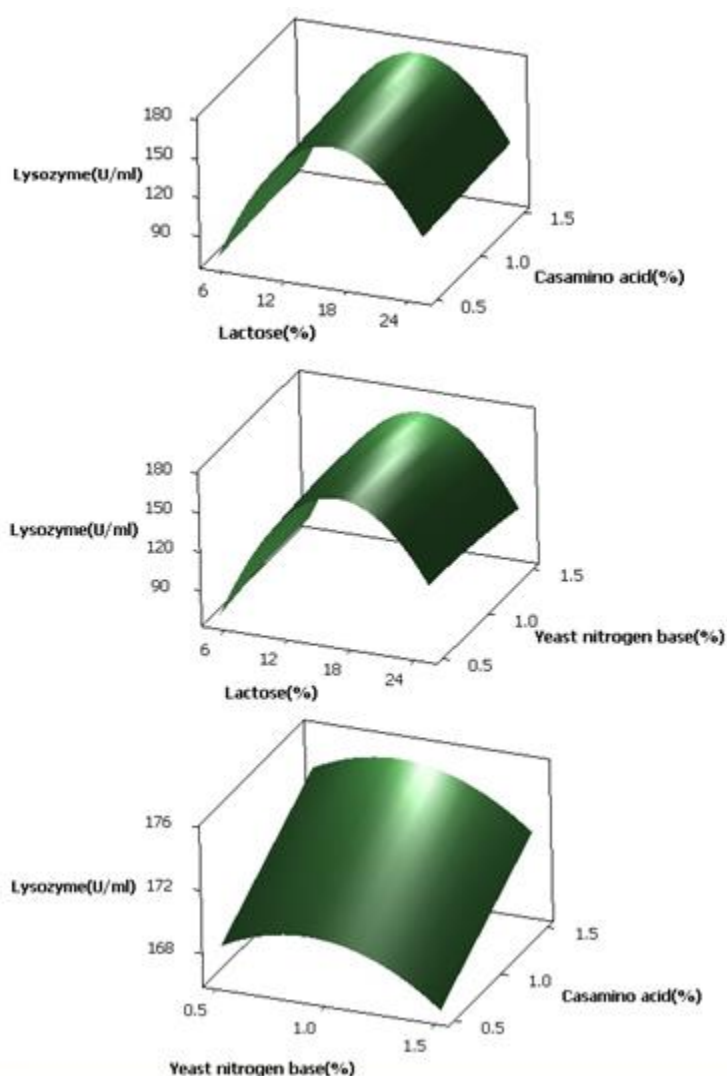
Figure 4.3 Response surface plots for human lysozyme production in biofilm reactor.

The regression models also show the effects of lactose, yeast nitrogen base, and casamino acid on human lysozyme and biomass productions. Eqn. 4.1 shows that lactose, yeast nitrogen base, casamino acid, their quadratic terms, and interaction of lactose and yeast nitrogen base concentrations have significant effects on model for human lysozyme production ($p<0.05$). Similarly, Eqn.4.2 shows that lactose, yeast nitrogen base, casamino acid, their quadratic terms, and yeast nitrogen base and casamino acid interaction have significant effects on the model for biomass production ($p<0.05$).

As a result of the optimization study, the required lactose, casamino acid, and yeast nitrogen base requirements (16, 1.2, and 0.8%, respectively) for maximum lysozyme production in biofilm reactor were found as lower than the requirements (22, 1.1, and 1%, respectively) for maximum biomass production. Although the biomass amount was not significantly different in the conditions which were optimum for biomass and lysozyme productions, the lysozyme production was significantly lower in the conditions for maximum biomass production when compared to the conditions for maximum lysozyme production. The reason of the decrease in lysozyme production may be related to production of other by products, which also caused decrease in pH of the fermentation medium. While the final pH of the medium was around 3.7 at the optimum conditions for lysozyme, the final pH was recorded as around 3 at the conditions optimum for biomass production.

Overall, higher lysozyme production amount was increased to 173 U/ml from 141 U/ml when the fermentation medium was optimized in biofilm reactor in addition to the optimizing growth parameters. The achieved lysozyme production was also significantly higher than the lysozyme production (110.3 U/ml) in suspended cell batch-fermentation reported by Huang and Demirci (2009). Moreover, increased maximum production rate and yield were achieved in biofilm reactor (4.07 U/ml/h and 1.33 lysozyme produced/lactose consumed) compared to suspended cell reactor (2.00 U/ml/h and 1.00 lysozyme produced/lactose consumed). Huang and Demirci (2009) determined the optimum lactose concentration as 9%, while in biofilm reactor the optimum lactose

concentration was found as 16%. The higher lactose concentration requirement may be related to the increase in the cell concentration in biofilm reactor.

4.6 Conclusion

Yeast nitrogen base was selected as the best nitrogen source for human lysozyme production, when compared to the yeast extract and corn steep liquor. Addition of salt caused inhibition in the lysozyme and biomass production. Moreover, 16% lactose, 1.2% casamino acid, 0.8% yeast nitrogen base were determined as optimum nutrient requirements of *K. lactis* K7 for human lysozyme production. Overall, the production level (173 U/ml lysozyme) increased by optimization of the fermentation medium when compared to the production (141 U/ml lysozyme) with original medium, which included 9% lactose, 0.67% yeast nitrogen base, and 1% casamino acid. Overall, the human lysozyme production by *K. lactis* K7 was increased to 173 U/ml, which is about 23% improvement in biofilm reactor and 57% improvement compared to the suspended-cell fermentation.

References

Archer, B. D., D. J. Jeenes, D. A. MacKenzei, G. Brightwell, N. Lambert, G. Lowe, S. E. Radford, and C. M. Dobson. 1990. Henn egg white lysozyme expressed in, and secreted from, *Aspergillus niger* is correctly processed and folded. *Biotechnology* 8: 741-745.

Choi, S., H. Paik, S. Lee, T. Nihira, and Y. Hwang. 2004. Enhanced productivity of human lysozyme by pH-controlled batch fermentation of recombinant Saccharomyces cerevisiae. *Journal of Bioscience and Bioengineering* 98(2): 132–135.

Cunningham, F. E., V. A. Proctor, and S. J. Goetsch. 1991. Egg-white lysozyme as a food preservative: An overview. *World's Poultry Science Journal* 47: 141-163.

Demirci, A., A. L. III Pometto. 1995. Repeated-batch fermentation in biofilm reactors with plastic-composite supports for lactic acid production. *Applied Microbiology Biotechnology* 44:585–589.

Demirci, A., A. L. III Pometto, and K-L.C. Ho. 1997. Ethanol production by Saccharomyces cerevisiae in biofilm reactors. *Journal of Industrial Microbiology and Biotechnology* 19: 299–304.

Demirci, A., T. Pongtharangkul, and A. L. Pometto III. 2007. Chapter 8: Applications of biofilm reactors for production of value-added products by microbial fermentation. In *Biofilms in the Food Environment,* 167-189. H. P. Blaschek, H. H. Wang, and M. E. Agle, ed. Iowa, USA: Blackwell Publishing and The Institute of Food Technologists.

Galaction, A. I., A. M. Lupaşteanu, and D. Caşcaval. 2010. Kinetic Studies on Alcoholic Fermentation Under Substrate Inhibition Conditions Using a Bioreactor with Stirred Bed of Immobilized Yeast Cells. *The Open Systems Biology Journal* 3: 9-20.

Huang, E. L., and A. Demirci. 2009. Enhanced human lysozyme production by *Kluyveromyces lactis*. *Food and Bioprocess Technology* 2: 222–228.

Iwata, T., R. Tanaka, M. Suetsugu, M. Ishibashi, H. Tokunaga, M. Kikuchi, and M. Tokunaga. 2004. Efficient secretion of human lysozyme from the yeast, *Kluyveromyces lactis*. *Biotechnology Letters* 26: 1803-1808.

Madigan, M. T., J. M. Martinko, and J. Parker. 2006. *Brock Biology of Microorganisms* (9th ed.), Prentice-Hall, Inc., Upper Saddle River, NJ.,

Maullu, C., G. Lampis, T. Basile, A. Ingiani, C. M. Rossolini, and R. Pompei. 1999. Production enriched lysozyme biomass from cheese industry by products. *Journal of Applied Microbiology* 86: 182-186.

Meyer, A. S. 2003. Antimicrobial enzymes. In *Food Preservation Techniques.*.P. Zeuthen and L. Bogh-Sorensen. CRC press.

Muraki, M., Y. Jigami, H. Tanaka, F. Kishimoto, H. Agui, and S. Ogino. 1985. Short Communication Expression of Synthetic Human Lysozyme Gene in *Escherichia coli*. *Agricultural and Biological Chemistry* 49 (9): 2829~2831.

Pichler, W. J., and P. Campi. 1992. Allergy to lysozyme/egg white-containing vaginal suppositories. *Annals of Allergy* 69(6): 521-525.

Pometto III, A.L., A. Demirci, and K. E. Johnson. 1997. Immobilization of
microorganisms on a support made of synthetic polymer and plant material. U.S.
Patent No. 5,595,893.

Pongtharangkul, T. and A. Demirci. 2006. Evaluation of culture medium for nisin
production in repeated-batch biofilm reactor, *Biotechnology Progress* 22(1): 217-
224.

Porro, D., M. Sauer, P. Branduardi, and D. Mattanovich. 2005. Recombinant Protein
Production in Yeasts. *Molecular Biotechnology* 31: 245-259.

Qureshi, N., B. A. Annous, T. C. Ezeji, P. Karcher, and I. S. Maddox. 2005. Biofilm
reactors for industrial bioconversion processes: employing potential of enhanced
reaction rates. *Microbial Cell Factories* 4(24): 1-21.

Richard, M. P., C. C. Ramesh, and M. S. Khem. 1965. A rapid and sensitive assay of
muramidase. *Proceedings of The Society for Experimental Biology and Medicine*
119: 384–386.

Rossolini, G. M., M. L. Riccio, E. Gallo, and C. L. Galeotti. 1992. *Kluyveromyces lactis*
rDNA as a target for multiple integration by homologous recombination. *Gene*
119: 75–81.

Salazar, O. and J. A. Asenjo. 2007. Enzymatic lysis of microbial cells. *Biotechnology
Letters* 9: 985–994.

Schekman, R. 1982. The secretory pathway in yeast. *Elsevier Biomedical Press* 0376
5067: 243-246.

Stewart, G. G. and I. Russel. 1987. Chapter 9: Biochemical and genetic control of sugar
and carbohydrate metabolism in yeasts. In *Yeast Biotechnology* 277-310. 277-
310. D. R. Berry, I. Russel, and G. G. Stewart, ed.. London: Allen & Unwin..

Tabbene, O., I. B. Slimene, K. Djebali, M. L. Mangoni, M. C. Urdaci, and F. Limam.
2009. Optimization of medium composition for the production of antimicrobial
activity by *Bacillus subtilis* B38. *Biotechnology Progress* 25(5): 1267-1274.

Tang, X. J., G.Q. He, Q.H. Chen, X.Y. Zhang, and M. A.M. Ali. 2004. Medium
optimization for the production of thermal stable b-glucanase by *Bacillus subtilis*

ZJF-1A5 using response surface methodology. *Bioresource Technology* 93: 175–181.

Wilken, L. and Z. L. Nikolov. 2006. Factors influencing recombinant human lysozyme extraction and cation exchange adsorption. *Biotechnology Progress* 22: 745-752.

Verduyn, C. 1991. Physiology of yeasts in relation to biomass yields. *Antonie Van Leeuwenhoek* 60: 325–353.

Yoshimura, K., A. Toibana, K. Kikuchi, M. Kobayashi, T. Hayakawa, K. Nakahama, M. Kikuchi, and M. Ikehara. 1987. Differences between *Saccharomyces cerevisiae* and *Bacillus subtilis* in secretion of human lysozyme. *Biochemical and Biophysical Research Communications* 145(2): 712-718.

CHAPTER 5

EFFECTS OF FED-BATCH AND CONTINUOUS FERMENTATIONS ON HUMAN LYSOZYME PRODUCTION BY *KLUYVEROMYCES LACTIS* K7 IN BIOFILM REACTORS

5.1 Abstract

Lysozyme is a lytic enzyme, which has antimicrobial activity. It has been used for food and pharmaceutical applications. Lysozyme production needs to be enhanced to meet its current public demand, because currently egg is used for commercial lysozyme production and it poses immunological problems and it is four-times less effective than human lysozyme. In our earlier study, biofilm reactors have been evaluated for human lysozyme production by *Kluyveromyces lactis* K7 in batch fermentation, which demonstrated significant improvement in production of human lysozyme. Therefore, this study was undertaken to evaluate fed-batch and continuous fermentations for the human lysozyme production by *K. lactis* K7 in biofilm reactor. Results showed that addition of lactose in the mid-log phase (177 U/ml) compared the late-log phase (174 U/ml) provided higher lysozyme production. However, addition nitrogen source addition did not provide any significant benefit. Moreover, fed-batch fermentation with glucose as initial carbon source and continuous lactose addition with 0.6 ml/min for 10 h demonstrated significantly higher lysozyme production (187 U/ml) compared to the batch fermentation (173 U/ml). In continuous fermentation, the biofilm reactor provided significantly higher productivity (7.5 U/ml/h) compared to the maximum productivity in suspended-cell bioreactor (4 U/ml/h). In conclusion, both fed-batch and continuous fermentations in biofilm reactors enhanced the production of human lysozyme.

5.2 Introduction

Lysozyme is an enzyme, which degrades the common cell wall polymer in Gram-positive and Gram-negative bacteria. Because of its antimicrobial properties, lysozyme has many applications in food and pharmaceutical industry (Schleifer and Otto, 1972). Commercially, lysozyme is currently produced from egg white (Naudi, 2000). However, individuals can be sensitive egg-white lysozyme due the allergic reactions to egg-white lysozyme (Pichler and Campi, 1992). Therefore, human lysozyme is preferred not only for non-allergic characteristics, but also because it has four times more activity (Naudi, 2000). Sequence analysis shows that lysozyme from chicken egg white is only 60% identical with human lysozyme, which indicates that antibodies against human and chicken egg white lysozyme are not same (Faure and Jollès, 1970).

Human milk is an important source for human lysozyme, but human breast milk is not a good source for commercial production of lysozyme (Yu et al., 2006). Therefore, several approaches have been for the expression of recombinant human lysozyme in plants, mice, bacteria, and yeasts (Yu et al, 2006; Muraki et al, 1985; Yoshimura et al., 1987; Choi et al., 2004; Iwata et al., 2004). *Kluyveromyces lactis* K7 is a genetically modified microorganism which produces human lysozyme with lactose as an inducer (Iwata et al., 2004). To date, human lysozyme production using *K. lactis* K7 has been studied in flask (Maullu et al., 1999), suspended-cell (Huang and Demirci, 2009)], and recently biofilm reactors (Chapters 3 and 4). Maullu et al. (1999) achieved five-fold higher human lysozyme production in cottage cheese whey, which includes 5.5% lactose compared to synthetic media (2% glucose, 0.67% yeast nitrogen base). Huang and Demirci (2009) studied the production of human lysozyme by batch and fed-batch fermentation in suspended cell bioreactor and concluded higher production amount in fed-batch fermentation (123.6 U/ml) than batch fermentation (110.3 U/ml). The production of human lysozyme in a biofilm reactor with plastic composite support (PCS) was evaluated (Chapter 3).

Biofilm reactors utilize passive immobilization of cells on to a solid support. Therefore, they provide increased biomass concentration, which can lead to high productivity. PCS includes polypropylene as a solid matrix and agricultural by-products to sustain cell growth. Therefore, in addition to high biomass concentration, biofilm reactors with PCS have many advantages including; operation stability, reduction of risk of washing the cells out at high dilution rates, low capital cost and operational cost, enhance resistance of microorganisms to extreme conditions, lower nutrient requirement, and have longevity and durability for long-term fermentation (Demirci et al., 2007). Many studies showed that using biofilm reactors with plastic composite support (PCS) can enhance production of ethanol, organic acid, enzymes, and bacteriocin (Demirci and Pometto 1995; Demirci et al., 1997). Because the properties of solid support, such as surface charge, hydrophobicity, porosity, roughness and density can affect biofilm formation. Different PCSs were evaluated (Chapter 3) and selected the PCS, which includes polypropylene, soybean hull, soybean flour, bovine albumin, and salts based on biofilm formation on PCS (CFU/g), human lysozyme production (U/ml), and absorption of lysozyme inside the support. Then, they optimized the growth conditions of *K. lacis* K7 and concluded that the optimum conditions for biomass and lysozyme productions were different (27 °C, pH 6, 1.33 vvm for biomass production; 25 °C, pH 4, no aeration for lysozyme production). After evaluating different pH and aeration shift strategies, the results showed that significantly higher lysozyme production amount (141 U/ml) was achieved at 25°C without pH and aeration control. Then, the fermentation media was optimized (Chapter 4) and achieved 173 U/ml lysozyme production in medium including 16.3% lactose, 1.2% casamino acid, 0.8 % yeast nitrogen base. As a result, higher lysozyme production was achieved in the biofilm reactor than suspended cell reactor and the production rate was doubled. However, the production can be further enhanced by the application of fed-batch and continuous fermentations, which eliminate substrate limitation. Gonzales Siso (1994) conducted fed-batch fermentation for the production of β-Galactosidase by *K. lactis* and extended maximum level of production on milk whey. While the maximum production level was achieved from 35 to 50 h in batch fermentation for the production of β-Galactosidase, with fed-batch fermentation with flow rate of 0.17 ml/h, biomass and

enzyme activity were at the maximum level of the batch culture from 30 up to 100 h. Paciello et al. (2010) evaluated fed-batch fermentation for glucoamylase production by *K. lactis* and observed that fed-batch mode avoid limitations in oxygen transfer, and achieved high cell density. Therefore, this study was undertaken to evaluate fed-batch and continuous fermentations in biofilm reactors to enhance human lysozyme production by *K. lactis* K7.

5.3 Materials and Methods

5.3.1   Microorganism and medium

Kluyveromyces lactis K7 (ATCC-MYA-413) was obtained from American Type Culture Collection (Manassas, VA). The cultivation medium included 2% (*w/v*) glucose, 1% (*w/v*) yeast extract, and 2% (*w/v*) peptone and the conditions were 25ºC for 24 h. The working culture was maintained on the agar slant, which contains the same composition as the cultivation medium and 2% (w/v) agar.

The fermentation base medium included 16.3% lactose, 1.2% casamino acid, and 0.8 % yeast nitrogen base as recommended by our earlier study (Chapter 4).

5.3.2   Plastic composite support (PCS) tubes

The PCS tubes were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw corotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Pometto et al. (1997). The composition of PCS tubes which were used in this study, included polypropylene (50% (*w/w*)), soybean hulls (35%), soybean flour (SF) (5%), salts (2 g/kg of sodium acetate, 1.2 g/kg of $MgSO_4 \cdot 7H_2O$, and 0.06 g/kg of $MnSO_4 \cdot 7H_2O$), yeast extract (5%), and dried bovine albumin (5%) (Chapter 3). After polypropylene and other ingredients of PCS were mixed, they were extruded at 13 rpm through a medium pipe die. The barrel temperatures were 200, 220, and 200°C and the die temperature was

165°C. The PCS tubes were produced in cylindrical shape with a wall thickness of 2.5 mm and an outer diameter of 10.5 mm.

5.3.3   Biofilm reactor set-up

A Sartorious Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel was used to construct the biofilm reactor with 12 PCS tubes (6.5 cm long) bound to the agitator shaft in a grid-like fashion, with six rows of two parallel tubes. Figure 5.1 shows the biofilm reactor design.
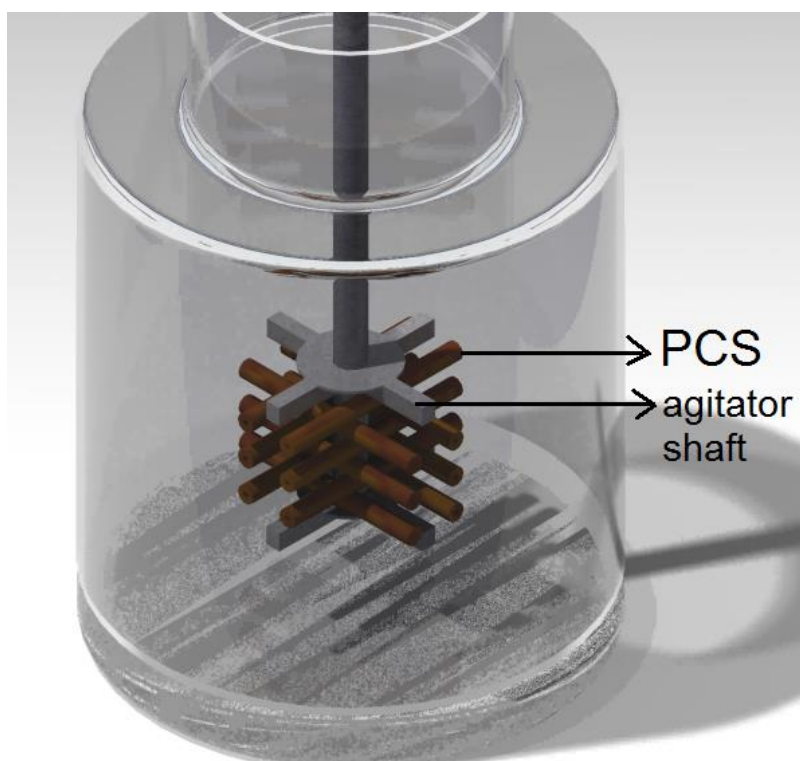
Figure 5.1 Biofilm reactor design with PCS tubes.

The reactor vessel with PCS was autoclaved with water at 121°C for 90 min. Then, 1.5 L of sterilized fermentation medium was added aseptically to the reactor vessel with PCS. For biofilm formation, a 24-h grown culture of *K. lactis* K7 (1% *v/v*) was inoculated. Then, five repeated-batch fermentations were performed at the conditions (no pH control, 25°C, 150 rpm, and no aeration) as recommended by our earlier study (Chapter 3).

5.3.4　　　Fed-batch fermentation

After five repeated batch fermentations were conducted to establish biofilm on the PCS tubes as described above, the fermentation was started by adding the base fermentation medium into biofilm reactor without inoculation of *K. lactis* K7, which already exists in the biofilm. The fermentation conditions were no pH control, 25ºC, 150 rpm, and no aeration as recommended by our earlier study (Chapter 3). Different fed-batch fermentation strategies were evaluated to determine the best time and concentration of extra carbon source addition as well as effects of nitrogen source addition, and glucose as a starting carbon source, and rate of extra carbon source addition. Specifically, the following strategies were evaluated:

i) *Lactose (500 g/l) addition at mid-log phase (28 h) and (late-log phase (38 h):* The initial base fermentation medium included 16.3% lactose, 1.2% casamino acid, and 0.8 % yeast nitrogen base . The consumed lactose was calculated up to 28 h and 38 h of fermentation and a sterile concentrated lactose solution (500 g/L) was added to increase the lactose concentration to back to ~140 g/l of lactose in the reactor.

ii) *Lactose and nitrogen addition:* After the best time was selected for lactose addition by the trials described above, nitrogen (1.2% casamino acid, 0.8 % yeast nitrogen base) was also added while adding the lactose to prevent from the diluting nitrogen source. The composition of initial base fermentation medium was kept the same as 16.3% lactose, 1.2% casamino acid, 0.8 % yeast nitrogen base.

iii) *Continuous lactose addition at a flow rate at between 28 and 38 h:* The lactose (400 g/l) addition was done at between mid-log and late-log phase. The flow rate was adjusted at 0.6 ml/min to make the lactose concentration ~140 g/l at 38 h. Similar to the previous runs, the fermentation was started with the base fermentation medium including 16.3% lactose, 1.2% casamino acid, and 0.8 % yeast nitrogen base.

iv) *Glucose (90 g/l) as an initial carbon source and lactose addition:* The initial fermentation medium included 90 g/l glucose, 1.2% casamino acid, and 0.8% yeast nitrogen base and lactose was added with different scenarios as listed below.

    a. Lactose (500 g/l) addition at 28 h to make the lactose level 16.3%.

    b. Lactose addition at different concentrations (160, 400, 500 g/l) at between 28 and 38 h at 0.6 ml/min flow rate.

After selecting the best fed- batch strategy, the same fed-batch fermentation strategy was evaluated in a suspended-cell bioreactor as a control. As soon as initial fermentation base medium was inoculated with a 24-h grown culture of *K. lactis* K7 (1% *v/v*), the fed-batch fermentation was started in a suspended cell bioreactor.

5.3.5   Continuous fermentation

Continuous fermentation was conducted at the pre-determined optimum conditions (no pH control, 25ºC, 150 rpm, and no aeration) from our previous study for batch fermentation (Chapter 3). The initial fermentation medium and the continuously added fermentation medium included 16.3% lactose, 1.2% casamino acid, 0.8 % yeast nitrogen base, which is the optimum medium determined (Chapter 4). The fermentation medium was continuously added and the effluent continuously collected from the reactor at the same rate. Different dilution rates (0.03-0.08 h^{-1}) were evaluated to determine optimum productivity in continuous fermentation in both suspended cell and biofilm reactors.

5.3.6   Analysis

5.3.6.1 Biomass

Biomass was determined by measuring optical density (OD) using a spectrophotometer (Beckman Coulter, Fullerton, CA) by obtaining absorbance at 600 nm, which was then converted to biomass concentration by using equation: : *Biomass* (g/l) $= 0.564 \times OD_{600}$ as suggested by (Huang and Demirci, 2009).

5.3.6.2 Lactose and glucose concentration

Lactose concentration determinations were conducted using a Waters's high pressure liquid chromatography (HPLC) equipped with a refractive index detector (Waters, Milford, MA). Lactose was separated using a Bio-Rad Aminex HPX-87H column (300×7.8 mm; Bio-Rad, Richmond, CA) with 0.8 ml/min of 0.012 N sulfuric acid as the mobile phase. The detector temperature and column temperature were maintained at 35 and 65ºC, respectively. Prior to the analysis, the fermentation samples were centrifuged for 2 min at 5200 g to separate the cells from the medium. Then, the samples were diluted 3-fold. Finally, the samples and the standard solutions were filtered with 0.2 µm PTFE membrane filters and injected to HPLC. The injection volume was 20 µl. Identification was done with comparison of retention times of compounds and external standard. For the quantification, a calibration curves were constructed by plotting peak areas versus concentrations of lactose and glucose standards (5, 10, 15, 20, 30 g/l).

5.3.6.3 Lysozyme activity

Lysozyme activity was determined using a method adapted from Richard et al. (1965). The assay procedure for lysozyme assay is as follows: A 0.5 mg/ml of *Micrococcus lysodeikticus* cell suspension (Sigma-Aldrich, St. Louis, MO) was prepared using a potassium phosphate buffer at pH 6.2. The assay solution consists of 600 µl of 0.5 mg/ml *M. lysodeikticus* cell suspension, 200 µl of 300 mM sodium chloride solution, and 400 µl of sample. The absorbances were measured every 10 s for 1 min at 540 nm. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 A_{540nm} change per minute. The slope of the linear regression line refers to the speed of lysis of *M. lysodeikticus* suspension, and the slope estimates the concentration of human lysozyme in unit per milliliter as shown below:

$$\text{Lysozyme (U/ml)} = (\text{slope} / 0.4 \text{ ml sample}) \times 1000 \quad \text{…………..……} \quad \text{Eqn. 5.1}$$

5.3.7   Statistical Analysis

The statistical analyses were performed using the MINITAB Statistical Software package (Version 15, Minitab Inc., State College, PA). Analysis of variance (ANOVA) and Tukey's test was performed to determine statistically significant differences between production amounts at different trails. A p-value of <0.05 was considered to be significant. All fermentation runs were conducted in duplicate.

5.4 Results and Discussion

Fed-batch and continuous fermentations were conducted to eliminate substrate limitation or product inhibition. The optimum growth conditions and medium composition for batch human lysozyme production by *K. lactis* K7 in a biofilm reactor with PCS were determined by our previous studies (Chapters 3 and 4). The fed-batch and continuous fermentation was conducted at the optimum conditions determined for batch fermentation, which are 25°C, no aeration, and no pH control with 150 rpm agitation.

5.4.1   Fed-batch fermentation

The fed-batch trials were based on the previously determined optimum medium composition for batch fermentation in a biofilm reactor (16.3% lactose, 1.2% casamino acid, 0.8% yeast nitrogen base). Earlier in Chapter 4, it was reported that high concentration of lactose cause inhibition in the production of lysozyme. Therefore, in fed-batch fermentation, the times for extra substrate addition were selected as late-log phase (38 h) and the mid-log phase (28 h) to allow *K. lactis* K7 the time to use the initial carbon source. For this reason, the lactose consumption was estimated from the batch fermentation data from our previous study (Chapter 4) until the carbon source addition time and additional lactose were added at the concentration of 500 g/l to bring the concentration back to about 140 g/l. The effects of different times on production are given in Table 5.1.

Table 5.1 Effects of time, lactose, and nitrogen sources on fed-batch fermentation.

Fed Batch Strategies	Lysozyme (U/ml)	Production rate (U/ml/h)	Biomass (g/l)
Control (Batch Fermentation)	173±0.89[b]	4.07±0.05[b]	2.30±0.23[b]
Feeding with lactose at 38 h	174±0.95[b]	4.10±0.08[b]	2.22±0.32[b]
Feeding with lactose at 28 h	177±0.82[a]	4.30±0.04[a]	2.42±0.29[a]
Feeding with lactose and nitrogen at 28 h	178±0.54[a]	4.38±0.05[a]	2.44±0.34[a]
Feeding with lactose, feeding rate 0.6 ml/min	178±0.43[a]	4.27±0.07[a]	2.44±0.25[a]

Different superscript letters within the same column shows the significant difference between treatments based on ANOVA test ($p<0.05$).

The fed-batch fermentation with carbon source addition at 28 h was shown in Figure 5.2. As a result, slightly higher human lysozyme production was observed when the carbon source was added at the mid-log phase than at the late-log phase. Then, at the same conditions, the same concentrations of yeast nitrogen base and casamino acid with initial medium were also added to the reactor at the 28 h of the fermentation to see the effect nitrogen source addition, but no significant increase in the human lysozyme production (178 U/ml) was observed (p≥0.05). Then, lactose was added continuously at between 28 and 38 h. The lactose consumption rate was calculated as 3±0.35 g/l/h at between 28 and 38 h. Therefore, the lactose feeding rate was adjusted as 0.6 ml/min at the 400 g/l concentration, which compensated the consumed lactose until 38 h and made the lactose concentration at around 140 g/l at 38 h. As a result of the addition of extra carbon with a rate, similar production amount (178U/ml) was observed when compared to the fed-batch fermentation with direct addition of extra lactose at 28 h (p≥0.05). Higher human lysozyme production rate (4.3 U/ml/h) was also achieved when lactose was added at 28 h compared to the batch fermentation and fed-batch fermentation with lactose addition at 38 h (4.1 U/ml/h) (p<0.05) and neither addition of nitrogen sources (4.38 U/ml/h) and continuous addition of lactose (4.27 U/ml/h) did not result in any significant increase in the production rate (p≥0.05). Moreover, although some lost in the human lysozyme production was reported toward the end of the fermentation (Chapter 4), surprisingly the

human lysozyme production did not show any lost during the fed-batch fermentation. The reason might be higher biomass concentration due to higher carbon source and the presence of the lactose in the 72 h of fermentation for human lysozyme production.
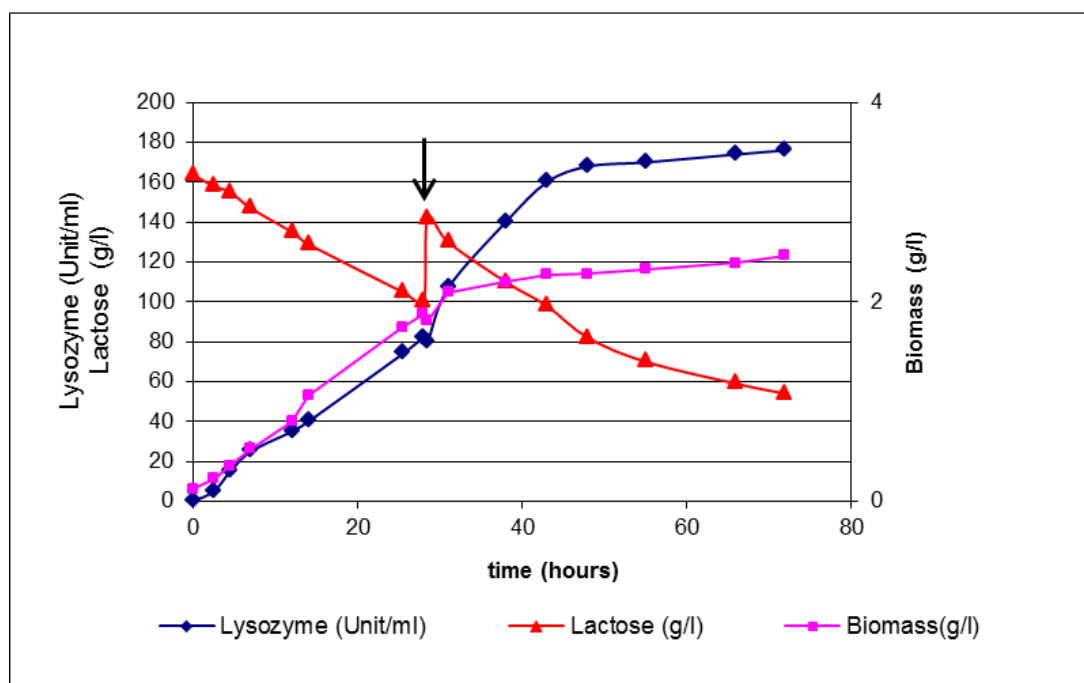
Figure 5.2 Fed-batch fermentation with lactose addition at 28 h. The arrow shows the lactose addition time.

A similar trend was also observed for biomass production. Higher biomass production was observed in fed-batch fermentation with lactose addition at 28 h and continuously than batch fermentation and fed-batch fermentation with lactose addition at 38 h (p<0.05). By fed-batch fermentation, addition of extra substrate in the mid-log phase provided extra substrate for microbial growth. As seen in Figure 5.2, there was still biomass production after 42 h while it was reported in Chapter 3 that the biomass stayed almost stable after 42 h in batch fermentation.

The fed-batch fermentation trials with glucose as the initial carbon source and addition of various lactose concentration feeds are shown in Table 5.2. Because *K. lactis* K7 has GAL7 promoter (Rossolini et al., 1992), lactose was fed to induce the production of human lysozyme. The human lysozyme production started when the lactose concentration was higher than glucose concentration at around 35 h of fermentation.

Table 5.2 Fed-batch fermentation with glucose as initial carbon source.

Fed Batch Strategies	Lysozyme (U/ml)	Production rate (U/ml/h)	Biomass (g/l)
Feeding lactose at 28 h	142 ± 0.95^b	5.2 ± 0.05^b	5.85 ± 0.55^b
Feeding with 160 g/l lactose, 0.6 ml/min	127 ± 0.95^c	2.15 ± 0.04^c	5.95 ± 0.35^b
Feeding with 400 g/l lactose, 0.6 ml/min	187 ± 0.95^a	5.9 ± 0.05^a	6.32 ± 0.6^a
Feeding with 500 g/l lactose, 0.6 ml/min	186 ± 0.95^a	5.9 ± 0.09^a	6.34 ± 0.4^a

Different superscript letters within the same column shows the significant difference between treatments based on ANOVA test ($p<0.05$).

At the first trial, the initial glucose concentration was 90 g/l and lactose was fed at the concentration of 500 g/l to make the concentration at around 16.3% at 28 h. Although the production rate (5.2 U/ml/h) was significantly higher than the batch fermentation (4.07 U/ml/h), lower production in fed-batch with initial glucose and direct lactose addition (142 U/ml) was observed when compared to the batch fermentation (173 U/ml/h) ($p<0.05$). The reason might be depletion of lactose after 72 h in fed-batch fermentation. Moreover, since there was still glucose at 28 h, addition of all lactose at one time might cause higher concentration of carbon source than *K. lactis* K7 can tolerate. Therefore, lactose at different concentrations was fed with the rate of 0.6 ml/min at between 28 and 38 h of fermentation. Feeding with lactose at the concentration of 160 g/l may have not provided enough lactose to achieve higher concentration of human lysozyme when compared to the batch fermentation ($p<0.05$). Therefore, in addition to production amount (127 U/ml), significantly lower production rate (2.15 U/ml/h) was observed in

fed-batch fermentation with glucose as initial substrate and continuous addition of 160 g/l lactose than the batch fermentation (p<0.05). Significantly higher human lysozyme production (187 U/ml/h) and production rate (5.9 U/ml/h) was observed when the fermentation started with 90 g/l glucose and then fed with 400 g/l lactose with a 0.6 ml/min feeding rate than the batch fermentation and other fed-batch fermentation trials (p<0.05). Moreover, significantly higher biomass production (6.32 g/l) in fed-batch fermentation with glucose as initial substrate and continuous 400 g/l lactose addition was observed when compared to biomass production (5.85 g/l) in fed-batch with glucose as initial substrate and direct lactose addition (p<0.05). However, no significant difference in the production levels was observed when the feeding concentration was increased to 500 g/l lactose from 400 g/l lactose (p≥0.05). Fed-batch fermentation with glucose as the initial carbon source and 400 g/l lactose addition at a rate of 0.6 ml/min at between 28 and 38 h is given in Figure 5.3. The production rate (5.9 U/ml/h) in fed-batch fermentation with glucose as the initial carbon source and continuous lactose addition was significantly higher than the batch fermentation (4 U/ml/h).

Finally, the fed-batch fermentation was performed in suspended cell bioreactor as a control. The fermentation was started with 90 g/l glucose and fed with 400 g/l lactose with 0.6 ml/min from 28 to 38 h. The biomass production reached to 5.97 g/l and human lysozyme production was 120 U/ml. In addition to biomass and lysozyme productions, maximum human lysozyme production rate (2.46 U/ml/h) was also significantly lower than the fed-batch fermentation in biofilm reactor (5.9 U/ml/h) (p<0.05). It is very clear that passive immobilized cells in biofilm reactor can cause such a significant difference. Huang and Demirci (2009) also conducted fed-batch fermentation for human lysozyme production in fed-batch fermentation. The fermentation started with 4.5% glucose and fed with 9% lactose and observed 4.14 g/l biomass, 100 U/ml of lysozyme and 1.6 U/ml/h of productivity at the end of 100 h of fermentation.
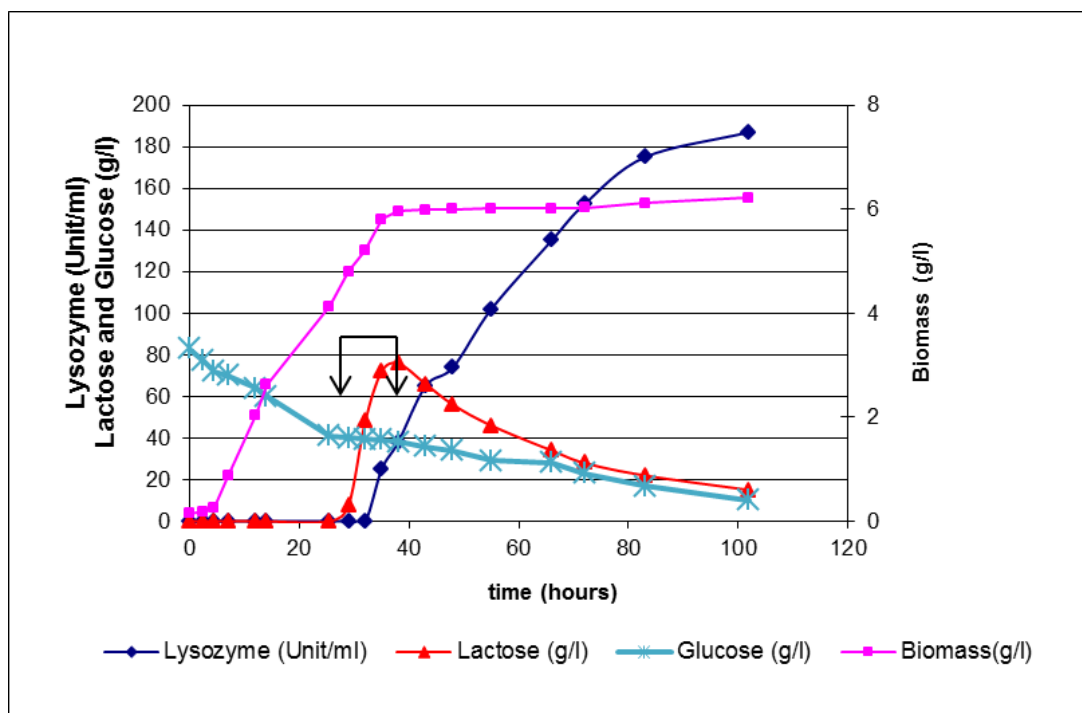
Figure 5.3 Fed-batch fermentation with 90 g/l glucose and feeding with 400 g/l lactose, feeding rate 0.6 ml/min at between 28 and 38 h. The lactose addition was done between the two arrows.

5.4.2 Continuous fermentation

Different dilution rates were evaluated to determine optimum dilution rate for human lysozyme production in continuous fermentation. Continuous fermentation was conducted in both suspended cell and biofilm reactors to see the effect of biofilm reactors. In continuous fermentation, the dilution rate should be adjusted to allow microorganisms enough time to grow and produce the product at higher rates. The cells can be washed out of the reactor at high dilution rates and the productivity can decrease. Figures 5.4 and 5.5 show the productivity and production levels at various dilution rates for biofilm and suspended-cell reactors, respectively.

Figure 5.4 Lysozyme production and productivity at various dilution rates in biofilm reactor.

Although biomass level in the suspended-cell bioreactor (1.95 g/l) was higher at 0.03 and 0.04 h^{-1} dilution rates than in the biofilm reactor (1.78 g/l), and because of the existence of passive immobilized cell on to the PCS tubes, the lysozyme production (141 U/ml) in the biofilm reactor was significantly higher than the production (107 U/ml) in the suspended cell bioreactor (p<0.05). While the biomass and lysozyme production started to decrease at 0.05 1/h dilution rate in suspended cell bioreactor, the decrease in the productions in the biofilm reactor were observed as the dilution rate reached to 0.06 h^{-1}.

While the optimum dilution rate in the biofilm reactor was determined as 0.055 h^{-1}, which provided 7.5 U/ml/h of human lysozyme productivity, the optimum dilution rate in suspended cell reactor was 0.04 h^{-1}, which provided 4.16 U/ml/h of human lysozyme productivity. Continuous fermentation provided significantly higher production rates in both the suspended cell bioreactor and the biofilm reactor when compared to batch

fermentation (p<0.05). It was also reported that the production rate in a biofilm reactor for batch mode was 4 U/ml/h (Chapter 4), while Huang and Demirci (2009) reported that the production rate in a suspended cell reactor in batch mode was 2 U/ml/h. Based on the batch fermentation data (Chapter 4), the maximum specific growth rate was 0.08 h^{-1} in biofilm reactor. At the dilution rate of 0.08 h^{-1}, the production rate was 3.6 U/ml/h in the biofilm reactor, but the production in the suspended cell reactor decreased to almost zero. The reason might be the passive immobilization of the cells on to the PCS tubes, which prevent washing the cells out of the reactor, and lower specific growth rate in suspended-cell bioreactor.
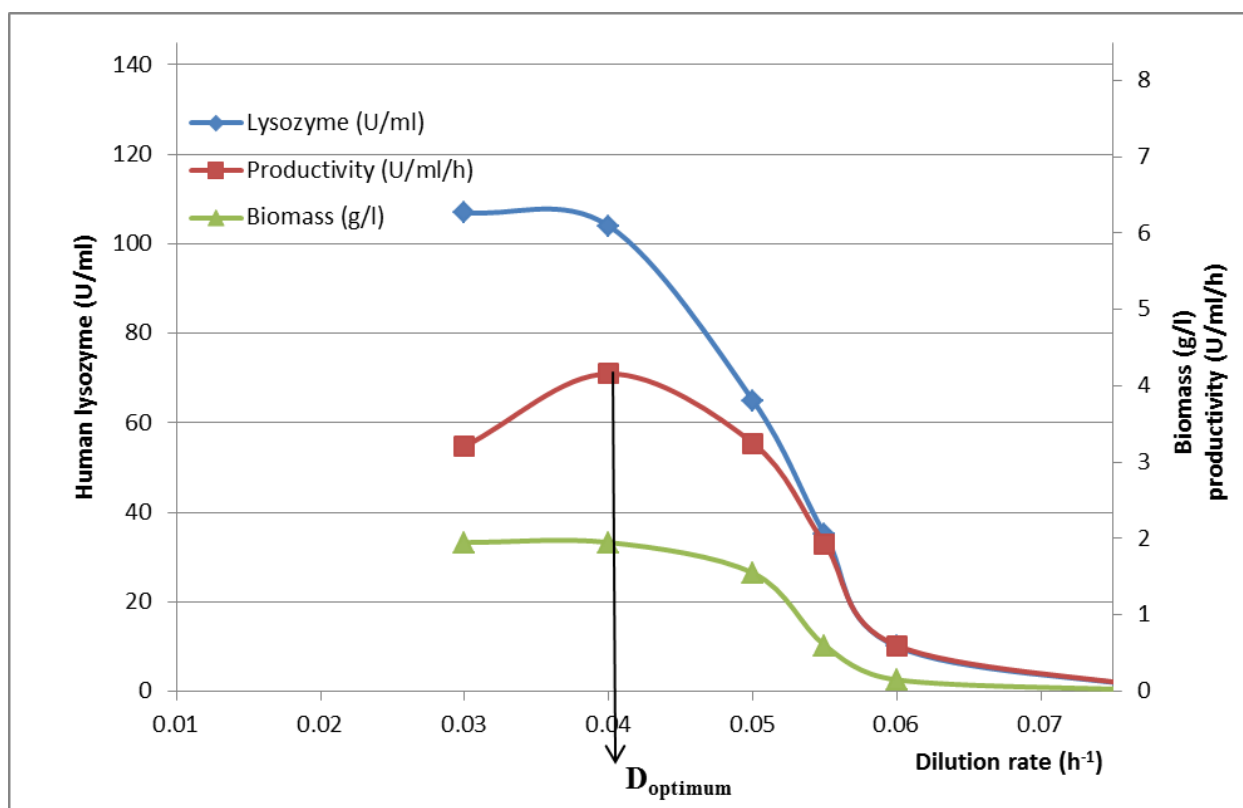
Figure 5.5 Lysozyme production and productivity at various dilution rates in suspended-cell reactor.

5.5 Conclusion

The initial carbon source, time of addition, and rate of carbon source addition were evaluated to find the best strategy for fed-batch fermentation for human lysozyme production in a biofilm reactor with PCS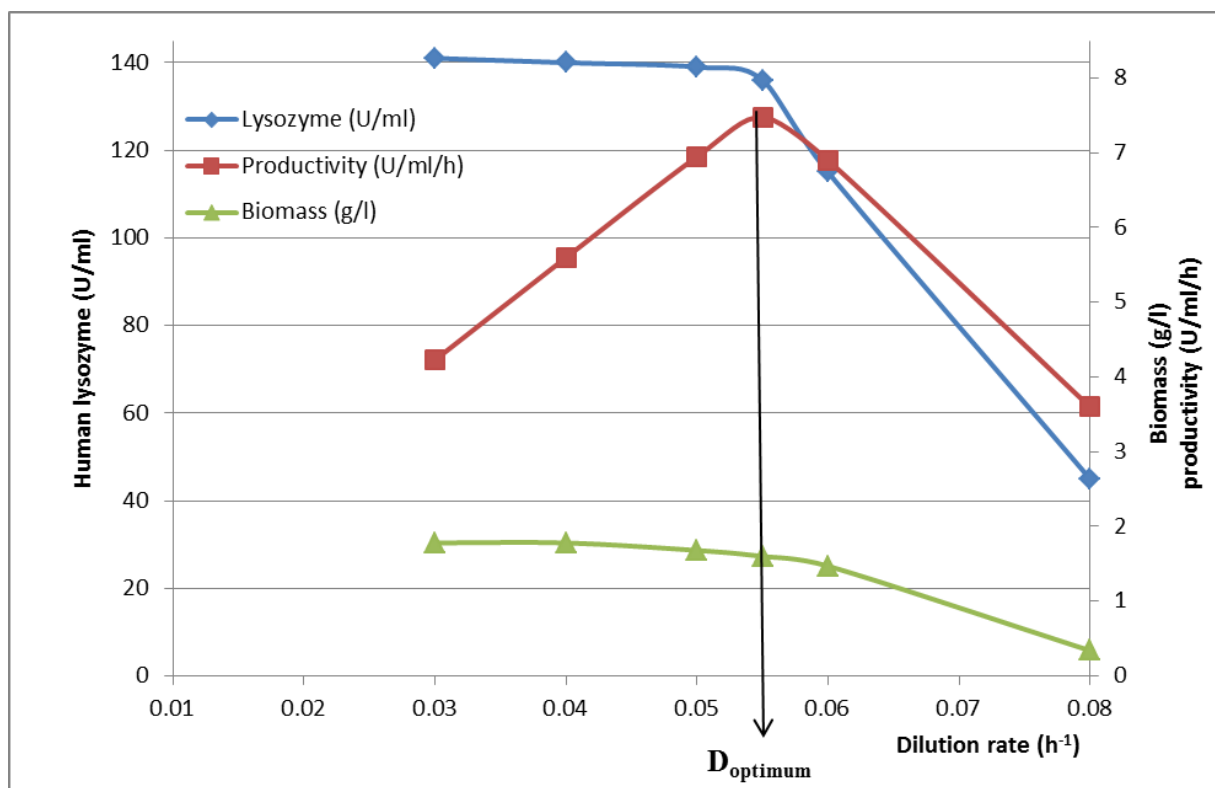. Overall, human lysozyme production and production rate were significantly increased by fed-batch fermentation with 90 g/l glucose and feeding with 400 g/l lactose at a rate 0.6 ml/min at between 28 and 38 h (187 U/ml, 5.9 U/ml/h) when compared to the production in batch fermentation (173 U/ml, 4 U/ml/h) ($p<0.05$). Continuous fermentation provided a significantly higher production rate (7.5 U/ml/h) than batch and fed-batch fermentations in the biofilm reactor. Moreover, a higher dilution rate was able to use for high productivity by the application of biofilm reactor (0.055 h^{-1} dilution rate) when compared to suspended-cell reactor (0.04 1/h dilution rate). In conclusion, human lysozyme production was enhanced by the application of biofilm reactors for fed-batch and continuous fermentations.

References

Choi, S., H. Paik, S. Lee, T. Nihira, and Y. Hwang. 2004. Enhanced productivity of human lysozyme by pH-controlled batch fermentation of recombinant *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering* 98(2): 132–135.

Demirci, A., A. L. III Pometto. 1995. Repeated-batch fermentation in biofilm reactors with plastic-composite supports for lactic acid production. *Applied Microbiology Biotechnology* 44: 585–589.

Demirci, A., A. L. III Pometto, and K-L.C. Ho. 1997. Ethanol production by Saccharomyces cerevisiae in biofilm reactors. *Journal of Industrial Microbiology and Biotechnology* 19: 299–304.

Demirci, A., T. Pongtharangkul, and A. L. Pometto III. 2007. Chapter 8: Applications of biofilm reactors for production of value-added products by microbial fermentation. In *Biofilms in the Food Environment,* 167-189. H. P. Blaschek, H. H. Wang, and M. E. Agle ed. Iowa, USA: Blackwell Publishing and The Institute of Food Technologists.

Gonzalez Siso, M. I. 1994. β-Galactosidase production by *Kluyveromyces lactis* on milk whey: Batch versus fed-batch cultures. *Process Biochemistry* 9: 565-568.

Huang, E. L., and A. Demirci. 2009. Enhanced human lysozyme production by *Kluyveromyces lactis. Food and Bioprocess Technology* 2: 222–228.

Faure, A. and P. Jollès. 1970. Immunological reactivity between human and bovine alpha-lactalbumins and several lysozymes of human origin and from bird egg whites. *Comptes Rendus Hebdomadaires des Seances de L Academie des Sciences. D: Sciences Naturelles* 271: 1916–1918.

Iwata, T., R. Tanaka, M. Seustsugu, M. Ishibashi, H. Tonkunga, M. Kikuchi, and M. Tokunga. 2004. Efficient secretion of human lysozyme from the yeast, *Kluyveromyces lactis*. *Biotechnology Letters* 26: 1803-1808.

Maullu, C., G. Lampis, T. Basile, A. Ingiani, C. M. Rossolini, and R. Pompei. 1999. Production enriched lysozyme biomass from cheese industry by products. *Journal of Applied Microbiology* 86: 182-186.

Muraki, M., Y. Jigami, H. Tanaka, F. Kishimoto, H. Agui, and S. Ogino. 1985. Short Communication Expression of Synthetic Human Lysozyme Gene in Escherichia coli. *Agricultural and Biological Chemistry* 49 (9): 2829~2831.

Naudi, A. S. 2000. *Natural Food Antimicrobial Systems*. New York, NY: CRC Press.

Paciello, L., F. Romano, E. de Alteriis, P. Parascandola, V. Romano. 2010. Glucoamylase by recombinant *Kluyveromyces lactis* cells: production and modelling of a fed batch bioreactor. *Bioprocess Biosyst Eng* 33: 525–532.

Pichler, W. J., and P. Campi. 1992. Allergy to lysozyme/egg white-containing vaginal suppositories. *Ann Allergy* 69(6): 521-525.

Pometto III, A.L., A. Demirci, and K. E. Johnson. 1997. Immobilization of microorganisms on a support made of synthetic polymer and plant material. U.S. Patent No. 5,595,893.

Richard, M. P., C. C. Ramesh, and M. S. Khem. 1965. A rapid and sensitive assay of muramidase. *Proceedings of The Society for Experimental Biology and Medicine* 119: 384–386.

Rossolini, G. M., M. L. Riccio, E. Gallo, and C. L. Galeotti. 1992. *Kluyveromyces lactis* rDNA as a target for multiple integration by homologous recombination. *Gene* 119: 75–81.

Schleifer, K.H. and Otto K. 1972. Peptidoglycan. Types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews* 36: 407–477.

Yoshimura, K., A. Toibana, K. Kikuchi, M. Kobayashi, T. Hayakawa, K. Nakahama, M. Kikuchi, and M. Ikehara. 1987. Differences between *Saccharomyces cerevisiae* and *Bacillus subtilis* in secretion of human lysozyme. *Biochemical and Biophysical Research Communications* 145(2): 712-718.

Yu, Z., Q. Meng, H. Yu, B. Fan, S. Yu, J. Fei, L. Wang, Y. Dai, and N. Li. 2006. Expression and bioactivity of recombinant human lysozyme in the milk of transgenic mice. *Journal of Dairy Sci*ence 89: 2911–2918.

CHAPTER 6

ONLINE RECOVERY OF HUMAN LYSOZYME PRODUCED BY *KLUYVEROMYCES LACTIS* K7 IN BIOFILM REACTOR

6.1 Abstract

Lysozyme is an antimicrobial compound, which has been used in both food and pharmaceutical industry. *Kluyveromyces lactis* K7 is a genetically modified microorganism, which is proposed to produce the human lysozyme commercially. Up to now, human lysozyme production was enhanced by the application of biofilm reactor with plastic composite (173 U/ml) compared to the suspended-cell bioreactor (110 U/ml). However, lysozyme has antimicrobial peptides, which might affect the growth of yeast cells during fermentation. As a result, high production potential of human lysozyme might be reduced. Therefore, a simultaneous fermentation and online recovery system may help to enhance the production. In order to test this strategy, the objective of this study was to select the best adsorption and desorption conditions for the resin, silicic acid, and evaluate the simultaneous fermentation and online recovery system for the production of human lysozyme *K. lactis* K7 in a biofilm reactor. For the adsorption, various percent ratios of silicic acid, temperature, and pH were evaluated, whereas temperature, pH, sodium dodecyl sulfate, NaCl, and ethanol as eluent were evaluated for desorption. The results demonstrated that the best adsorption (95.6% adsorption) was obtained at 25°C, pH 4, and 25% silicic acid ratio. The best desorption parameters were determined as 25°C, pH 6.2, and 5% sodium dodecyl sulfate with 1 M NaCl and 20% ethanol as eluent. Four repetitive desorption process provided up to 98% desorption. Finally, the simultaneous fermentation and online recovery system with tangential flow microfilter module improved the production of human lysozyme to 280.4 U/ml, which is a 63% improvement compared to the biofilm bioreactor without the online recovery system.

6.2 Introduction

Lysozyme is an antimicrobial agent, which is called as muramidases or N-acetylmuramideglycanohydrolases (Naudi, 2000). Lysozyme consists of 129 amino acids cross-linked by 4 disulfide bridges (Gill and Holley, 2000). The natural substrate of lysozyme is a murein layer (peptidoglycan), which is a common cell wall polymer. Lysozyme catalyzes the hydrolysis (the insertion of a water molecule) of the β (1→4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in murein layer. By this enzymatic activity, lysozyme is an effective antimicrobial agent especially against Gram-positive bacteria. Moreover, lysozyme peptides were found to be active against Gram-negative bacteria and the fungus *Candida albicans* with limited success (Ibrahim et al., 2001; Ibrahim et al., 1996). Düring et al. (1999) reported that the antimicrobial activity of the peptides may be related to the cationic nature.

In nature, lysozyme is found in many species. The chicken egg contains about 0.3-0.4 g of lysozyme (Naudi, 2000). Body fluids such as human tears and saliva contain 2.6 mg/ml, and 0.13 mg/ml of lysozyme, respectively (Naudi, 2000). Moreover, Maga et al. (1998) reported that lysozyme is present at 400 μg/ml in human milk, which is higher than other mammalian milk such as cow milk (0.13 μg/ml), goat milk (0.25 μg/ml), or sheep milk (0.10 μg/ml). Among these, chicken egg is the only source for the commercial production of lysozyme so far. However, chicken egg lysozyme can be allergic to some people and has lower activity compared to the human lysozyme. Therefore, human lysozyme gene was transferred into *Kluyveromyces lactis* to produce human lysozyme via fermentation route (Rossolini et al., 1992). The lysozyme produced by *K. lactis* K7 is secreted through the cell wall into fermentation broth during fermentation. The secretion sequence is endoplasmic reticulum- Golgi - vesicle-cell surface (Schekman, 1982).

Up to now, human lysozyme production by *K. lactis* K7 was studied in shake-flask, suspended cell, and biofilm reactors. Maullu et al. (1999) produced human lysozyme in cottage cheese whey (5.5% lactose) in shake-flask. Huang and Demirci (2009) studied the production of human lysozyme by batch fermentation (110.3 U/ml) and fed-batch

fermentation in suspended cell bioreactor (123.6 U/ml). Then, we have produced human lysozyme in a biofilm reactor with a plastic composite support, which provided passive immobilization of the cells onto support yielding higher microbial populations in the bioreactor (Chapters 3 and 4). The growth conditions of *K. lacis* K7 in biofilm reactor was optimized (Chapter 3) and concluded that the optimum conditions for biomass and lysozyme productions were different (27 °C, pH 6, 1.33 vvm for biomass production; 25 °C, pH 4, no aeration for lysozyme production). After evaluating different pH and aeration shift strategies, significantly higher lysozyme was produced (141 U/ml) at 25°C without pH and aeration. Then, the fermentation medium was optimized (Chapter 4) and at 173 U/ml lysozyme in a medium including 16.3% lactose, 1.2% casamino acid, 0.8% yeast nitrogen base. Since lysozyme has antimicrobial peptides which may affect the cell growth or might be degraded by the proteases produced during fermentation, the production can be further enhanced if the lysozyme is extracted during fermentation. For example; Pongtharangkul and Demirci (2007) significantly enhanced nisin production (7,445 IU/ml) by using an online recovery system with silicic acid as an adsorbent when compared to a batch fermentation without an online recovery (1,897 IU/ ml). They suggested that the reason of increase in the production might be higher biomass density due to application of tangential flow microfilter module. Moreover, Liu et al. (2010) proposed an online recovery system with foam during fermentation of *Lactococcus lactis subsp. lactis* ATCC 11454 for nisin production. Ttotal nisin production (4,870 ±180 IU/ml) was increased by 30.3% with online foam separation. Therefore, the aim of this study was to select the best adsorption and desorption conditions for human lysozyme recovery and implement a simultaneous fermentation and recovery system.

6.3 Materials and Methods

6.3.1   Microorganism and medium

Kluyveromyces lactis K7 (ATCC-MYA-413) was obtained from American Type Culture Collection (Manassas, VA). The cultivation medium included 2% (*w/v*) glucose, 1% (*w/v*) yeast extract, and 2% (*w/v*) peptone and the conditions were 25ºC for 24 h. The

working culture was maintained on the agar slant withthe same composition with cultivation medium and 2% (w/v) agar.

The fermentation medium included 16.3% lactose, 1.2% casamino acid, and 0.8 % yeast nitrogen base as recommended by our earlier study (Chapter 4).

6.3.2   Plastic composite support (PCS) tubes

The PCS tubes were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw corotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Pometto et al. (1997). The composition of PCS tubes, which were used in this study, included polypropylene (50% (*w/w*)), soybean hulls (35%), soybean flour (SF) (5%), salts (2 g/kg of sodium acetate, 1.2 g/kg of $MgSO_4 \cdot 7H_2O$, and 0.06 g/kg of $MnSO_4 \cdot 7H_2O$), yeast extract (5%), and dried bovine albumin (5%) (Chapter 3). After polypropylene and other ingredients of PCS were mixed, they were extruded at 13 rpm through a medium pipe die. The barrel temperatures were 200, 220, and 200 °C and the die temperature was 165°C. The PCS tubes were produced as cylindrical shape with a wall thickness of 2.5 mm and an outer diameter of 10.5 mm (Figure 6.1A).

6.3.3   Biofilm reactor set-up and biofilm formation

A Sartorious Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel was used to construct biofilm reactor, which had 12 PCS tubes (6.5 cm long) bound to the agitator shaft in a grid-like fashion, with six rows of two parallel tubes (Figure 6.1B). The reactor vessel with PCS was autoclaved with water at 121°C for 90 min. Then, 1.5 L of sterilized fermentation medium was added aseptically to the reactor vessel with PCS. For biofilm formation, a 24-h grown culture of *K. lactis* K7 (1%, v/v) was inoculated. Then, five repeated-batch fermentations were performed at the conditions with no pH control, 25ºC, 150 rpm, and no aeration as recommended by our earlier study (Chapter 3).

Figure 6.1 Plastic composite support (A), Biofilm reactor set up with plastic composite support (B).

6.3.4   Batch Fermentation for human lysozyme production

After formation of biofilm on the PCS tubes, the batch fermentation was conducted without inoculation of fresh culture since there was already immobilized *K. lactis* K7 on the PCS tubes. The fermentation conditions were no pH control, 25ºC, 150 rpm, and no aeration as recommended (Chapter 3). The fermentation started as soon as the fresh fermentation medium was transferred to biofilm reactor.

6.3.5   Recovery of human lysozyme from fermentation medium

The online recovery system with silicic acid as the resin (mesh size 100, Sigma Chemical, St. Louis, MO , reported by Pongtharangkul and Demirci (2007), was used with some modifications. The recovery included two steps; adsorption of the lysozyme onto silicic acid and desorption of the lysozyme from silicic acid. The best adsorption and desorption conditions were selected in the shake flask study. To select the best conditions, the fermentation medium, which was taken in the end of 72h of fermentation from the biofilm reactor, was centrifuged at 4500 rpm for 30 min to obtain cell-free fermentation medium. The cell free medium (~pH 4.15) was analyzed to determine the initial lysozyme activity.

6.3.5.1 Adsorption condition selection

In the first step, silicic acid was added into 25 ml of cell free fermentation medium and incubated at certain temperature for 1 h at 300 rpm. Then, the medium and silicic acid were separated from each other by centrifugation at 4500 rpm. The recovered silicic acid resin was washed in 25 ml of deionized water for 5 min to rinse off the left-over fermentation medium residues. After re-centrifugation, the solution is decanted. The parameters, which were evaluated to select the best adsorption conditions, were the pH of fermentation medium (pH 4 and 6), the temperature of the incubation of fermentation medium with silicic acid (25, 50, and 90°C), and the concentration of silicic acid (5, 10, 15, 20, 25, and 30% (weight of silicic acid/volume of fermentation medium)). First, the adsorption was conducted for each evaluated pH at every evaluated temperature to see their main and interaction effects. After the best temperature and pH were selected, at selected temperature and pH conditions, the adsorption process was conducted at different ratios of silicic acid. The adsorbed lysozyme was calculated by subscribing the remained lysozyme in the medium (R) after adsorption process from the initial lysozyme amount in the cell free fermentation medium (I) as shown in Eqn 6.1.

$$\text{Adsorption (\%)} = 100 \times (I\text{-}R) \, / \, I \quad \text{.....................................} \quad \text{Eqn. 6.1}$$

6.3.5.2 Desorption condition selection

In the desorption step, the silicic acid was re-suspended in 25 ml of the desorption eluents and incubated at 25°C for 30 min at 300 rpm. The incubation was continued for ten more minutes at various temperatures. Finally, the eluent was centrifuged to separate the silicic acid and was analyzed to determine the activity of the desorbed human lysozyme. The desorption eluents evaluated were Eluent 1: 1 M NaCl+20% ethanol; Eluent 2: 1 M NaCl+20% ethanol+1% sodium dodecyl sulfate; Eluent 3: 1 M NaCl+20% ethanol+3% sodium dodecyl sulfate; Eluent 4: 1 M NaCl+20% ethanol+5% sodium dodecyl sulfate; and Eluent 5: 1 M NaCl+20% ethanol+7% sodium dodecyl sulfate. Also, two pHs (pH 3 and 6) and three temperatures (25, 50, and 90°C) were evaluated for 10 min incubation time. Finally, the best desorption condition and eluent were selected by comparing the desorption percentages for each evaluated pH at every evaluated temperature to observe their main and interaction effects on desorption. The higher lysozyme activity in the final eluent (F) was the indication of the best desorption condition, which was calculated by multiplying 100 by the division of the lysozyme activity in the final eluent (F) by the initial lysozyme amount in the cell free fermentation medium (I) as shown in Eqn 6. 2.

$$\text{Desorption (\%)} = 100 \times F / I \ \text{……………………………..} \quad \text{Eqn. 6.2}$$

Finally, consecutive desorption process was conducted. Each desorption was conducted at the selected conditions, and the % desorption was calculated as the percent of initial lysozyme amount in the fermentation medium.

6.3.6 Simultaneous online recovery and fermentation

After selection of the best adsorption and desorption conditions, simultaneous recovery and fermentation were conducted. The biofilm reactor with PCS were connected to a tangential flow filter unit equipped with hydrophilic polyvinylidene membrane (0.45 μm nominal pore size, Pellicon® XL Durapore HVMP, Millipore, Bedford, MA), which separated the cells from the fermentation broth before the adsorbent column (Figure 6.2).
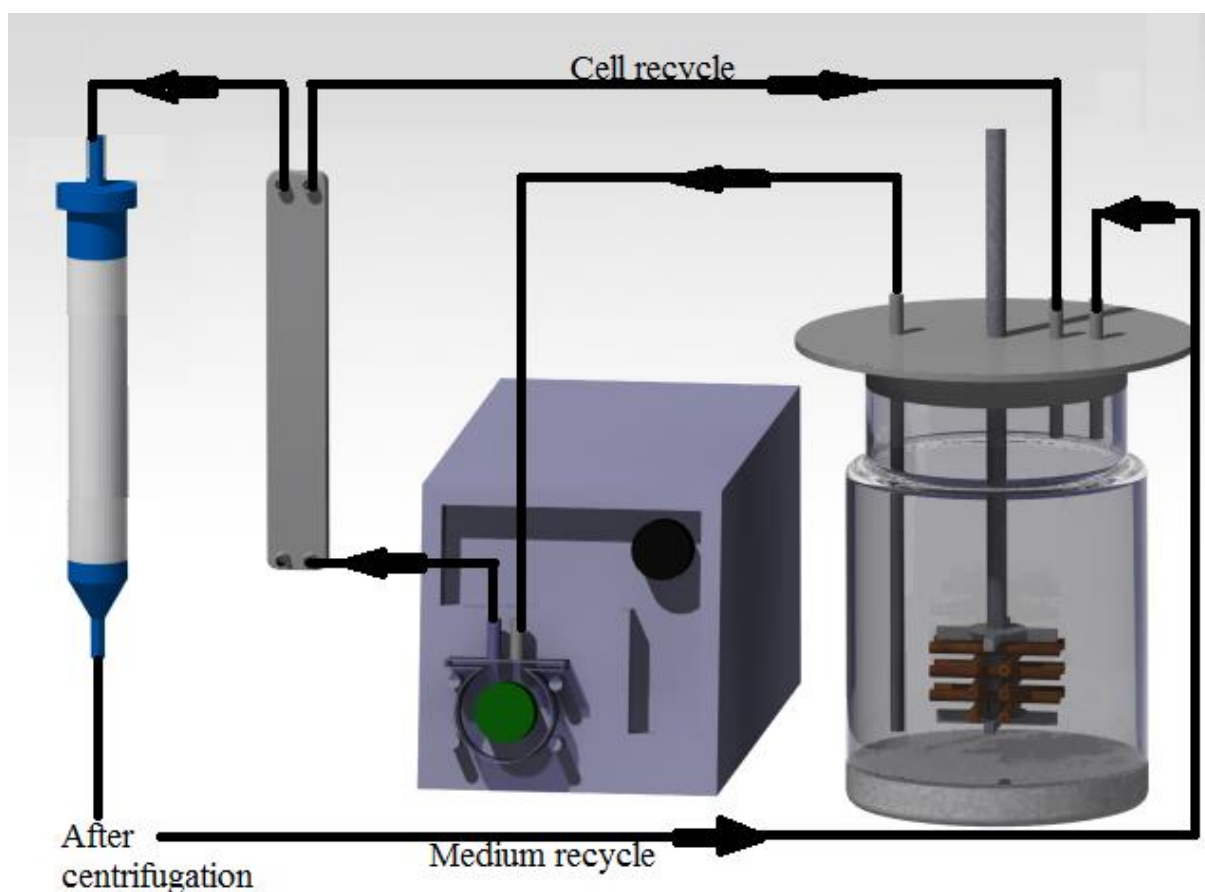
Figure 6.2 Simultaneous fermentation and recovery system.

The cells from the filtration unit and fermentation broth from the adsorbent column were recycled to the reactor. The simultaneous recovery started at 24h of fermentation and the adsorbent column was replaced with a column with fresh adsorbent every 10 h until the end of the fermentation. First, the fermentation medium was fed to a microfiltration unit with a 2 ml/min flow rate. The retentate, which includes cells, was recycled to the reactor and the permeate was fed to the adsorption column. Then, the cell-free fermentation broth was recycled to the reactor. Concentrations of human lysozyme in the biofilm reactor and in the eluent (after desorption) was measured. The summation of the human lysozyme in the reactor and in the eluent (after desorption) at certain time of fermentation was the produced human lysozyme in the system.

6.3.7 Analysis

6.3.7.1 Biomass

Biomass was determined by measuring optical density (OD) using a spectrophotometer (Beckman Coulter, Fullerton, CA) by obtaining absorbance at 600 nm, which was then converted to biomass concentration by using Eqn. 6.3 as suggested by Huang and Demirci (2009).

$$\textit{Biomass} \text{ (g/l)} = 0.564 \times OD_{600} \text{ ....} \quad \text{Eqn 6.3}$$

6.3.7.2 Lactose concentration

Lactose concentration was determined using a Waters's high pressure liquid chromatography (HPLC) equipped with a refractive index detector (Waters, Milford, MA). Lactose was separated using Bio-Rad Aminex HPX-87H column (300×7.8 mm; Bio-Rad, Richmond, CA) with 0.8 ml/min of 0.012 N sulfuric acid as the mobile phase. The detector temperature and column temperature were maintained at 35ºC and 65ºC, respectively. Prior to the analysis, the fermentation samples were centrifuged for 2 min at 5,200 x g to separate the cells from the medium. Finally, the samples and the standard solutions were filtered with 0.2 μm PTFE membrane filters and injected to the HPLC. The injection volume was 20 μl. Identification was done by comparing retention times of test compounds and the external standard. For quantification, calibration curves were constructed by plotting peak areas versus concentrations of lactose standards (5, 10, 15, 20, 30 g/l).

6.3.7.3 Lysozyme activity

Lysozyme activity was measured using a method adapted from Richard et al. (1965). The procedure for the lysozyme assay is as follows: A 0.5 mg/ml of *Micrococcus lysodeikticus* cell suspension (Sigma-Aldrich, St. Louis, MO) was prepared using potassium phosphate buffer at pH 6.2. The assay solution consists of 600 μl of 0.5 mg/ml *M. lysodeikticus* cell suspension, 200 μl of 300 mM sodium chloride solution, and 400 μl

sample. The absorbances were measured every 10 s for 1 min at 540 nm. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 A_{540nm} change per minute. The slope of the linear regression line refers to the speed of lysis of *M. lysodeikticus* suspension, and the slope estimates the concentration of human lysozyme in unit per milliliter as shown in Eqn. 6.4.

$$\text{Lysozyme (U/ml)} = (\text{slope} / 0.4 \text{ ml sample}) \times 1000 \quad .... \quad \text{Eqn 6.4}$$

6.3.8 Statistical Analysis

The statistical analyses were performed by using MINITAB Statistical Software package (Version 15, Minitab Inc., State College, PA). Analysis of variance (ANOVA) and Tukey's test was performed for investigating statistically significant differences at different trails. A *p*-value of <0.05 was considered to be significant. All fermentation runs were conducted in duplicate.

6.4 Results and Discussion

To evaluate online recovery system, the best adsorption and desorption conditions were studied to enhance human lysozyme production.

6.4.1 Selection of the best adsorption and desorption conditions

The adsorption and desorption conditions was evaluated for the recovery of human from the fermentation medium using silicic acid as adsorbent. Silicic acid was selected as an adsorbent because silicic acid is non-toxic, so it can be used as an adsorbent for the products, which have applications in food and pharmaceutical industries (Wijntje et al., 2005). Moreover, it has been reported that silicic acid doesn't adsorb the nutrients from the medium, which can be recycled to the reactor (Wijntje et al., 2005; Pongtharangkul and Demirci, 2007). Adsorption of the compounds onto silicate based compounds includes electrostatic and hydrophobic interactions (Wijntje et al., 2005; Coventry et al., 1996). Firstly, the effects of pH and temperature on adsorption were evaluated for adsorption of human lysozyme to the silicic acid (5%). Figure 6.3 shows the interaction

plot of effects of pH and temperature on adsorption of produced human lysozyme on to silicic acid. The adsorption decreased significantly as the temperature increased at both pH 4 and 6 ($p<0.05$).
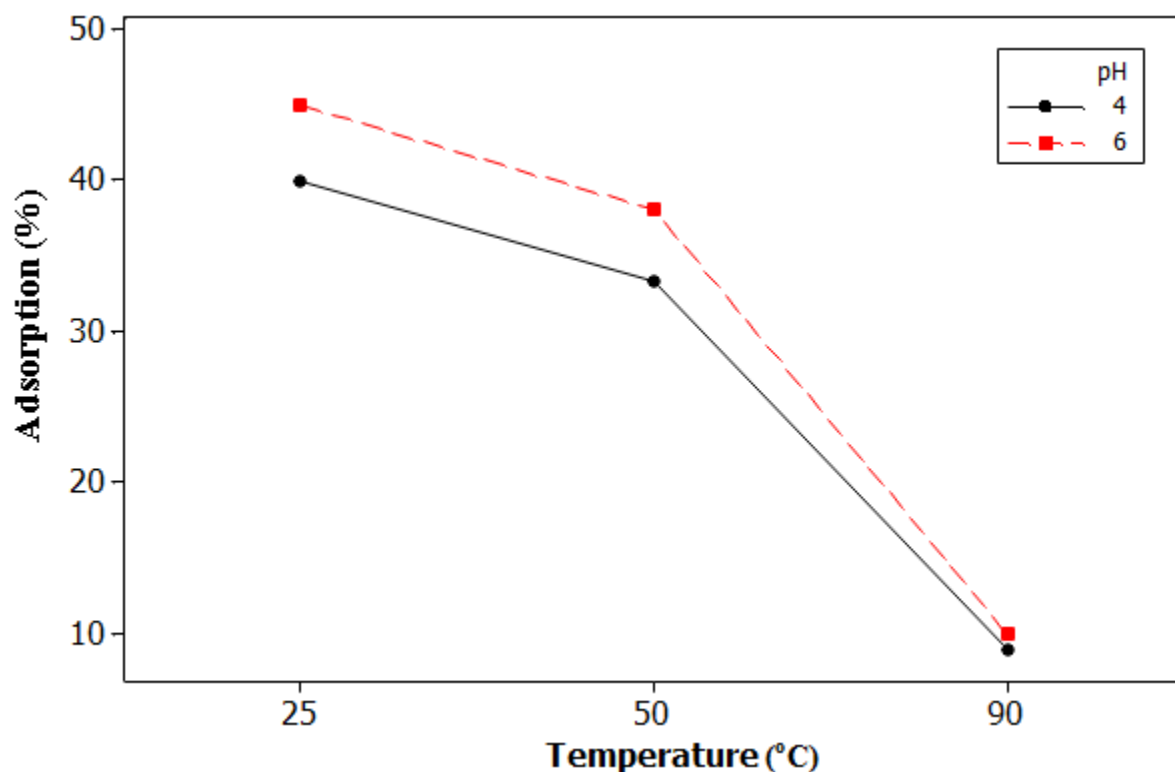
Figure 6.3 Interaction plot of pH and temperature on adsorption (%).

When the temperature was 25°C, the adsorption was 45% and while the temperature increased to 90°C, the adsorption decreased to 12% at pH 6. The decrease of the adsorption with increasing temperature may be related to the breakage of the hydrogen bonds (Pongtharangkul and Demirci, 2007). Although, the adsorption at pH 6 was slightly higher than the adsorption at pH 4, the difference was not significant at all temperature conditions, which might be related to inactivation of human lysozyme activity at high temperatures. Moreover, the difference between the adsorptions at each pH level decreased as the temperature increased to 90°C. The reason of the slight increase

in the adsorption at high pH might be related reduced net charges on lysozyme. Similar trends were also observed for the adsorption of bacteriocins to silica based compounds (Janes et al., 1998; Pongtharangkul and Demirci, 2007). However, in this study, pH 4 was selected as the best condition at 25°C, which achieved 40% adsorption, because the the fermentation medium was around at pH 4 and no-pH change during the recovery process can be more feasible for a simultaneous fermentation and recovery system. Conventry et al. (1996) reported optimal adsorption of bacteriocins on to diatomic calcium silicate at the fermentation medium pH of 4.5. The adsorption increases if the electrostatic interactions between the Micro-Cel particles and the bacteriocin molecules were increased. Conventry et al. (1996) stated that the adsorption may decrease if the pH is higher than the isoelectric point of the bacteriocin, which cause negatively charged peptides (such as pH>10).If the pH is lower than 2, this ca cause less negatively charged silica surface.
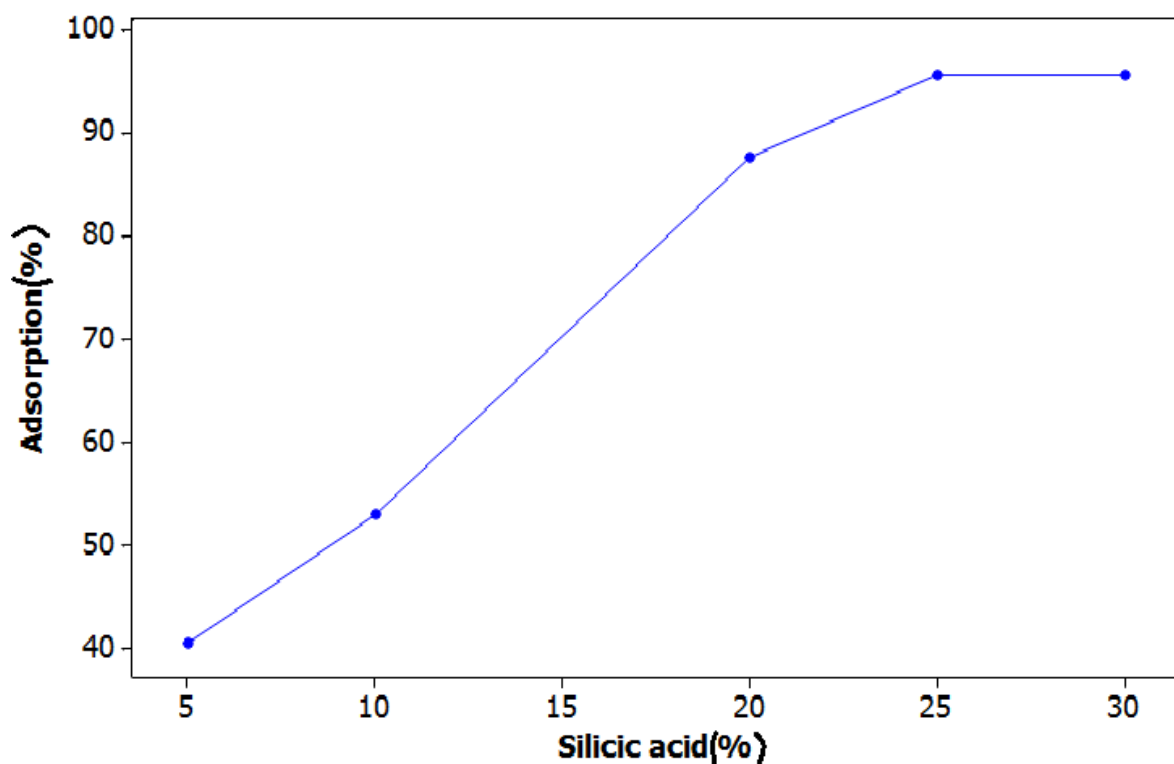
Figure 6.4. Effects of silicic acid concentration on adsorption (%).

Then, since only 40% of adsorption was achieved with 5 % of silicic acid, higher percent ratios of silicic acid were evaluated (Figure 6.4). The silicic acid ratio significantly affected on human lysozyme adsorption process ($p<0.05$). When the silicic acid concentration was increased from 5% to 10, to 20, to 25 %, the adsorption increased to 53%, 87.6%, 95.6%, respectively. However, no significant increase was observed as the silicic acid increased further to 30% ($p\geq0.05$). The maximum holding capacity of silicic acid achieved in this study was 6.57 U/g silicic acid at a 25% ratio.

After selection of the best adsorption conditions, the recovery process started at the selected adsorption conditions, which were 25% silicic acid, pH 4, and 25°C. Desorption of human lysozyme from the silicic acid was conducted at different conditions. Conditions that weaken the hydrophobic and electrostatic interactions between the silicic acid and human lysozyme should be targeted for optimum desorption. For this purpose different eluents, the pH and the temperature of the desorption process were evaluated. The desorption process was conducted with the eluents at both pH 4 and pH 6; Eluent 1: 1 M NaCl+20% ethanol, Eluent 2: 1 M NaCl+20% ethanol+1% sodium dodecyl sulfate, Eluent 3: 1 M NaCl+20% ethanol+3% sodium dodecyl sulfate, Eluent 4: 1 M NaCl+20% ethanol+5% sodium dodecyl sulfate, and Eluent 5: 1 M NaCl+20% ethanol+7% sodium dodecyl sulfate. The composition of desorption eluent is an important factor to weaken the hydrogen bonds. Another important factor to improve desorption is pH, which affects the electrostatic interaction. NaCl and ethanol has been used as desorption eluents because of their ability to destroy hydrogen bonds and because they can be used in the food and pharmaceutical industry because of their nontoxic nature (Pongtharangkul and Demirci, 2007). Moreover, the hydrophobic interaction between the adsorbent material and product may be influenced by the surfactant (Coventry et al., 1996; Pongtharangkul and Demirci, 2007). Sodium dodecyl sulfate is a safe surfactant because it has been widely used as food grade emulsifier (Kralova and Sjöblom, 2009). As shown in Figure 6.5, desorption increased as the sodium dodecyl sulfate increased to 5%. Only 5% desorption was achieved with the Eluent 1, which did not include sodium dodecyl sulfate. Then, desorption significantly increased to 17.45% when the eluent included 1% sodium

dodecyl sulfate (p<0.05). Desorption increased further to 27.68, 35.17, 38.99, and 42.69% as the sodium dodecyl concentration was increased to 2, 3, 4, and 5%. Further increases in the sodium dodecyl sulfate concentration did not improve the desorption ($p \geq 0.05$) at either pH 3 and 6.2. Desorption was slightly higher at pH 3 than desorption at pH 6. For example, while desorption was 42.69 % at pH 6.2 and 44.14% at pH 3 ($p \geq 0.05$). Conventry et al. (1996) also reported that sodium dodecyl sulfate increased the effectiveness of the desorption process: 100% of piscicolin (51,200 AU/ml) desorption was achieved by increasing the ratio of sodium dodecyl sulfate from 0.1 to 1% (w/v).
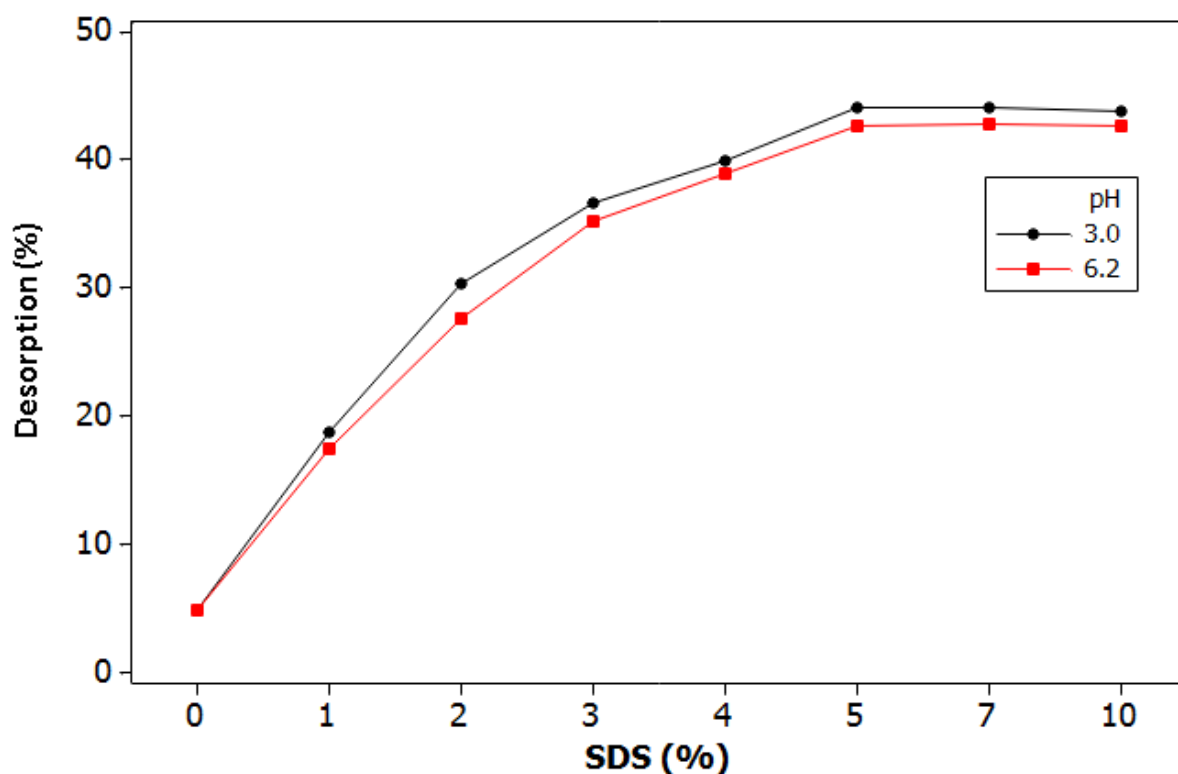
Figure 6.5 Interaction plot of pH and sodium dodecyl sulfate (SDS) (%) on desorption (%).

After selection of the best eluent, the desorption process was conducted at different temperatures (25, 50, and 90°C) at each pH (4.0 and 6.2) with the selected eluent (Figure 6.6). Although it has been reported that the heat treatment during desorption may cause breakage of the hydrogen bonds and enhance the desorption process (Pongtharangkul and Demirci, 2007; Janes et al. 1998), desorption of lysozyme from the silicic acid did not significantly change (p$\geq$0.05) when the temperature increased from 25°C to 50°C at either pH 3 and 6.2. However, the increase in temperature to 90°C caused a significant decrease in the desorption of lysozyme (p<0.05). The reason might be inactivation of the lysozyme activity at 90°C. At all evaluated temperatures, the desorption at pH 3 was slightly higher than pH 6.2.
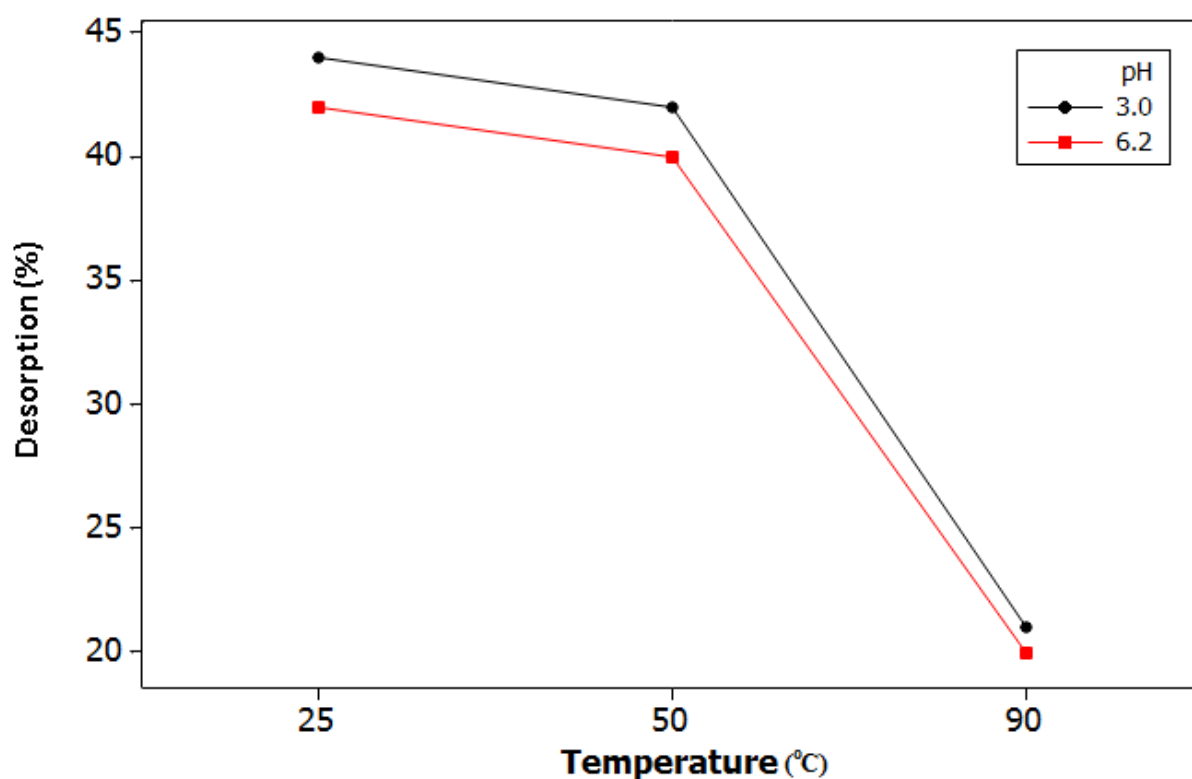
Figure 6.6 Interaction plot of pH and temperature on desorption (%).

Pongtharangkul and Demirci (2007) and Janes et al. (1998) enhanced the desorption process of bacteriocins by reducing the pH to 3. On the other hand, Conventry (1996) achieved 100% desorption by addition of sodium dodecyl sulfate at pH 6.5. Because the surfactant concentration was able to increase the effectiveness of the desorption process unlike pH, Conventry et al. (1996) suggested that the hydrophobic interactions between Micro-Cel particles and bacteriocin (piscicolin) molecules are more important than the electrostatic interactions. In this study, the pH decrease did not cause a significant increase in desorption and the eluent with 5% sodium dodecyl sulfate was already at pH 6.2. Therefore, this pH was selected for desorption of human lysozyme from the silicic acid.
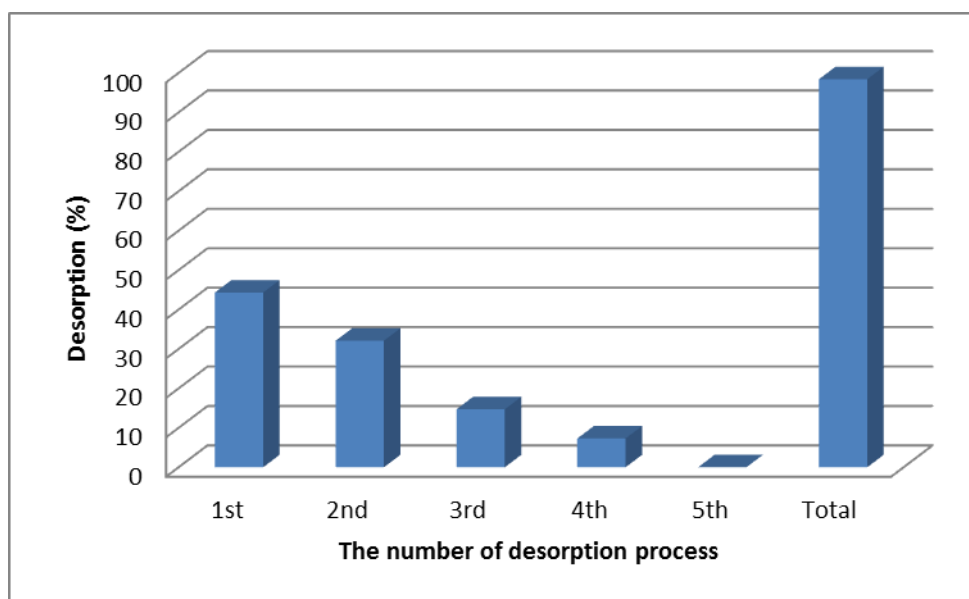
Figure 6.7 Effects of repetitive desorption process on total recovery.

Finally, repetitive desorption was evaluated to find out whether desorption can be improved or not (Figure 6.7). Each desorption step was performed at the pre-determined optimum conditions; i.e., pH 6.2, 5% sodium dodecyl sulfate, and $25^{o}C$. The second desorption process provided 32% desorption of the initial lysozyme, while the third and the fourth steps achieved 14.7 and 7.3 % of desorption, respectively. The repetition of

desorption process for the fifth time did not provide any further lysozyme desorption. As a result of four repetitive desorption processes, total of 98.4% desorption was achieved.

6.4.2 Simultaneous online recovery and fermentation for human lysozyme production in biofilm reactor

After selection of best adsorption and desorption conditions for the recovery of human lysozyme from the fermentation medium, simultaneous online recovery and fermentation were conducted. Figure 6.8 shows human lysozyme production with and without recovery system.
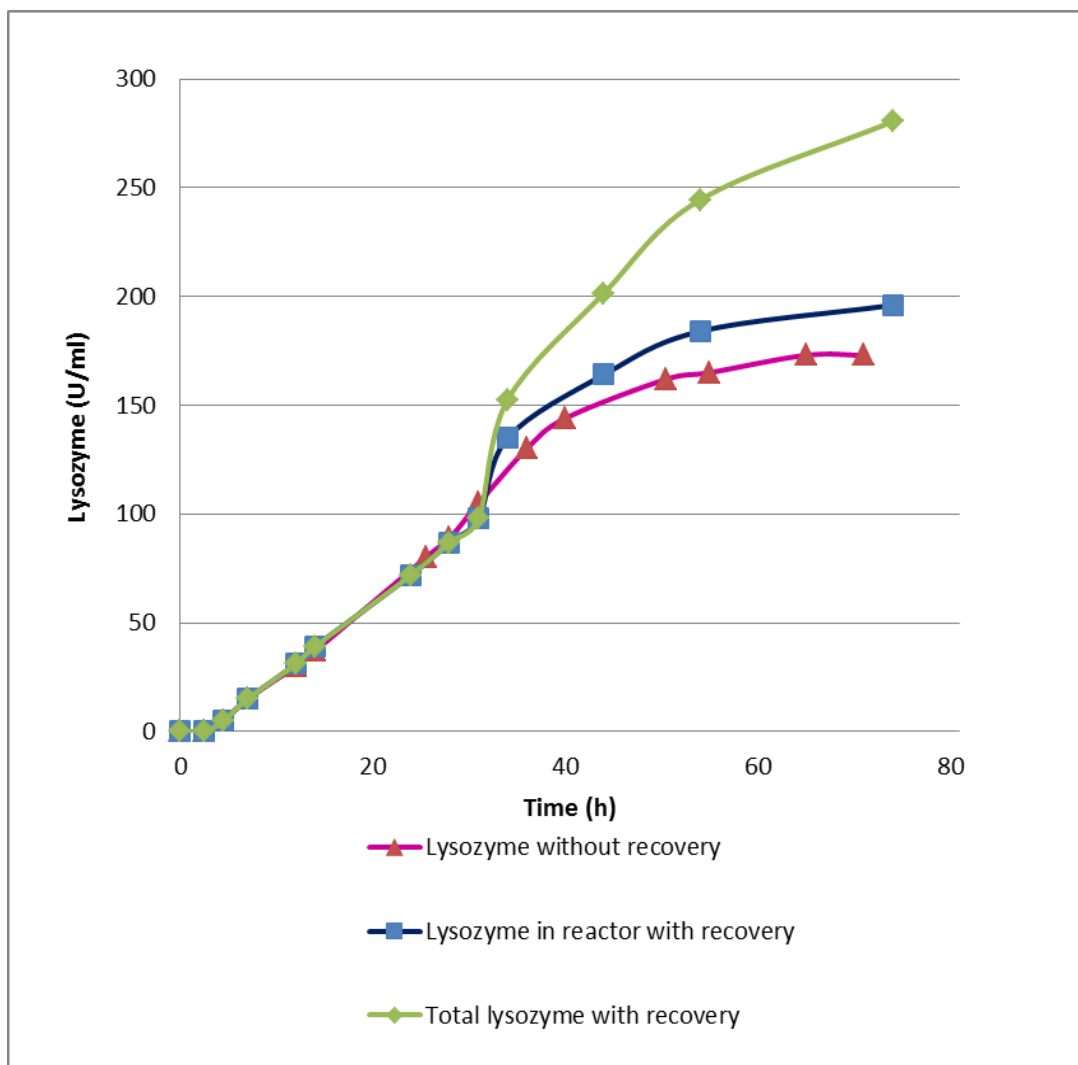
Figure 6.8 Simultaneous recovery and fermentation after 24 h.

The online recovery was started at 24 h of fermentation and the adsorbent column was replaced with the fresh column every 10 h. The adsorption column included 75 g of silicic acid to have the 25% (w/v) silicic acid ratio as determined earlier, because 300 ml of permeate was fed to the adsorbent column for 10 h. As a result, the significantly higher lysozyme production of lysozyme (164 U/ml) in the reactor was observed at 44 h compared to fermentation without recovery (150 U/ml) ($p < 0.05$). The reason for the increase in lysozyme production after 44 h might be the reduced effect of antimicrobial proteins on the growth of *K. lactis* K7, because a significantly higher biomass density was observed after 31 h ($p < 0.05$). At the end of the fermentation, the biomass was 4.04 g/l with the recovery system, while the biomass in the fermentation without recovery was 2.18 g/l. Overall, at the end of the fermentation, total human lysozyme (recovered and the produced lysozyme in the reactor at 74 h) was calculated as 280.4 U/ml, while the production without the online recovery system was 173 U/ml. This represents a 63% increase in the production of human lysozyme by *K .lactis* K7 in biofilm reactor was achieved by simultaneous recovery and fermentation system.

6.5 Conclusion

The recovery conditions for human lysozyme produced by *K. lactis* K7 in a biofilm reactor were evaluated. The best adsorption conditions were determined to be 25°C, pH 4, and a 25% silicic acid ratio. Adsorption at these conditions was 95.6%. The best desorption conditions 25°C, pH 4, and 5% sodium dodecyl sulfate with 1 M NaCl and 20% ethanol as the eluent. In both adsorption and desorption processes, while the temperature significantly affected the results, pH was not significant. When simultaneous fermentation and recovery process with tangential flow microfilter module was studied, these systems provided a 63 and 47% increase in the production of human lysozyme and biomass, respectively.

References

Coventry, M. J., J. B. Gordon, M. Alexander, M. W. Hickey, J. Wan. 1996. A food-grade
        process for isolation and partial purification of bacteriocins of lactic acid that used

diatomite calcium silicate. *Applied and Environmental Microbiology* 62(5):1764 – 1769.

Düring, K., P. Porsch, A. Mahn, O. Brinkmann, and W. Gieffers.1999. The non-enzymatic microbicidal activity of lysozymes. *Federation of European Biochemical Societies Letters* 449: 93–100.

Gill, A. O., and R. A. Holley. 2000. Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA. *Food Research International* 33: 83-90.

Huang, E. L., and A. Demirci. 2009. Enhanced human lysozyme production by *Kluyveromyces lactis. Food and Bioprocess Technology* 2: 222–228.

Ibrahim, H. R., S. Higashiguchi, M. Koketsu, L. R. Juneja, M. Kim, T. Yamamoto, Y.Sugimoto, and T. Aoki. 1996.Partially Unfolded lysozyme at neutral pH agglutinates and kills Gram-negative and Gram-positive bacteria through membrane damage mechanism. *Journal of Agricultural and Food Chemistry* 44: 3799−3806.

Ibrahim, H. R., T. Matsuzaki, and T. Aoki. 2001. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. *Federation of European Biochemical Societies Letters* 506: 27-32.

Janes, M. E., R. Nannapaneni, A. Proctor, M. G. Johnson. 1998. Rice hull ash and silicic acid as adsorbents for concentration of bacteriocins. *Applied Environmental Microbiol*ogy 64(11): 4403– 4409.

Kralova, I and J. Sjöblom. 2009. Surfactants Used in Food Industry: A Review. *Journal of Dispersion Science and Technology* 30: 1363–1383.

Liu, W., H. J. Zheng, Z. L. Wu, and Y. F. Wang. 2010. Effects of pH profiles on nisin fermentation coupling with foam separation. *Applied Microbiology and Biotechnology* 85(5): 1401-1407.

Maga, E. A., G. B. Anderson, J. S. Cullor, W. Smith, and J. D. Murray. 1998. Antimicrobial properties of human lysozyme transgenic mouse milk. *Journal of Food Protection* 61(1): 52-56.

Maullu, C., G. Lampis, T. Basile, A. Ingiani, C. M. Rossolini, and R. Pompei. 1999. Production enriched lysozyme biomass from cheese industry by products. *Journal of Applied Microbiology* 86: 182-186.

Naudi, A. S. 2000. *Natural Food Antimicrobial Systems*. New York, NY: CRC Press.

Pometto III, A.L., A. Demirci, and K. E. Johnson. 1997. Immobilization of microorganisms on a support made of synthetic polymer and plant material. U.S. Patent No. 5,595,893.

Pongtharangkul, T. and A.Demirci. 2007. Online recovery of nisin during fermentation and its effect on nisin production in biofilm reactor. *Applied Microbiology and Biotechnology* 74(3): 555-562.

Richard, M. P., C. C. Ramesh, and M. S. Khem. 1965. A rapid and sensitive assay of muramidase. *Proceedings of The Society for Experimental Biology and Medicine* 119: 384–386.

Rossolini, G. M., M. L. Riccio, E. Gallo, and C. L. Galeotti. 1992. *Kluyveromyces lactis* rDNA as a target for multiple integration by homologous recombination. *Gene* 119: 75–81.

Schekman, R. 1982. The secretory pathway in yeast. *Elsevier Biomedical Press* 0376 5067: 243-246.

CHAPTER 7

STABILITY OF HUMAN LYSOZYME IN CELL FREE FERMENTATION MEDIUM PRODUCED BY *KLUYVEROMYCES LACTIS* K7 IN BIOFILM REACTOR

7.1 Abstract

For downstream processing of produced human lysozyme, understanding of the stability of lysozyme at various conditions is needed to make sure the activity at maximum level during processing or storage at the room or colder temperatures. Therefore, three factor Box-Behnken design was used to observe the effects of the combination of the pH, temperature, and time. Moreover, the storage stability was also observed for 30 days. As a result, lysozyme activity in cell free fermentation medium was stable for 30 days at all temperatures (4, 10, and 25°C). Also, pH and the interaction of time and temperature were found as significant effects on produced human lysozyme activity. The activity of human lysozyme was decreased as the pH decreased to 3.5 and increased to 9. Moreover, greater temperature caused decrease in the activity over time. The lysozyme activity decreased more dramatically when the temperature increased at pH 9 versus pH 3.5.

7.2 Introduction

Human lysozyme is an enzyme (EC 3.2.1.17) with 130 amino acids and a molecular weight of 14.5 kD (Bethell, 2006). Human lysozyme is an $\alpha+\beta$ protein (single polypeptide chain) with five α-helixes and five β-strands, and consists of two domains. The active site is located in the cleft positioned between the two domains (Takano et al., 1995). The amino acid side-chains glutamic acid 35 (Glu35) and aspartate 52 (Asp52) of lysozyme plays roles for enzymatic activity. Glu35 protonates the glycosidic oxygen by acting as a proton donor to the glycosidic bond, cleaving the C-O bond in the substrate.

Asp52 acts as a nucleophile to generate a glycosyl enzyme intermediate so it can only react with a water molecule to give the product of hydrolysis, leaving the enzyme unchanged (Vocadlo et al., 2001). The natural substrate of lysozyme is the peptidoglycan polymer (GlcNAc-MurNAc)n found in most bacterial cell walls. In addition to antibacterial activity, it also has antiviral, antifungal and antiparasitic activities (Naudi, 2000).

Human lysozyme is present in human biological fluids and secretions (milk, tears, saliva, genital, nasal and bronchial secretions) (Bethell, 2006). Although human lysozyme has advantages in terms of non-allergic nature, and high enzymatic activity, currently commercial production of lysozyme is from chicken eggs. For the commercial production of human lysozyme, human lysozyme gene was expressed in many host cells. Up to now, human lysozyme production by *K. lactis* K7 has been studied in shake flask (Maullu et al., 1999), suspended cell (Huang and Demirci, 2009), and biofilm reactors (Chapters 3 and 4). Biofilm reactors enhanced human lysozyme production by using biofilm reactor with plastic composite support which provided passive immobilization of the cells.

After fermentation, the human lysozyme needs to be recovered from the broth for commercial applications. To optimize the timing of the recovery process, the stability of the human lysozyme activity in cell free fermentation medium needs to be determined because the enzymatic activity of lysozyme is affected by many factors, such as pH, temperature, ionic strength, etc. For example; Hughey and Johnson (1987) reported that egg white lysozyme is heat resistant at low pHs. Yang et al. (2011) reported that the activity of human lysozyme, which was extracted from transgenic milk, did not change when the temperature varied from 25 to 60°C, but the activity was slightly higher at around 40°C. Egg-white lysozyme activity is maximized at around pH 5 and the activity decreases when the pH is close to 3.8 and 6.7 (Naudi, 2000). Lysozyme activity is also affected by salt concentration and this affects the pH spectrum. Yang et al. (2011) stated that a salt concentration of 0.05 M provided lysozyme activity in broader range of pH values (pH 5-9) than salt concentration of 0.1 M. To our knowledge, there has been no

study on the stability of human lysozyme produced by *K. lactis* K7 in a biofilm reactor against time, temperature, and pH in the literature. Therefore, the aim of this study was to determine the effects of pH and temperature at different times on the activity of human lysozyme produced by *K. lactis* K7 in a biofilm reactor. Moreover, the storage stability of produced human lysozyme at different temperatures in cell-free fermentation broth was determined to determine the maximum holding time before the recovery process.

7.3 Materials and Methods

7.3.1   Microorganism and medium

Kluyveromyces lactis K7 (ATCC-MYA-413) was obtained from American Type Culture Collection (Manassas, VA). The cultivation medium included 2% (*w/v*) glucose, 1% (*w/v*) yeast extract, and 2% (*w/v*) peptone and the growth conditions were 25ºC for 24 h. The working culture was maintained on the agar slant, which contains the same composition with cultivation medium and 2% (w/v) agar.

The fermentation medium included 16.3% lactose, 1.2% casamino acid, and 0.8% yeast nitrogen base as recommended by our earlier study (Chapter 4).

7.3.2   Plastic composite support (PCS) tubes

The PCS tubes were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Pometto et al. (1997). The composition of PCS tubes, which were used in this study, included polypropylene (50% (*w/w*)), soybean hulls (35%), soybean flour (SF) (5%), salts (2 g/kg of sodium acetate, 1.2 g/kg of $MgSO_4 \cdot 7H_2O$, and 0.06 g/kg of $MnSO_4 \cdot 7H_2O$), yeast extract (5%), and dried bovine albumin (5%) (Chapter 3). The PCS tubes were produced as cylindrical shape with a wall thickness of 2.5 mm and an outer diameter of 10.5 mm.

7.3.3   Biofilm reactor set-up and biofilm formation

A Sartorious Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel was used to construct biofilm reactor, which had 12 PCS tubes (6.5 cm long) bound to the agitator shaft in a grid-like fashion, with six rows of two parallel tubes. The reactor vessel with PCS was autoclaved with water at 121°C for 90 min. Then, 1.5 L of sterilized fermentation medium was added aseptically to the reactor vessel with PCS. For biofilm formation, a 24-h grown culture of *K. lactis* K7 (1% *v/v*) was inoculated. Then, five repeated-batch fermentations were performed at the conditions (no pH control, 25ºC, 150 rpm, and no aeration) as recommended by our earlier study (Chapter 3).

7.3.4   Batch Fermentation for human lysozyme production

After formation of biofilm on the PCS tubes, the batch fermentation was conducted without inoculation of fresh culture since there was already immobilized *K.lactis* K7 on the PCS tubes. The fermentation conditions were no pH control, 25ºC, 150 rpm, and no aeration as recommended (Chapter 3). The fermentation started as soon as the fresh fermentation medium was transferred to biofilm reactor.

7.3.5   Stability of produced human lysozyme

The fermentation medium, which was taken in the end of 72 h of fermentation from the biofilm reactor, was centrifuged at 3400x*g* for 30 min to obtain cell-free fermentation broth for stability tests. The cell-free fermentation broth was kept in sterilized flasks and kept sterile during the stability tests to avoid any activity decrease by the possible reaction on the microorganisms, caused by the contamination.

7.3.5.1 Storage stability

The cell free fermentation broth was stored at 4, 10, and 25°C for 30 days. The human lysozyme activity of the medium was monitored during storage.

7.3.5.2 Temperature and pH effects on stability

Three factors Box-Behnken response surface design was used to see the effects of temperature, pH during the time, and their interactions on human lysozyme activity. Temperature, pH, and time were evaluated at the following rages; 25-100°C, pH 3.5-9, 0-120 min, respectively. The experimental design was given in Table 1. The pH of the cell-free fermentation medium was adjusted by using concentrated HCl or 4 N NaOH. Then the incubated samples were analyzed for human lysozyme activity.

7.3.6 Lysozyme activity measurement

Lysozyme activity was measured using a method adapted from Richard et al. (1965). The procedure for lysozyme assay can be described briefly as follows: A 0.5 mg/ml of *Micrococcus lysodeikticus* cell suspension (Sigma-Aldrich, St. Louis, MO) was prepared using potassium phosphate buffer at pH 6.2. The assay solution consists of 600 µl of 0.5 mg/ml *M. lysodeikticus* cell suspension, 200 µl of 300 mM sodium chloride solution, and 400 µl sample. The absorbances were measured every 10 s for 1 min at 540 nm. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 A_{540nm} change per minute. The slope of the linear regression line refers to the speed of lysis of *M. lysodeikticus* suspension, and the slope estimates the concentration of human lysozyme in unit per milliliter as shown in Eqn 7.1

$$\text{Lysozyme (U/ml)} = (\text{slope} / 0.4 \text{ ml sample}) \times 1000 \quad \dotsb \quad \text{Eqn. 7.1}$$

7.3.7 Statistical analysis

The statistical analyses were performed by using MINITAB Statistical Software package (Version 15, Minitab Inc., State College, PA). Analysis of variance (ANOVA) and Tukey's test was performed for investigating statistically significant differences at different trails. A *p*-value of <0.05 was considered to be significant. All experiments were conducted in duplicate.

7.4 Results and Discussion

An evaluation of the storage stability of cell-free medium is important to adjust the time before the recovery process. Therefore, the storage stability of human lysozyme in cell-free fermentation medium was evaluated at 4, 10, 25°C for 30 days (Figure 7.1) at around pH 4. During 30 days of storage, lysozyme activity did not change at 4, 10°C. However, slight decrease in the lysozyme activity (from 172 U/ml to 166 U/ml) was observed at 25°C, but the decrease was not significant (p$\geq$0.05).
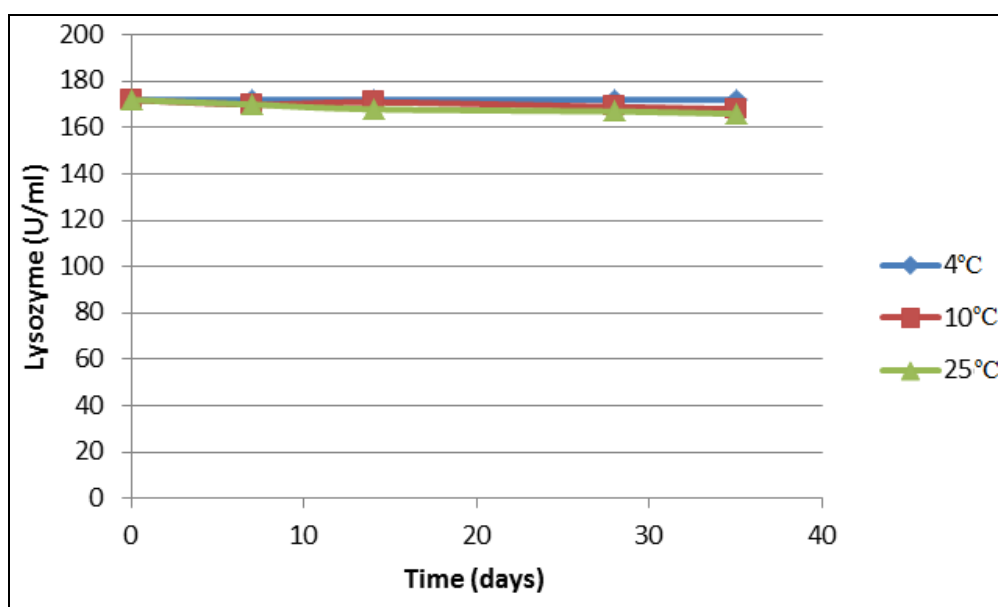
Figure 7.1 Storage stability of produced human lysozyme at various temperatures.

Then, the effects of pH, temperature, time, and their interactions on human lysozyme activity were evaluated using three factors in the Box-Behnken design. The temperature, pH, and time were evaluated at the ranges of 25-100°C, 3.5-9, 5-120 min, respectively. The human lysozyme activity results for various combinations are presented in Table 7.1.

Table 7.1 Effects of pH and temperature during the time on human lysozyme activity.

Time (h)	pH	Temperature (°C)	Lysozyme (U/ml)
62.5	3.50	100.0	5.00
120.0	3.50	62.5	20.00
120.0	6.25	100.0	15.00
120.0	9.00	62.5	0.00
120.0	6.25	25.0	122.00
62.5	9.00	100.0	0.00
5.0	3.50	62.5	45.00
62.5	6.25	62.5	83.00
5.0	6.25	25.0	118.00
5.0	9.00	62.5	35.00
5.0	6.25	100.0	90.00
62.5	3.50	25.0	62.00
62.5	9.00	25.0	55.00
62.5	6.25	62.5	83.00
62.5	6.25	62.5	80.00

As a result, a second order polynomial model was obtained (Eqn. 7.2). The sample variations of 99.5% of lysozyme activity were attributed to the factors stated in the model. The predictive capability of the model was found as 91.8%. The adjusted R^2, which is correlation strength between response and predicted value, was 98.5%.

Lysozyme (U/ml) = -155.3+ 91.9×pH + 0.41×time - 0.72×temperature – 7.5×pH^2

$\qquad$ - 0.009×time×temperature **……………………………..** Eqn. 7.2

Table 7.2 shows the analysis of variance results for the models. The model was significant and the lack of fit was insignificant at the 95% confidence interval. While

temperature and time by themselves was not significantly effective on lysozyme activity ($p \geq 0.05$), their interaction effect was significant ($p < 0.05$). Lysozyme was also significantly affected by the pH with a quadratic relationship ($p < 0.05$). For example, while the lysozyme activity was 62 U/ml at 25°C, pH 3.5 after 62.5 min, 55 U/ml of lysozyme activity was observed at 25°C, pH 9 after 62.5 min. On the other hand, the lysozyme activity (122 U/ml) was higher at the same temperature (25°C) after 120 min when the incubation pH was 6.25 than the activity at pH 3.5 and 9.

Table 7.2. Analysis of variance for lysozyme response surface quadratic model obtained from the experimental design.

Source	Degree of freedom	Sum of squares	F value	p value
Regression	9	23662.7	102.90	0.000
Linear	3	9991.8	140.43	0.000
Quadratic	3	12084.7	157.66	0.000
Interaction	3	1586.3	20.69	0.003
Residual	5	127.7		
Lack of fit	3	121.7	13.53	0.0700
Pure error	2	6		
Total	14	23790.4		

Figure 7.2 shows the response surface plots for the effects of pH, temperature, and time on human lysozyme activity. At 62.5°C after incubation for 120 min, although 20 U/ml lysozyme activity was observed at pH 3.5, there was no lysozyme activity at pH 9. Cunningham et al. (1991) reviewed that egg-white lysozyme is heat stable in acidic solutions (pH 5.29, 100°C, 30 min).
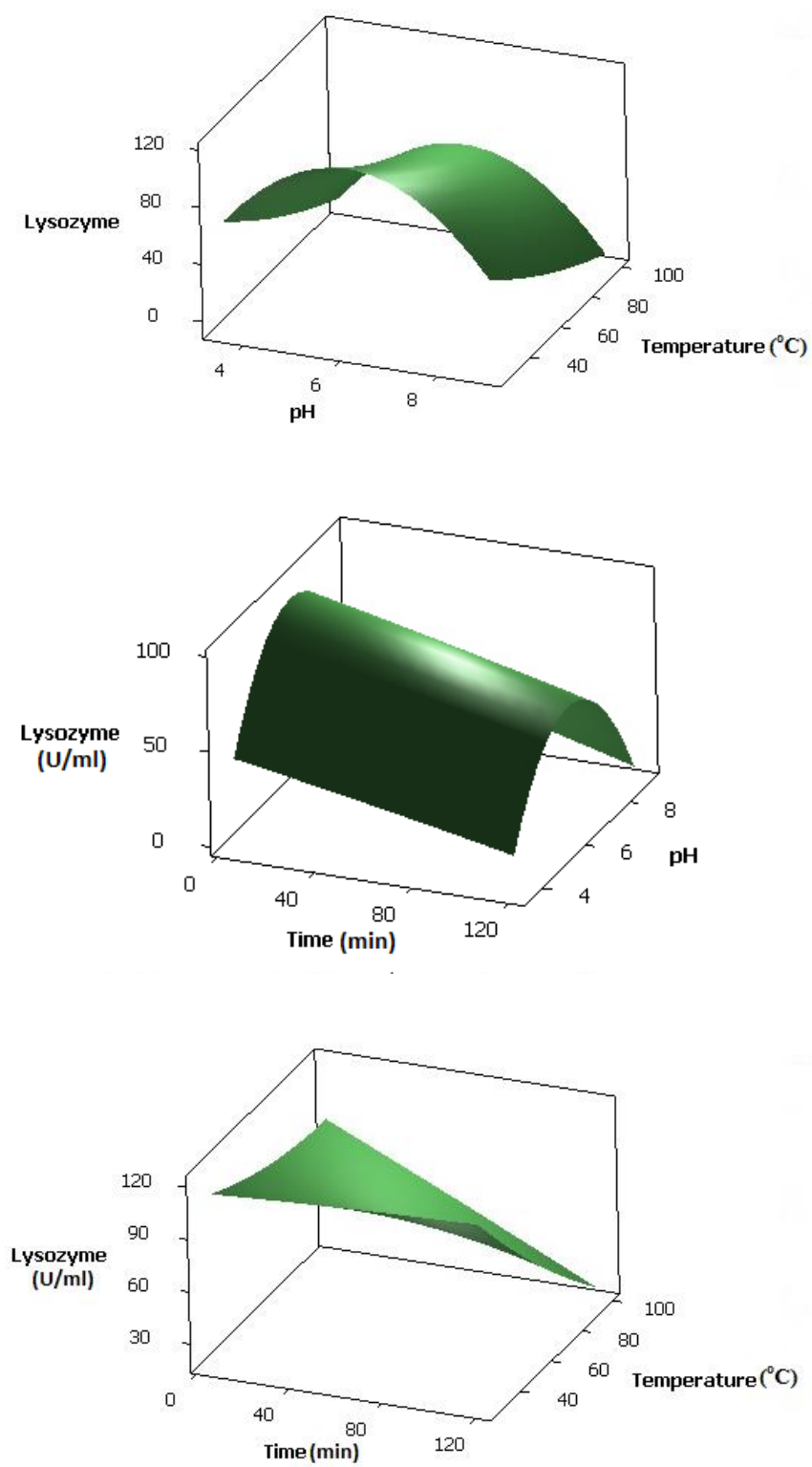
Figure 7.2. Effects of pH, temperature, time, and their interaction on produced human lysozyme.

Yang et al. (2011) also reported more than a 20% decrease after incubation at 100°C for 5 min at pH 6.24 in the human lysozyme activity, which was extracted from the transgenic milk. Moreover, Bethell (2006) investigated the stability of human lysozyme from transgenic rice and concluded that while human lysozyme at pH 7.4 was stable at 60º C and 72º C for 15 min, it lost its activity after 5 min at 85º C and almost immediately at 100º C. Tomizawa et al. (1994) suggested that irreversible inactivation of lysozyme occurs at 100°C and pH 4 as a result of the accumulation of chemical reactions (isomerization of Asp-Gly, deamidation of Asn, racemization of Asp and Asn, and cleavage of the AspX peptide bond).

7.5 Conclusion

Lysozyme activity in a cell free fermentation medium was stable for 30 days at temperatures of 4, 10, and 25°C. The human lysozyme activity was evaluated at various pH, temperature, and times. Activity lost at higher pH was significantly higher than the lower pH at high temperatures. Also, the activity was high at 25°C, and a decrease in lysozyme activity at both 62.5^{o}C and 100°C were observed at all pH levels. As a future study, the effect of ionic strength effect can be evaluated for the stability of human lysozyme.

References

Bethell, D. R. 2006. Generally recognized as safe notification for lysozyme (human) from rice for use as an ingradient in infant and pre-term formulas and pediatric medical foods. Sacramento,CA: Ventria Biosciences.

Cunningham, F. E., V. A. Proctor, and S. J. Goetsch. 1991. Egg-white lysozyme as a food preservative: An overview. *World's Poultry Science Journal* 47: 141-163.

Huang, E. L., and A. Demirci. 2009. Enhanced human lysozyme production by *Kluyveromyces lactis. Food and Bioprocess Technology* 2: 222–228.

Hughey, V. L., and E. A. Johnson. 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Applied and Environmental Microbiology* 53(9): 2165-2170.

Maullu, C., G. Lampis, T. Basile, A. Ingiani, C. M. Rossolini, and R. Pompei. 1999. Production enriched lysozyme biomass from cheese industry by products. *Journal of Applied Microbiology* 86: 182-186.

Naudi, A. S. 2000. *Natural Food Antimicrobial Systems*. New York, NY: CRC Press.

Pometto III, A.L., A. Demirci, and K. E. Johnson. 1997. Immobilization of microorganisms on a support made of synthetic polymer and plant material. U.S. Patent No. 5,595,893.

Richard, M. P., C. C. Ramesh, and M. S. Khem. 1965. A rapid and sensitive assay of muramidase. *Proceedings of The Society for Experimental Biology and Medicine* 119: 384–386.

Takano, K., K. Ogasahara, H. Kaneda, Y. Yamagata, S. Fujii, E. Kanaya, M. Kikuchi, M. Oobatake, and K. Yutani.1995. Contribution of Hydrophobic Residues to the Stability of Human Lysozyme: Calorimetric Studies and X-ray Structural Analysis of the Five Isoleucine to Valine Mutants. *Journal of Molecular Biology* 254: 62–76

Tomizawa, H., H. Yamada, and T. Imoto. 1994. The Mechanism of Irreversible Inactivation of Lysozyme at pH 4 and 100 "C. *Biochemistry* 33: 13032-13037.

Vocadlo, D. J., G. J. Davies, R. Laine, and S. G. Withers. 2001. Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate. *Nature:* 412 (6849): 835-838.

Yang, B., J. Wang, B. Tang, Y. Liu, C. Guo, P. Yang, T. Yu, R. Li, J. Zhao, L. Zhang, Y. Dai, and N. Li. 2011. Characterization of Bioactive Recombinant Human Lysozyme Expressed in Milk of Cloned Transgenic Cattle. *PLoS ONE* 6(3): 1-10.

CHAPTER 8

MODELING OF HUMAN LYSOZYME PRODUCTION
BY *KLUYVEROMYCES LACTIS K7* IN BIOFILM REACTOR

8.1 Abstract

Human lysozyme production by microbial fermentation is getting attention due to its non-allergic nature and higher enzyme activity compared to the conventional chicken egg lysozyme. The batch fermentation for human lysozyme production by *K. lactis* K7 in biofilm reactor was described by primary models. Since all biomass production, human lysozyme production, and lactose consumption followed sigmoidal pattern in plots, the modified Gompertz and modified Logistic models were evaluated to describe the biomass production, lysozyme production, and lactose consumption. While the biomass production described better with modified Logistic model, the human lysozyme production and lactose consumption were described better by modified Gompertz models. The R^2 for all models for biomass production, human lysozyme production, and lactose consumption was 0.99. The root mean square errors for biomass production, human lysozyme production, and lactose consumption were 0.046, 7.1, and 3.4, respectively. These models can provide information about the kinetic parameters and they can be a useful tool to control the batch fermentation of *K. lactis* K7 for human lysozyme production in a biofilm reactor.

8.2 Introduction

Lysozyme is a natural antimicrobial compound, which was discovered in 1922 by Alexander Fleming (Naudi, 2000). Lysozyme is an enzyme, which is a type of murein hydrolases. Murein hydrolases is the common group that digests peptidoglycans of bacteria. Because of the bactericidal effect, lysozyme has been of interest in the food, pharmaceutical, and cosmetic industries. Lysozyme is an attractive natural antimicrobial compound, because it is harmless to humans and is already produced naturally in the

body and many foods, such as milk and turnips. The FAO/WHO and several countries including Austria, Belgium, Denmark, Finland, France, Germany, Italy, The United Kingdom, Spain, and Australia have acknowledged the non-toxicity of lysozyme (Naudi, 2000). Therefore, it is needed to be produced commercially to meet its current demand. *Kluyveromyces lactis* K7 is a genetically modified microorganism, which can produce human lysozyme in the presence of lactose.

Up to now, human lysozyme production using *K. lactis* K7 has been studied in flask by Maullu et al. (1999), in suspended-cell reactor by Huang and Demirci (2009), and in biofilm reactors (Chapters 3 and 4). Maullu et al. (1999) produced human lysozyme in cottage cheese whey. Huang and Demirci (2009) produced lysozyme in batch fermentation and evaluatuated different growth parameters. Biofilm reactor can enhance the production by increasing the cell concentration via passive immobilization of cells on to solid support. Biofilm formation and detachment of the cells is a dynamic process. When the growth is in balance with the detachment, the system reaches to pseudo-steady state and at this stage the thickness of the biofilm is considered as maximum (Demirci et al., 2007). Therefore, suspended growth of cells also occurs in the biofilm reactor. The growth conditions of *K. lacis* K7 in bioreactors was optimized (Chapter 3) at 25°C, pH 4 with no aeration. The fermentation media was optimized (Chapter 4) resulting in 173 U/ml lysozyme production at 16.3% lactose, 1.2% casamino acid, and 0.8 % yeast nitrogen base.

Industrial fermentation systems need tools to control the process. Kinetic models can provide information to describe the fermentation process at certain times and help to design and predict production during the process. Sigmoidal equations, such as modified Logistic and modified Gompertz, have been widely used to describe the fermentation systems since they include all lag, log, and stationary phases. Arroyo et al. (2005) used a modified Gompertz model to describe the growth of *Pichia anomala*, a strain of yeast associated with olive fermentation to determine maximum specific growth rate, the lag phase period, and maximum specific growth rate at evaluated salt concentration,

temperature, and pH. Mu et al. (2006) described the kinetics of batch anaerobic hydrogen production by mixed anaerobic cultures with a modified Gompertz equation. Chowdhury et al. (2007) also reported that both modified Gompertz and Logistic model equations were fitted well to the experimental data for the growth of *Pediococcus acidilactici* H and the production of bacteriocin (pediocin). Moreover, Zajsek and Gorsek (2010) described batch kefir fermentation for ethanol production by mixed natural microflora by Gompertz equations, which was able to give the growth of lactic acid bacteria, the lag time, the specific production rate and the maximum ethanol mass concentration and describe the formation of ethanol as the fermentation proceeded.

In the literature, there is no study on modeling of human lysozyme production by microbial fermentation. Therefore, this study was undertaken to evaluate the modified Gompertz and modified Logistic models to describe the production of suspended biomass and human lysozyme productions, lactose consumption during batch fermentation for human lysozyme production by *K. lactis* K7 in a biofilm reactor.

8.3 Materials and Methods

8.3.1   Microorganism and medium

Kluyveromyces lactis K7 (ATCC-MYA-413) was obtained from American Type Culture Collection (Manassas, VA). The cultivation medium included 2% (*w/v*) glucose, 1% (*w/v*) yeast extract, and 2% (*w/v*) peptone and the conditions were at 25ºC for 24 h. The working culture was maintained on the agar slant, which contains the same composition with cultivation medium and 2% (w/v) agar.

The fermentation base medium included 16.3% lactose, 1.2% casamino acid, and 0.8 % yeast nitrogen base as recommended by our earlier study (Chapter 4).

8.3.2   Biofilm reactor set-up

Sartorious Biostat B Plus bioreactor (Allentown, PA) equipped with a 2-L vessel was used to construct biofilm reactor, which had 12 Plastic composite support (PCS) tubes (6.5 cm long) bound to the agitator shaft in a grid-like fashion, with six rows of two parallel tubes. The PCS tubes were manufactured in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw corotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) (Pometto et., 1997). The composition of PCS tubes, which were used in this study, included polypropylene (50% (*w/w*)), soybean hulls (35%), soybean flour (SF) (5%), salts (2 g/kg of sodium acetate, 1.2 g/kg of $MgSO_4 \cdot 7H_2O$, and 0.06 g/kg of $MnSO_4 \cdot 7H_2O$), yeast extract (5%), and dried bovine albumin (5%) (Chapter 3).

The reactor vessel with PCS was autoclaved with water at 121°C for 90 min. Then, 1.5 L of sterilized fermentation medium was added aseptically to the reactor vessel with PCS. For biofilm formation, a 24-h grown culture of *K. lactis* K7 (1% *v/v*) was inoculated. Then, five repeated-batch fermentations were performed at the conditions (no pH control, 25ºC, 150 rpm, and no aeration) as recommended by our earlier study (Chapter 3).

8.3.3   Batch fermentation in biofilm reactor

Batch fermentations were conducted in the biofilm reactor without inoculation, because it had already stable biofilm in it. The growth conditions were 25ºC, no pH control, 150 rpm, and no aeration as suggested by our earlier study (Chapter 3). Samples were taken during fermentation periodically for about 80 hours during fermentation and analyzed to determine the amounts of lysozyme, biomass, and lactose. The fermentation run was done in triplicate. After each batch, the spent fermentation medium in the reactor was pumped aseptically out of the reactor and the fresh sterile medium was pumped in to the biofilm reactor and another batch has been started.

8.3.4   Analysis

8.3.4.1 Biomass

Suspended biomass was determined by measuring optical density (OD) using a spectrophotometer (Beckman Coulter, Fullerton, CA) by obtaining absorbance at 600 nm, which was then converted to biomass concentration by using Eqn 8.1 (Huang and Demirci (2009).

$Biomass$ (g/l) = $0.564 \times OD_{600}$ **………………………………..**   Eqn. 8.1

8.3.4.2 Lactose concentration

 Lactose concentration determinations were conducted using a Waters's high pressure liquid chromatography (HPLC) equipped with a refractive index detector (Waters, Milford, MA). Lactose was separated using Bio-Rad Aminex HPX-87H column (300×7.8 mm; Bio-Rad, Richmond, CA) with 0.8 ml/min of 0.012 N sulfuric acid as mobile phase. The detector temperature and column temperature were maintained at 35 and 65ºC, respectively. Prior to the analysis, the fermentation samples were centrifuged for 2 min at 5200 g to separate the cells from the medium. Then, the samples were diluted 3-fold. Finally, the samples and the standard solutions were filtered with 0.2 µm PTFE membrane filters and injected to HPLC. The injection volume was 20 µl. Identification was done with comparison of retention times of compounds and external standard.

8.3.4.3 Lysozyme activity

Lysozyme activity measurement was carried out using a method adapted from Richard et al. (1965). The procedure for lysozyme assay is as follows: A 0.5 mg/ml of *Micrococcus lysodeikticus* cell suspension (Sigma-Aldrich, St. Louis, MO) was prepared using potassium phosphate buffer at pH 6.2. The assay solution consists of 600 µl of 0.5 mg/ml *M. lysodeikticus* cell suspension, 200 µl of 300 mM sodium chloride solution, and 400 µl sample. The absorbances were measured every 10 s for 1 min at 540 nm. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 A_{540nm} change per

minute. The slope of the linear regression line refers to the speed of lysis of *M. lysodeikticus* suspension, and the slope estimates the concentration of human lysozyme in unit per milliliter by Eqn 8.2.

$$\text{Lysozyme (U/ml)} = (\text{slope} / 0.4 \text{ ml sample}) \times 1000 \dots\dots\dots\dots \text{ Eqn. 8.2}$$

8.3.5 Mathematical models

Primary models for biomass and human lysozyme production and consumption of lactose were developed using the average data obtained from two batch fermentations; one used for the model and the other set used for the validation of model.

8.3.5.1 Modeling of biomass production

The modified Logistic (Eqn. 8.3) and modified Gompertz (Eqn. 8.4) models, which were developed by Pearl and Reed (1920) and further modified by Zwietering et al. (1990) with the parameters with biological meaning, were used to describe the biomass production.

$$X_t = \frac{A}{\left(1 + exp\left(\frac{4\mu_m}{A}(\lambda - t) + 2\right)\right)} \quad \dots\dots\dots\dots\dots\dots\dots \text{ Eqn. 8.3}$$

$$X_t = A\ exp\left(-exp\left(\frac{\mu_m}{A}\ e\ (\lambda - t) + 1\right)\right) \quad \dots\dots\dots\dots\dots\dots \text{ Eqn. 8.4}$$

where X_t (g/l) is the biomass at certain time (t), μ_m is the maximum growth rate, A is the maximum biomass level, λ is the lag time (Figure 2.9), which is the x intercept of the tangent line on the growth curve, where the specific growth rate is at the maximum level.

8.3.5.2 Modeling of human lysozyme production

The modified Logistic (Eqn. 8.5) and modified Gompertz (Eqn. 8.6) equations were used to model human lysozyme production by substituting the human lysozyme production parameters in Eqn.8.3 and Eqn. 8.4, respectively.

$$P_t = \frac{A}{\left(1 + exp\left(\frac{4\mu_{m\text{-}p}}{A}(\lambda - t) + 2\right)\right)} \quad \dots\dots\dots\dots\dots\dots\dots \quad \text{Eqn. 8.5}$$

$$P_t = A\, exp\left(-exp\left(\frac{\mu_{m\text{-}p}}{A}\, e\, (\lambda - t) + 1\right)\right) \quad \dots\dots\dots\dots\dots \quad \text{Eqn. 8.6}$$

where P_t (U/ml) is the human lysozyme production at certain time (t), $\mu_{m\text{-}p}$ is the maximum human lysozyme production rate, A is the maximum human lysozyme production level, λ is the lag time, which is the x intercept of the tangent line on the production curve, where the production rate is at the maximum level.

8.3.5.3   Modeling of lactose consumption

The consumed lactose was described by the modified Logistic (Eqn. 8.7) and modified Gompertz (Eqn. 8.8) equations by substituting the lactose consumption parameters in Eqns 8.3 and 8.4, respectively, and substituting them from the maximum lactose concentration, which give the residual lactose concentration at time t (h).

$$S_t = A - \frac{A}{\left(1 + exp\left(\frac{4\mu_{m\text{-}s}}{A}(\lambda - t) + 2\right)\right)} \quad \dots\dots\dots\dots\dots\dots \quad \text{Eqn. 8.7}$$

$$S_t = A - A\ exp\left(-exp\left(\frac{\mu_{m-s}}{A}\ e\ (\lambda - t) + 1\right)\right) \quad \text{.....................} \quad \text{Eqn. 8.8}$$

where S_t (g/l) is the lactose concentration at certain time (t), μ_{m-s} is the maximum lactose consumption rate, A is the maximum lactose consumption level, λ is the lag time, which is the x intercept of the tangent line on the consumption curve, where the consumption rate is at the maximum level.

8.3.6 Statistical analysis

The statistical analyses were performed by using MINITAB Statistical Software package (Version 15, Minitab Inc., State College, PA). The fitted models were validated for the goodness-of-fit by root mean square error (RMSE) and mean absolute error (MAE) between the calculated data from the model and experimental data. Moreover, the intercept and R^2 of the regression line, which was obtained by plotting experimental data versus the calculated data from the model.

8.4 Results and Discussion

Two batch fermentations were performed for human lysozyme production by *K. lactis* K7 in biofilm reactor. The data from one batch was used to construct primary models for biomass production, human lysozyme production, and lactose consumption. The data from the second batch was used for validation of the models.

8.4.1 Modeling of biomass production

Sigmodial models; modified Logistic and modified Gompertz models were used to describe the lag, log, and stationary phases of the growth of *K. lactis* K7. The maximum growth rate, lag time, maximum biomass production amount were calculated from the experimental data as: 0.08 g/l/h, 6 h, 2.18 g/l, respectively. These values were used in Eqns 8.3 and 8.4 to build the modified Logistic and modified Gompertz models for the biomass production.

Figure 8.1 shows the graphical comparisons of the actual growth curve with modified logistic and modified-Gompertz models by plotting both the experimental data and the predicted values obtained from the models.
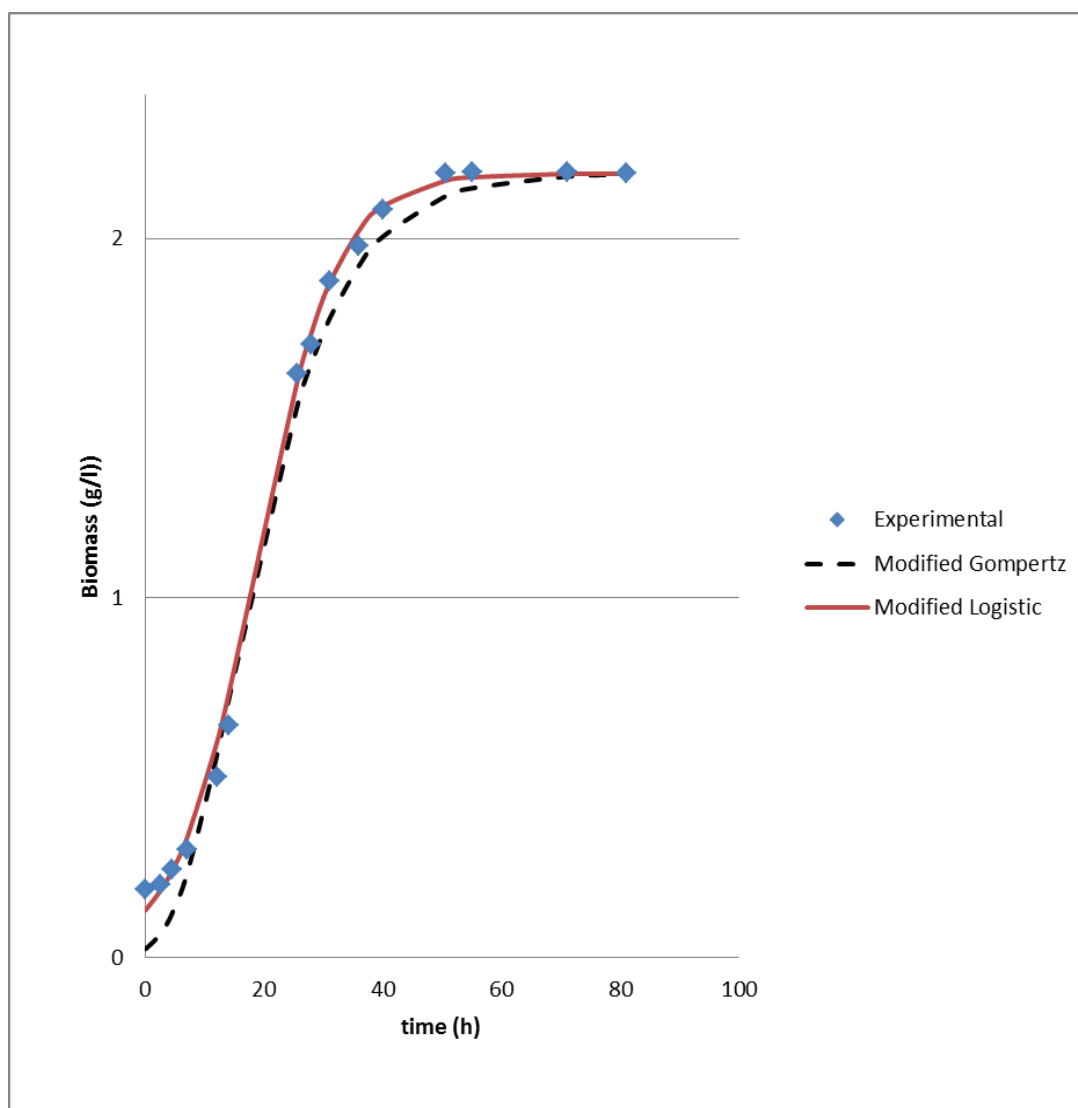
Figure 8.1. Growth curve of *K. lactis* K7 fitted with the modified Logistic and modified Gompertz equations.

Table 8.1 shows the comparison via regression through origin of experimental and predicted values. The resulting R^2 for both models was 0.99, which was obtained by plotting experimental versus predicted values and calculating the (Figure 8.2). Linear regression line that fits the data points was generated. The linear equation that define this trend was "Experimental=a×Predicted+b". The intercept (b) and the slope (a) were close to 0 and 1, respectively, which means that the models were able to describe the biomass production successfully (Zhao et al., 2001).
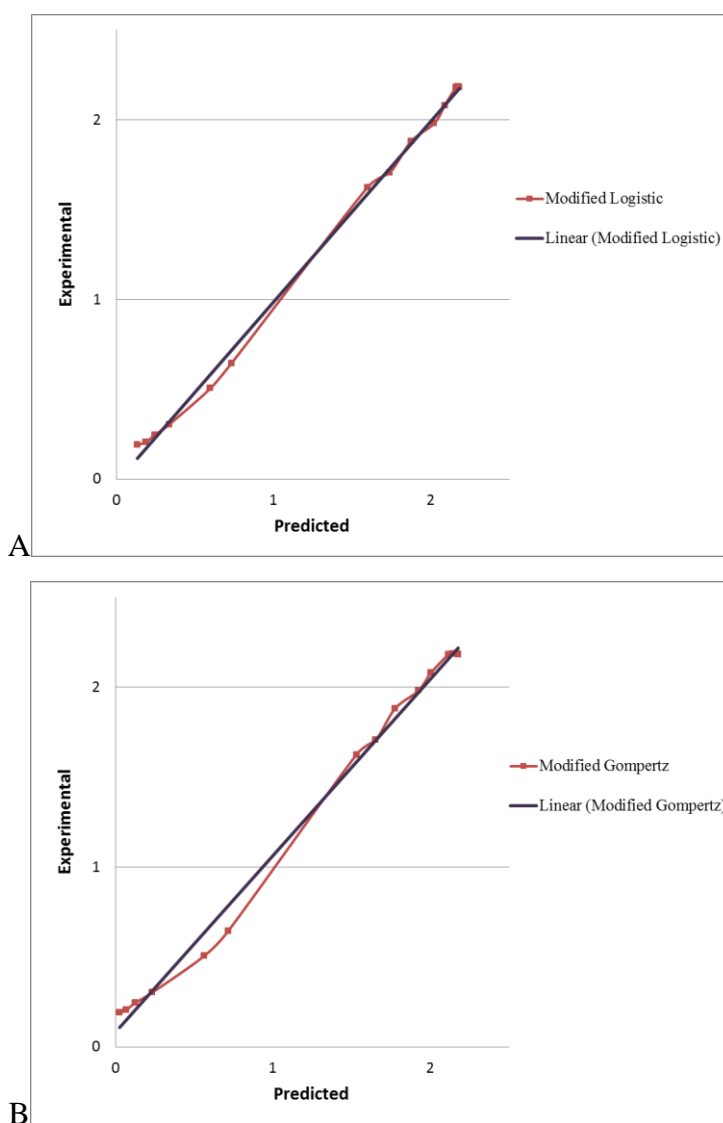
Figure 8.2 Experimental versus predicted values with trend lines for both models for biomass production. (A. For modified Logistic model, B. For modified Gompertz model).

The MAE and RMSE were evaluated (Table 8.1). While the MAE shows how far estimates or forecasts are from actual values, RMSE, which gives a relatively high weight to large errors, is the square root of average of squared errors. The greater difference between MAE and RMSE shows the greater variance in the individual errors in the sample. If they are close, all the errors are at the same magnitude. Moreover, lower MAE and RMSE means the model describes the data well. The RMSE and MAE for the modified Logistic model were lower than the modified Gompertz model for biomass production and their values were close to each other, which means all the errors during lag, log, and stationary phases were close to each other because the modified Gompertz model estimated lower biomass value than the actual value in the end of lag and log phases. In addition to biomass amount, the production rate between each time interval $[(X_{t2}-X_{t1})/(t_2-t_1)]$ was calculated from the experimental and predicted data to see if the models are able to describe the production rate at each time interval. Both modified Gompertz model (0.012 RMSE and 0.005 MAE) and modified Logistic model (0.011 RMSE and 0.003 MAE) described well the production rate between each time intervals.

Table 8.1 Validation of models for biomass production.

Model	RMSE	MAE	R^2	Slope	Intercept
Modified Logistic	0.046	0.011	0.99	1.01	-0.11
Modified Gompertz	0.080	0.050	0.99	1.04	-0.01

8.4.2 Modeling of human lysozyme production

Since human lysozyme production is growth associated and follows a sigmoidal trend, the modified Logistic and modified Gompertz models were adjusted to describe the human lysozyme production. The experimental data, which was obtained from the two fermentation runs, was used to calculate the maximum production rate (4.07 g/l/h), lag time (5 h), maximum production (173 U/ml). Then, Eqns 8.5 and 8.6 were used to build the modified Logistic and modified Gompertz models for human lysozyme production,

respectively. Figure 8.3 shows the experimental and predicted data, which was obtained from the constructed modified Logistic and modified Gompertz models.
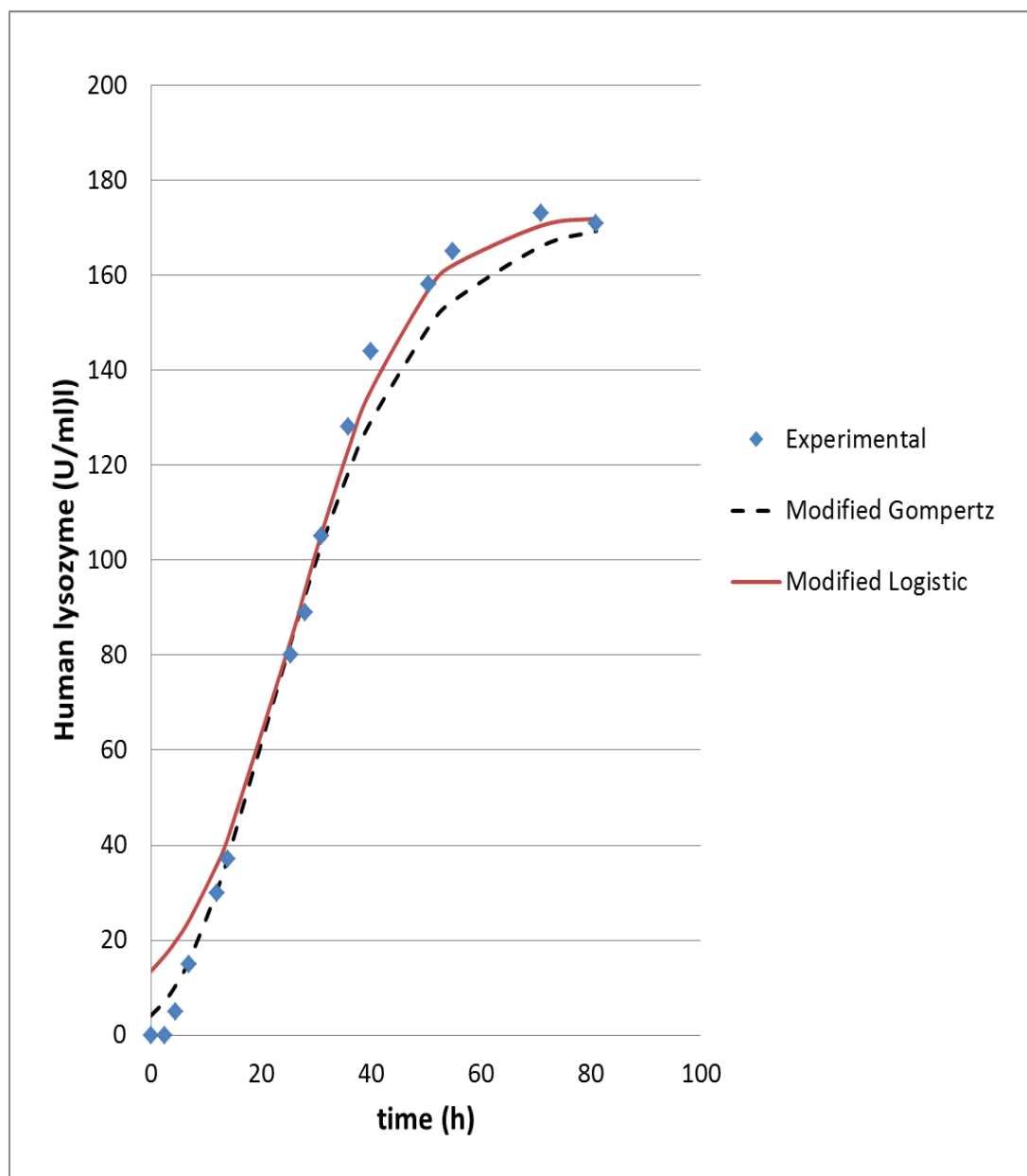
Figure 8.3 Experimental and predicted data by the modified Logistic and modified Gompertz equations for human lysozyme production.

While the modified Logistic models overestimated the production in the lag phase, the modified Gompertz model slightly underestimated the production at beginning of the stationary phase. Table 8.2 shows the validation of the models for human lysozyme production. The linear regression trend lines were shown in Figure 8.4 A and B. Although both model had 0.99 R^2, the intercept of the modified Gompertz model was closer to 0 than modified Logistic model (Table 8.2).

A

B
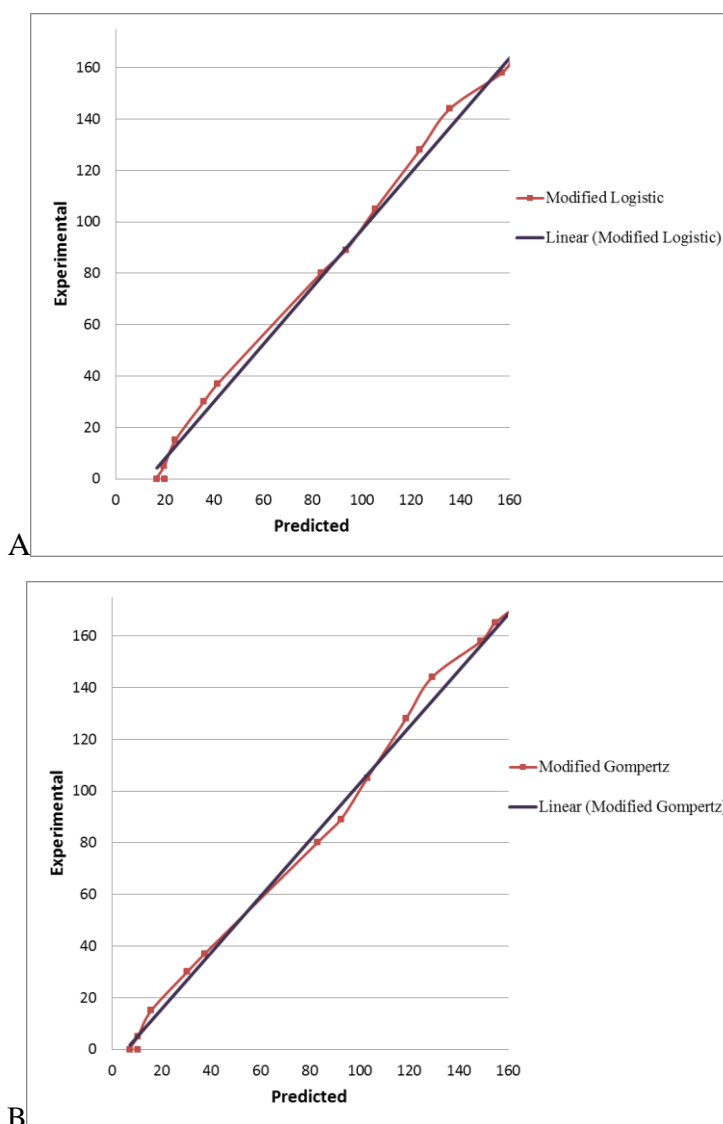
Figure 8.4 Experimental versus predicted values for models for lysozyme production (A. For modified Logistic model, B. For modified Gompertz model).

Moreover, because MAE and RMSE were also lower for modified Gompertz model than modified logistic model, modified Gompertz model was selected as a better model to describe the human lysozyme production than modified Logistic model. It might also be concluded that since the difference between RMSE and MAE was higher in modified Gompertz model, the errors during lag, log, and stationary phases were more different than each other than modified Logistic model. The production rate between each time interval $[(P_{t2}-P_{t1})/(t_2-t_1)]$ was also calculated from the experimental and predicted data to see if the models are able to describe the production rate at each time interval for lysozyme production. The RMSE and MAE for modified Logistic model were 0.96 and 0.57, respectively. Similarly, modified Gompertz model provided lower RMSE (1.00) and MAE (0.52) for the production rate at each time interval than the production amount. Therefore, we can state that the models were able to describe better the production rate for each time interval than the lysozyme production production.

Table 8.2 Validation of models for human lysozyme production.

Model	RMSE	MAE	R^2	Slope	Intercept
Modified Logistic	8.8	4.1	0.99	1.10	-12.99
Modified Gompertz	7.1	1.52	0.99	1.08	-4.87

8.4.3 Modeling of lactose consumption

The human lysozyme fermentation medium was also analyzed to determine the lactose concentration at certain times. Lactose consumption also showed a sigmoidal curve, so the modified Logistic and modified Gompertz models were modified to describe the lactose consumption by *K. lactis* K7 for human lysozyme production in biofilm reactor. Figure 8.3 shows the experimental and fitted data by the models for lactose consumption.

The maximum consumption rate (2.4 g/l/h), lag time (6 h), maximum lactose concentration (143 g/l) were calculated from the experimental data and substituted in to the Eqns 8.7 and 8.8 to build the modified Logistic and modified Gompertz models,

respectively. As it can be clearly seen from Figure 8.5, the modified Logistic model underestimated the lactose concentration at the beginning of the fermentation.
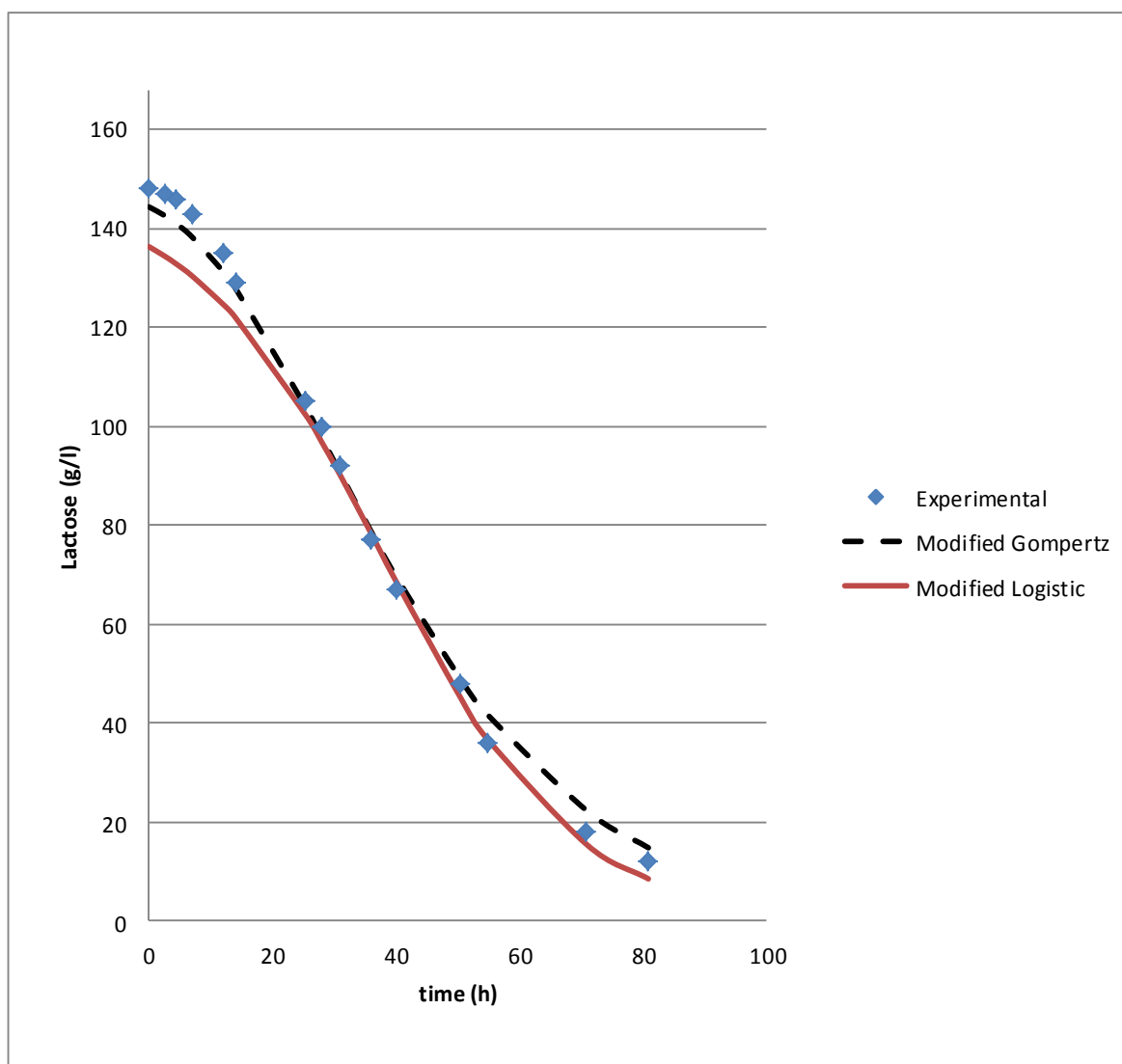
Figure 8.5 Experimental and predicted data by the modified Logistic and modified Gompertz equations for lactose consumption.

The linear regression trend line was generated by plotting experimental versus predicted values and the linear regression equation was obtained to define the relation between the values (Figure 8.6). The R^2 for both models was 0.99 while the slope was closer to 1 in the modified Gompertz model than modified Logistic model (Table 8.3).
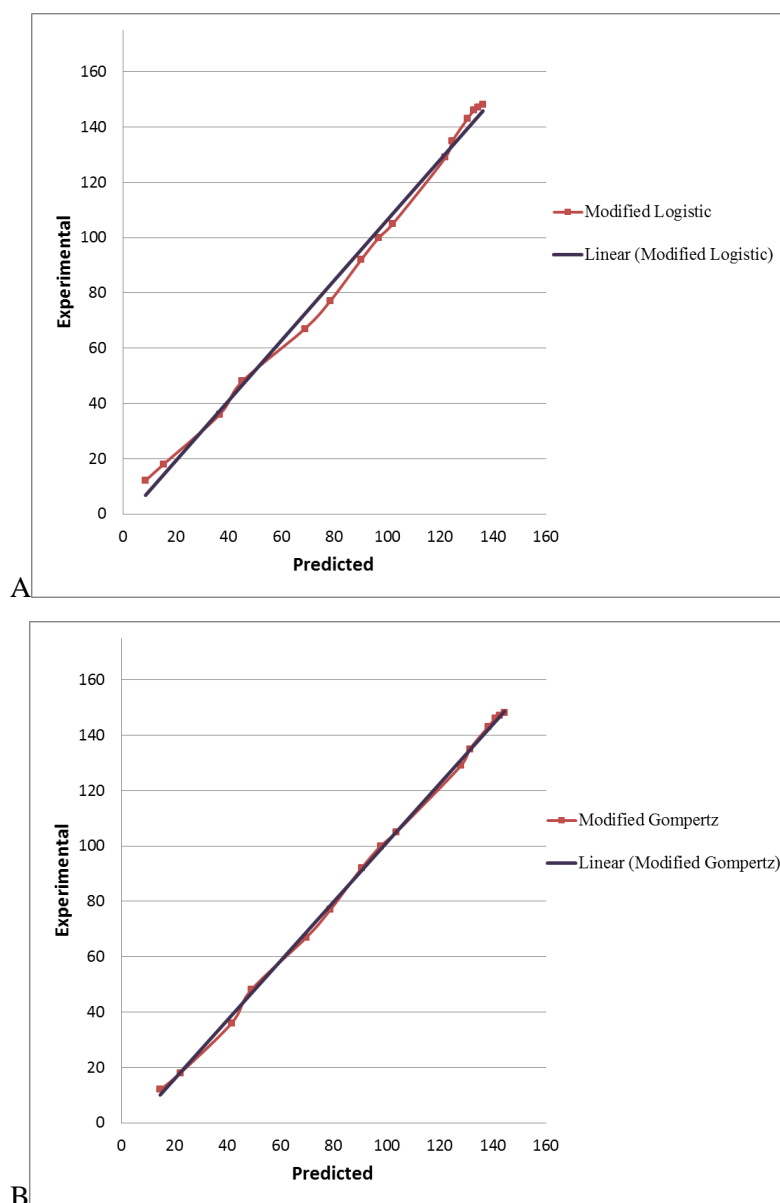
Figure 8.6 Predicted versus experimental values for models for lactose consumption. (A. For modified Logistic model, B. For modified Gompertz model.)

Moreover, the RMSE and MAE were lower for the modified Gompertz model than modified Logistic model. Therefore, it was concluded that the modified Gompertz model described successfully the lactose consumption better than the modified Logistic model. The lactose consumption rate at each time interval $[(S_{t1}-S_{t2})/(t_2-t_1)]$ was calculated for experimental and predicted data. Both the modified Gompertz model (0.52 RMSE and 0.17 MAE) and modified Logistic model (0.59 RMSE and 0.22 MAE) described well the consumption rate at each time intervals.

Table 8.3 Validation of models for lactose consumption.

Model	RMSE	MAE	R^2	Slope	Intercept
Modified Logistic	7.4	5.37	0.99	2.75	0.91
Modified Gompertz	3.4	0.63	0.99	0.93	5.55

8.5 Conclusion

The modeling of batch fermentation for human lysozyme production by *K. lactis* K7 in a biofilm reactor was investigated. Modified Gompertz and modified Logistic models were described for human lysozyme production, biomass production, and lactose consumption. The model that best described the biomass production was the modified Logistic model with 0.99 R^2 and a 0.046 RMSE. The modified Gompertz model described human lysozyme production and lactose consumption better than the modified Logistic model. The R^2 for both human lysozyme production and lactose consumption was 0.99 and the RSME values for human lysozyme production and lactose consumption were 7.1 and 3.4, respectively. As a future study, secondary models can be built to describe batch fermentation for human lysozyme production by *K. lactis* K7 in a biofilm reactor at different environmental conditions.

References

Arroyo, F. N., M. C. Duran Quintana, and A. Garrido Fernandez. 2005. Evaluation of Primary Models To Describe the Growth of *Pichia anomala* and Study of Temperature, NaCl, and pH Effects on Its Biological Parameters by Response Surface Methodology. *Journal of Food Protection* 68(3): 562–570.

Chowdhury, B. R., R. Chakraborty, U. R. Chaudhuri. 2007. Validity of modified Gompertz and Logistic models in predicting cell growth of *Pediococcus acidilactici* H during the production of bacteriocin pediocin AcH. *Journal of Food Engineering* 80: 1171–1175.

Demirci, A., T. Pongtharangkul, and A. L. Pometto III. 2007. Chapter 8: Applications of biofilm reactors for production of value-added products by microbial fermentation. In *Biofilms in the Food Environment,* 167-189. H. P. Blaschek, H. H. Wang, and M. E. Agle ed. Iowa, USA: Blackwell Publishing and The Institute of Food Technologists.

Huang, E. L., and A. Demirci. 2009. Enhanced human lysozyme production by *Kluyveromyces lactis. Food and Bioprocess Technology* 2: 222–228.

Maullu, C., G. Lampis, T. Basile, A. Ingiani, C. M. Rossolini, and R. Pompei. 1999. Production enriched lysozyme biomass from cheese industry by products. *Journal of Applied Microbiology* 86: 182-186.

Mu, Y., G. Wang, Han-Q. Yu. 2006. Kinetic modeling of batch hydrogen production process by mixed anaerobic cultures. *Bioresource Technology* 97: 1302–1307.

Naudi, A. S. 2000. *Natural Food Antimicrobial Systems*. New York, NY: CRC Press.

Pearl, R. and L. J. Reed. 1920. On the rate of growth of the population of the United States since 1970 and its mathematical representation. *Proceedings of the National Academy of Sciences of the United States of America* 6 (6): 275–288.

Pometto III, A.L., A. Demirci, and K. E. Johnson. 1997. Immobilization of microorganisms on a support made of synthetic polymer and plant material. U.S. Patent No. 5,595,893.

Richard, M. P., C. C. Ramesh, and M. S. Khem. 1965. A rapid and sensitive assay of muramidase. *Proceedings of The Society for Experimental Biology and Medicine* 119: 384–386.

Zajsek, K. and A. Gorsek. 2010. Modelling of batch kefir fermentation kinetics for ethanol production by mixed natural microflora. *Food and Bioproducts Processing* 88: 55-60.

Zhao, L., Y. Chen, and D. W. Schaffner. 2001. Comparison of logistic regression and linear regression in modeling percentage data. *Applied and Environmental Microbiology* 67(5): 2129–2135.

Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. Van‟t Riet. 1990. Modeling of the bacterial growth curve. *Applied Environmental MicrobiolOgy* 56: 1875-1881.

CHAPTER 9

CONCLUSIONS AND SCOPE FOR FUTURE RESEARCH

This study aimed to enhance human lysozyme production by *K. lactis* K7 using a biofilm reactor with PCS and different fermentation strategies. The Figures 9.1 and 9.2 show the production levels and the production rates in each fermentation processes that have been conducted in this study.

First, different plastic composite supports with different compositions were evaluated in terms of their absorption, biofilm formation, and human lysozyme production. The PCS, which included polypropylene (50% (*w/w*)), soybean hulls (35%), soybean flour (SF) (5%), salts (2 g/kg of sodium acetate, 1.2 g/kg of $MgSO_4 \cdot 7H_2O$, and 0.06 g/kg of $MnSO_4 \cdot 7H_2O$), yeast extract (5%), and dried bovine albumin (5%), was selected for human lysozyme production. Then, the optimum combination of growth parameters was determined by using a three-factor Box –Behnken design of response surface. The results suggested that the optimum conditions for biomass and lysozyme productions were different (27 °C, pH 6, 1.33 vvm for biomass production; 25 °C, pH 4, no aeration for lysozyme production). Therefore, different pH and aeration shift strategies were tested to increase the biomass at the first step and then secrete the lysozyme after the shift. Without pH control, a gradual decrease in the pH of fermentation broth was observed and the fermentation at 25 °C without pH and aeration control provided the highest lysozyme production amount (141 U/ml) among evaluated pH and aeration shift strategies (p <0.05) (Chapter 3).

After evaluation of the growth conditions, the fermentation medium composition was evaluated for human lysozyme production by *K. lactis* K7 in a biofilm reactor. Yeast nitrogen base was selected as the best nitrogen source for human lysozyme production, when compared to the yeast extract and corn steep liquor. Addition of salt inhibited the

lysozyme and biomass production. Moreover, 16% lactose, 1.2% casamino acid, 0.8% yeast nitrogen base were determined as optimum nutrient requirements of *K. lactis* K7 for human lysozyme production. As a result, 173 U/ml of lysozyme production was achieved with this fermentation medium. Moreover, higher production rate (4.07 U/ml/h) was obtained compared to the suspended-cell fermentation (2 U/ml/h) because of the increase in the cell density in the reactor (Chapter 4).

Then, fed-batch and continuous fermentation modes were evaluated for human lysozyme production by *K. lactis* K7 in a biofilm recator. Lysozyme production and production rate were significantly increased by fed-batch fermentation (187 U/ml, 5.9 U/ml/h) with 90 g/l glucose and feeding with 400 g/l lactose at a feeding rate of 0.6 ml/min at between 28 and 38 h because glucose caused increase in the cell growth and then lactose induced the human lysozyme production. Different dilution rates were evaluated for continuous fermentation. As a result, continuous fermentation with 0.055 h^{-1} dilution rate provided a significantly higher production rate (7.5 U/ml/h) than batch and fed-batch fermentations in biofilm reactor. Moreover, a higher dilution rate was able to use for high productivity by the application of a biofilm reactor (0.055 h^{-1} dilution rate) when compared to a suspended-cell reactor (0.04 h^{-1} dilution rate) because of the immobilized cells in the biofilm reactor (Chapter 5).

Since lysozyme has antimicrobial peptides, which might affect the growth of yeast cells, a simultaneous fermentation and online recovery system was designed to enhance human lysozyme production. To enhance recovery of lysozyme from the fermentation medium, adsorption and desorption of human lysozyme from the silicic acid, which was used as an adsorbent, were evaluated. The best adsorption (95.6% adsorption) was obtained at 25°C, pH 4, and a 25% silicic acid ratio. The conditions including 25°C, pH 6.2, and 5% sodium dodecyl sulfate with 1 M NaCl and 20% ethanol as the eluent composition provided 42% desorption. Four consecutive desorption processes increased recovery up to 98%. Then, the simultaneous fermentation and online recovery system with tangential

flow micro-filter module yielded a total of 280.4 U/ml of human lysozyme production was achieved (Chapter 6).

The storage stability before downstream processing and effect of temperature, pH, and time were evaluated. It was found out that lysozyme activity in cell free fermentation medium was stable for 30 days at all evaluated temperatures (4, 10, and 25°C). Also, the human lysozyme activity was at a maximum level at 25°C, decreased at 62.5^{o}C, and 100°C at all pH levels (Chapter 7).

Finally, batch fermentation for human lysozyme production by *K. lactis* K7 in a biofilm reactor was described by primary models. The modified Gompertz and modified Logistic models were used to describe human lysozyme production, biomass production, and lactose consumption, since all of them followed a sigmoidal pattern. The model that best described the biomass production was modified Logistic model with 0.99 R^2 and 0.046 RMSE. The modified Gompertz model described the human lysozyme production (0.99 R^2 and 7.1 RMSE) and lactose consumption (0.99 R^2 and 3.4 RMSE) better than the modified Logistic model (Chapter 8).

In conclusion, biofilm reactors enhanced human lysozyme production by *Kluyveromyces lactis* K7. Optimization of growth parameters, fermentation medium, application of fed-batch and continuous fermentation, and a simultaneous recovery system further enhanced the productivity. Moreover, the batch production was modeled and the stability of lysozyme in the fermentation medium was evaluated to give baseline information for the commercial production.
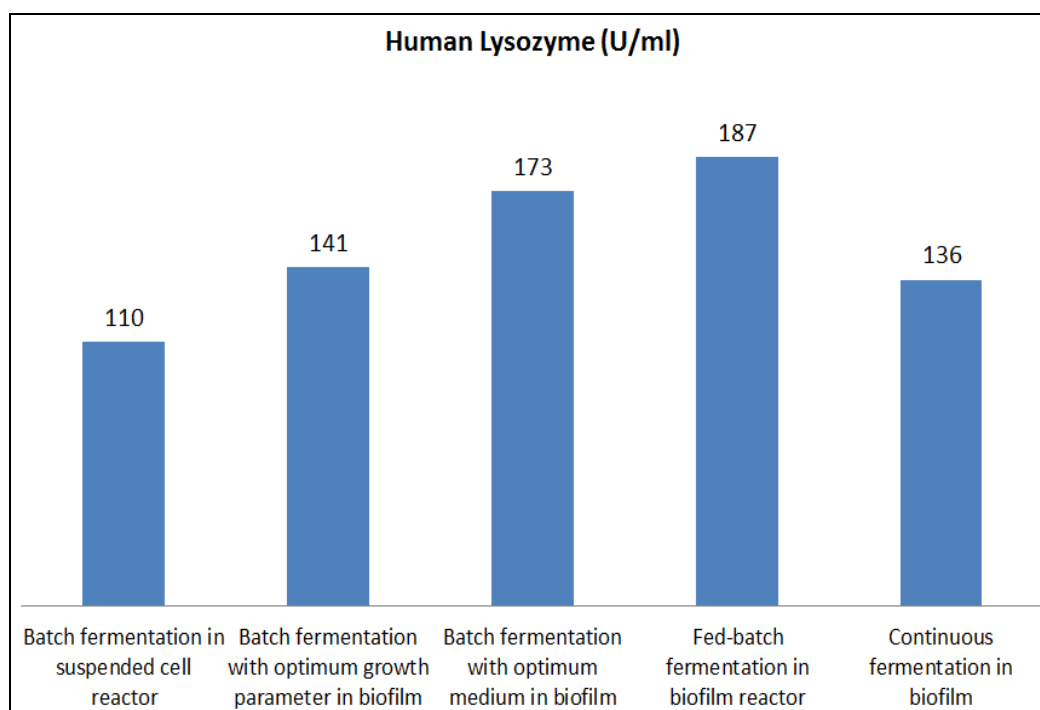
Figure 9.1. Summary of improvements in human lysozyme production achieved in this study.
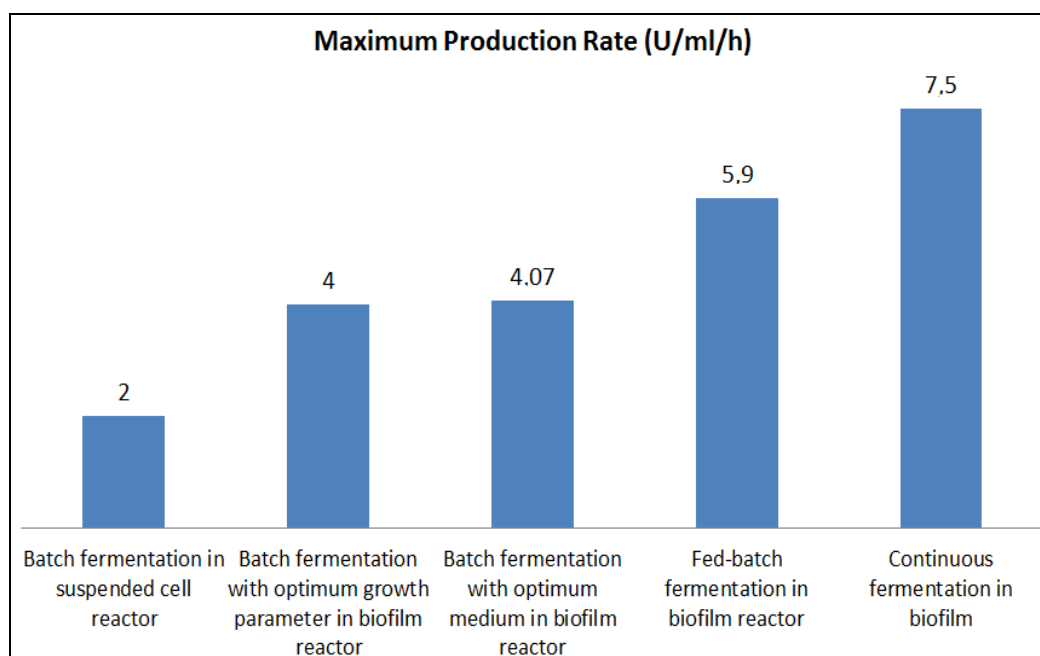
Figure 9.2. Summary of improvements in human lysozyme production rate achieved in this study.

As the recommendations for future studies, the design of the biofilm reactor can be changed to increase surface area of the support. Therefore, much more microorganism can attach to the surface. Although human lysozyme is a growth associated product, the optimum conditions for the production of human lysozyme by *K. lactis* K7 and cell growth are different. Therefore, increasing the biofilm formation can help to increase cell density in the reactor with batch fermentation mode at the optimum condition for human lysozyme production. Moreover, a strain, which can form more biofilm on the supports, can be genetically modified to produce human lysozyme more effectively in a biofilm reactor.

As a future study, different compositions of PCS, which have much more nitrogen content, can also be developed to reduce the need for a nitrogen source in the fermentation medium because PCS have a moderate nitrogen-leaching rate and the nitrogen content in the PCS can be sufficient for several batches. Moreover, continuous fermentation medium can also be evaluated since the medium, which left the reactor during continuous fermentation, still had unconsumed nutrients. Therefore, the cost of the fermentation can be deceased. The online recovery system can also be developed to process continuously because although this process reduced the product inhibition and allow much more cell growth, the decrease in the production rate at 80 h can be related the depletion of the nutrients. Continuous fermentation will provide fresh fermentation medium while the cell recycle and online recovery occur simultaneously.

Furthermore, the effectiveness of the adsorption process can be increased by removing the excess water from the fermentation medium, which will be recovered for human lysozyme if the recovery process will not be an online system. The removing of the water can help to increase in the hydrophobic interactions between the silicic acid and human lysozyme and this process can provide higher adsorption (%). In addition, different adsorbents and eluents can be evaluated for the effectiveness of the recovery process. The purification of the lysozyme from the recovery eluent also needs to be developed, because in this study the remaining sodium dodecyl sulfate content in the final eluent was

not determined and the removal of it is important if the human lysozyme will be used in the food industry. There is a maximum legal limit for sodium dodecyl sulfate concentration in food formulations. The characterization of the purified human lysozyme can be determined to provide information to industry about the stability of the produced lysozyme at different pH and temperatures for their processing systems.

Moreover, the fed-batch and continuous fermentations can be described by kinetic models and secondary models can be developed to further describe the fermentation in different environmental conditions. The support can be enhanced for large scale reactors and scale-up studies are needed. Since each microorganisms and production has its own characteristics, biofilm reactor with PCS can be evaluated for different antimicrobial compound productions.

REFERENCES

American College of Allergy, Asthma & Immunology. 2010. Egg Allergy. Available at; http://www.acaai.org/allergist/allergies/Types/food-allergies/types/Pages/egg-allergy.aspx.

Aeschlimann, A. and U. von Stockar. 1991. Continuous production of lactic acid from whey permeate by *Lactobacillus helveticus* in a cell-recycle reactor. *Enzyme and Microbial Technology* 13(10): 811–816.

Agle, M.E. 2007. Biofilms in food industry. In *Biofilms in the Food Environment*, 3-19. H. H. Wang,M. Agle, and E. Meredith, ed. Iowa: USA: Blackwell Publishing.

Andrews, G. F., and J. P. Fonta. 1989. A fluidized-bed continuous bioreactor for lactic acid production. *Applied Biochemistry and Biotechnology* 20-21(1): 375-390.

Archer, D. B., I. N. Roberts, and D. A. MacKenzie. 1990. Heterologous protein secretion from Aspergillus niger in phosphate-buffered batch culture. *Applied Microbiology and Biotechnology* 34: 313-315.

Arroyo, F. N., M. C. Duran Quintana, and A. Garrido Fernandez. 2005. Evaluation of Primary Models To Describe the Growth of *Pichia anomala* and Study of Temperature, NaCl, and pH Effects on Its Biological Parameters by Response Surface Methodology. *Journal of Food Protection* 68(3): 562–570.

Atkinson, B.andE. L. Swilley. 1963. A mathematical model for the trickling filter. In Proceeding. 18th Industrial Waste Conference, Purdue University, Lafayatte: IN, 706–737.

Bester, B. H. and S. H. Lombard. 1990. Influence of lysozyme on selected bacteria associated with Gouda cheese. *Journal of Food Protection* 53(4): 306-311.

Blondeau, K., H. Boze, G. Jung, G. Moulin, and P. Galzy. 1994. Physiological approach to heterologous human serum albumin production by *Kluyveromyces lactis* in chemostat culture. *Yeast* 10: 1297-1303.

Boltz, J. P., E. Morgenroth, D. Brockmann, G. T. Daigger, M. Henze, B. Rittmann, K. H. Sørensen, I. Takàcs, P. A. Vanrolleghem, M. van Loosdrecht M. 2012. Framework for biofilm model calibration protocol. In: Proceedings 3rd

IWA/WEF Wastewater Treatment Modelling Seminar (WWTmod2012). Mont-Sainte-Anne, Québec: Canada, 143-146.

Burmølle, M., S. W. Jeremy, R. Dhana , H. H. Lars,J. S. Søren, and K. Staffan. 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Applied and Environmental Microbiology* 72(6): 3916–3923.

Cao, N., J. Du, C. S. Gong, G. T. Tsao. 1996. Simutaneous production and recovery of fumaric acid from immobilized *Rhizopus oryzae* with a rotary biofilm contactor and an adsorption column. *Applied Environmental Microbiology* 62: 2926–2931.

Cao, N., J. Du, C. S. Gong, and G. T. Tsao. 1997. Production of fumaric acid by immobilized Rhizopus using rotary biofilm contactor. *Applied Biochemistry Biotechnology* 63–65: 387–394.

Capdeville, B. and J. L. Rols. 1992. Introduction to biofilms in water and wastewater treatment. In Biofilms – Science and Technology, 13-20. L. F. Melo, T. R. Bott, M. Fletcher, B. Capdeville, ed. Dordrecht: The Netherlands: Kluwer Academic Publishers.

Casey, E., B. Glennon, and G. Hamer. 1999. Review of membrane aerated biofilm reactors. *Resources, Conservation and Recycling* 27: 203–215.

CDC. 2011. Estimates of Foodborne Illness in the United States. Available at: http://www.cdc.gov/foodborneburden. Accessed 23 May 2012.

Characklis, W.G., K. C. Marshall. 1990. Biofilms: A basis for an interdisciplinary approach. In *Biofilms in the Food Environment*, 3-17. H. H. Wang,M. Agle, and E. Meredith, ed. Iowa: USA: Blackwell Publishing.

Chen, Jyh-P., Kuo-W. Wu, H. Fukuda. 2008. Bioethanol production from uncooked raw starch by immobilized surface-engineered yeast cells. *Applied Biochemistry and Biotechnology* 145: 59–67.

Cheng, K., J. M. Catchmark, and A. Demirci. 2009a. Enhanced production of bacterial cellulose by using a biofilm reactor and its material property analysis. *Journal of Biological Engineering* 3(12): 1-10.

Cheng, K., J. M. Catchmark, and A. Demirci. 2009b. Effects of plastic composite support and pH profiles on pullulan production in a biofilm reactor. *Applied Microbiology Biotechnology* 86: 853–861.

Cheng, K., A. Demirci, and J. M. Catchmark. 2010. Advances in biofilm reactors for production of value-added products. *Applied Microbiology Biotechnology* 87:445–456.

Choi, S., H. Paik, S. Lee, T. Nihira, and Y. Hwang. 2004. Enhanced productivity of human lysozyme by pH-controlled batch fermentation of recombinant *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering* 98(2): 132–135.

Chowdhury, B. R., R. Chakraborty, U. R. Chaudhuri. 2007. Validity of modified Gompertz and Logistic models in predicting cell growth of *Pediococcus acidilactici* H during the production of bacteriocin pediocin AcH. *Journal of Food Engineering* 80: 1171–1175.

Cochran, K. J.. 2006. Combined fermentation and recovery using expanded chromatography. Worcester Polytechnic Institute.

Cunningham, F. E., V. A. Proctor, and S. J. Goetsch. 1991. Egg-white lysozyme as a food preservative: An overview. *World's Poultry Science Journal* 47: 141-163.

Degeest, B. and L. De Vuyst. 1999. Indication that the nitrogen source influences both amount and size of exopolysaccharides produced by *Streptococcus thermophilus* LY03 and modelling of the bacterial growth and exopolysaccharide production in a complex medium. *Applied Environmental Microbiology* 65: 2863–2870.

Demirci, A., A. L. III Pometto, and K. E. Johnson. 1993. Lactic acid production in a mixed culture biofilm reactor. *Applied Environmental Microbiology* 59: 203- 207.

Demirci, A., A. L. III Pometto. 1995. Repeated-batch fermentation in biofilm reactors with plastic-composite supports for lactic acid production. *Applied Microbiology Biotechnology* 44: 585–589.

Demirci, A., A. L. III Pometto, and K-L.C. Ho. 1997. Ethanol production by Saccharomyces cerevisiae in biofilm reactors. *Journal of Industrial Microbiology and Biotechnology* 19: 299–304.

Demirci, A., T. Pongtharangkul, and A. L. Pometto III. 2007. Chapter 8: Applications of biofilm reactors for production of value-added products by microbial fermentation. In *Biofilms in the Food Environment,* 167-189. H. P. Blaschek, H. H. Wang, and M. E. Agle ed. Iowa, USA: Blackwell Publishing and The Institute of Food Technologists.

De Vuyst, L., F. Vanderveken, S. Van de Ven, and B. Degeest. 1998. Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in milk medium and evidence for their growth-associated biosynthesis. *Journal of Applied Microbiology* 84: 1059-1068.

Dharmadhikari, M. 2011. Yeast Autolysis. Available at: http://www.extension.iastate.edu. Accessed 19 July 2011.

Dumsday, G.J., B. Zhou, S. Buhmann, G. A. Stanley, and N. B. Pamment. 1997. Continuous ethanol production by *Escherichia Coli* KO11 in continuous stirred tank and fluidized bed fermenters. *Australasian Biotechnology* 7(5): 300-303.

Düring, K., P. Porsch, A. Mahn, O. Brinkmann, and W. Gieffers.1999. The non-enzymatic microbicidal activity of lysozymes. *Federation of European Biochemical Societies Letters* 449: 93–100.

Enzminger, J.D. and J.A. Asenjo. 1986. Use of cell recycle in the aerobic fermentative production of citric acid by yeast. *Biotechnology Letters* 8(1): 7-12.

Faure, A. and P. Jollès. 1970. Immunological reactivity between human and bovine alpha-lactalbumins and several lysozymes of human origin and from bird egg whites. *Comptes Rendus Hebdomadaires des Seances de L Academie des Sciences. D: Sciences Naturelles* 271: 1916–1918.

Flemming, Hans-C. and J. Wingender. 2010. The biofilm matrix. *Nature Reviews Microbiology* 8: 623-633.

Gemili, S., E. S. Umdu, N. Yaprak, F. I. Ustok, F. Y.G. Yener, C. M. Gucbilmez, S. A. Altinkaya, and A. Yemenicioglu. 2007. Partial purification of hen egg white lysozyme by ethanol precipitation method and determination of the thermal stability of its lyophilized form. *Turkish Journal of Agriculture and Forestry* 31: 125-134.

Gilby, C. 2001. Lysozyme – a natural way to manage fermentation risks?. Available at: http://www.cix.co.uk/~cgilby/lysozyme1.pdf. Accessed on 16 September 2010.

Gill, A. O. and R. A. Holley. 2000. Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA. *Food Research International* 33: 83-90.

Gonzalez-Siso, M. I., M. A. Feire-Picos, E. Ramil, M. Gonzalez-Dominguez, A. R. Torres, and M. E. Cerdan. 2000. Respirofermentative metabolism in *Kluyveromyces lactis*: insights and perspectives. *Enzyme and Microbial Technology* 26: 699-705.

Gupta, D. K., K. von Figura, and A. Hasilik. 1987. Conditions for areliable application of lysoplate method in the determination of lysozyme. Clinica Chimica Acta 165: 73-82.

Hardjito, L., P. F. Greenfield, and P. L. Lee. 1993. Recombinant protein production via fed-batch culture of the yeast Saccharomyces cerevisiae. *Enzyme and Microbial Technology* 15: 120-126.

Henson, M. A. Group. 2013. Dynamics and Control of Biochemical Reactorst. Department of Chemical Engineering, University of Massachusetts Amherst. Available at: http://www.ecs.umass.edu/che/henson_group/research/bioreactor.html. Accesed 25 May 2014.

Ho, G. K. L., A. L. Pometto, , P. N. Hinz, and A. Demirci. 1997a. Nutrient leaching and end product accumulation in plastic composite support for L-(+)-lactic aicd biofilm fermentation. *Applied Environmental Microbiology* 63: 2524–2532.

Ho, G. K. L., A. L. Pometto, P. N. Hinz, J. S. Dickson, and A. Demirci. 1997b. Ingredient selection for plastic composite supports used for l-(+)-lactic acid biofilm fermentation by *Lactobacillus casei* subsp *rhamnosus*. *Applied and Environmental Microbiology* 63: 2516–2523.

Huang, J., S. Nandi, L. Wu, D. Yalda, G. Bartley, R. Rodriguez, B. Lonnerdal, and N. Huang. 2002. Expression of natural antimicrobial human lysozyme in rice grains. *Molecular Breeding* 10: 83–94.

Huang, E. L., and A. Demirci. 2009. Enhanced human lysozyme production by *Kluyveromyces lactis. Food and Bioprocess Technology* 2: 222–228.

Hughey, V. L., and E. A. Johnson. 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Applied and Environmental Microbiology* 53(9): 2165-2170.

Hui, Y. S., A. A. Amirul, R. M. A. Yahya, and M. N. M. Azizan. 2010. Cellulase production by free and immobilized *Aspergillus terreus. World Journal of Microbiology and Biotechnology* 26: 79–84.

Hutkins, R. W. 2006. *Microbiology and technology of fermented foods*. Iowa: US: Blackwell Publishing.

Horiuchi, J-I., K. Tabata, T. Kanno, M. Kobayashi. 2000. Continuous acetic acid production by a packed bed bioreactor employing charcoal pellets derived from waste mushroom medium. *Journal of Biosence and Bioengineering* 89(2): 126-130.

Ibrahim, H. R., S. Higashiguchi, M. Koketsu, L. R. Juneja, M. Kim, T. Yamamoto, Y.Sugimoto, and T. Aoki. 1996. Partially Unfolded Lysozyme at Neutral pH Agglutinates and Kills Gram-Negative and Gram-Positive Bacteria through Membrane Damage Mechanism. *Journal of AgricUltural and Food Chemistry.* 44: 3799−3806.

Ibrahim, H. R., T. Matsuzaki, and T. Aoki. 2001. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. *Federation of European Biochemical Societies Letters* 506: 27-32.

Islam, R., J. Kite, A. S. Baker, A. Ching, and M. R. Islam. 2006. Affinity purification of hen egg lysozyme using sephadex G75. *African Journal of Biotechnology* 5(20): 1902-1908.

Iwata, T., R. Tanaka, M. Suetsugu, M. Ishibashi, H. Tokunaga, M. Kikuchi, and M. Tokunaga. 2004. Efficient secretion of human lysozyme from the yeast, Kluyveromyces lactis. *Biotechnology Letters* 26: 1803-1808.

Jacobsen, S., J. D. Mikkelsen, and J. Hejgaard. 1990. Characterization of two antifungal endochitinases from barley grain. *Plant Physiology* 79: 554-562.

Jayaraman, V. K. 1992. The solution of hollow-fiber bioreactor design equations. *Biotechnology Progress* 8 (5): 462–464.

Jenzano, J. W., S. L. Hogan, and R. L. Lundblad. 1986. Factors influencing measurement of human salivary lysozyme in lysoplate and turbidietric assays. *Journal of Clinical Microbiology* 24(6): 963-967.

Jenzano, J. W. and R. L. Lundblad. 1988. Effects of polyamines on turbidimetric and lysoplate assays for lysozyme. *Journal of clinical microbiology* 26(1): 34-37.

Jolles, P., and J. Jolles. 1984. What's new in lysozyme research? *Molecular and Cellular Biochemistry* 63: 165 189.

Jolles, P. 1996. *Lysozymes: Model Enzymes in Biotechnology and Biology*. Basel; Boston; Berlin; Birkhauser.

Jackson, L. R., L. J. Trudel, and N. S. Lipman. 1999. Small-Scale Monoclonal Antibody Production *In Vitro*: Methods and Resources. *Proceedings of the Production of Monoclonal Antibodies Workshop*, Bologna, Italy.

Kang, S. W., S. W. Kim, and J. S. Lee. 1995. Production of cellulase and xylanase in a bubble column using immobilized *Aspergillus niger* KKS. Applied Biochemistry and Biotechnology 53: 101–106.

Keevil, C.W., J. T. Walker. 1992. Normarski DIC microscopy and image analysis of biofilms. *Binary* 4: 93–85.

Khiyami, M. A., A. L. Pometto, W. J. Kennedy. 2006. Ligninolytic enzyme production by *Phanerochaete chrysosporium* in plastic composite support biofilm stirred tank bioreactors. *Journal of Agricultural and Food Chemistry* 54(5): 1693-8.

Kiers, J., A. M. Zeeman, M. Luttik, C. Thiele,J. I. Castrillo,H. Y. Steensma,J. P. Van Dijken, and J. T. Pronk.1997. Regulation of alcoholic fermentation in batch and chemostat cultures of *Kluyveromyces lactis*CBS 2359. *Yeast* 14(5): 459-469.

Körstgens, V., H. C. Flemming, J. Wingender, and W. Borchard. 2001. Influence of calcium ions on the mechanical properties of a model biofilm of mucoid *Pseudomonas aeruginosa*. *Water Science and Technolology* 43: 49–57.

Kumar, C. G., and S. K. Anand. 1998. Review Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology* 42: 9–27.

Kumaravel, V. and S. R. Gopal. 2010. Immobilization of *Bacillus amyloliquefaciens* MBL27 cells for enhanced antimicrobial protein production using calcium alginate beads. *Biotechnology and Applied Biochemistry* 57: 97-103.

Kunduru, R. M. and A. L. III Pometto. 1996a. Evaluation of plastic composite supports for enhanced ethanol production in biofilm reactors. *Journal of Industrial Microbiology* 16: 241–248.

Kunduru, R. M. and A. L. III Pometto. 1996b. Continuous ethanol production by Zymomonas mobilis and Saccharomyces cerevisiae in biofilm reactors. *Journal of Industrial Microbiology* 16: 249–256.

Landini, P., D. Antoniani, J. G. Burgess, and R. Nijland. 2010. Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Applied Microbiology and Biotechnology* 86: 813–823.

Leitea, J. A. C., B. S. Fernandesa, E. Pozzia, M. Barbozab, and M. Zaiata. 2008. Application of an anaerobic packed-bed bioreactor for the production of hydrogen and organic acids. *International Journal of Hydrogen Energy* 33: 579 – 586.

Luedeking, R. and E. L. Piret. 1959. A kinetic study of the lactic acid fermentation. *Journal of Biochemical and Microbiological Technology and Engineering* 1: 393-412.

Liu, W., H. J. Zheng, Z. L. Wu, and Y. F. Wang. 2010. Effects of pH profiles on nisin fermentation coupling with foam separation. *Applied Microbiology and Biotechnology* 85(5): 1401-1407.

Liu, D., Y. Chen, F. Y. Ding, T. Zhao, J. L. Wu, T. Guo, Heng-F. Ren, Bing-B. Li, Huan-Q. Niu, Z. Cao, Xiao-Q. Lin, Jing-J. Xie, Xue-J. He, and Han-J. Ying. 2014. Biobutanol production in a Clostridium acetobutylicum biofilm reactor integrated with simultaneous product recovery by adsorption. *Biotechnology for Biofuels* 7(5): 1-13.

Lloyd, J. R., T. R. Hirst, and A. W. Bunch. 1997. Hollow-fibre bioreactors compared to batch and chemostat culture for the production of a recombinant toxoid by a marine Vibrio. *Applied Microbiology and Biotechnology* 48(2): 155-161.

Lopez-Pedemonte, T. J., A. X. Roig-Sagues, A. J. Trujillo, M. Capellas, and B. Guamis. 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. *Journal of Dairy Science* 86: 3075–3081.

MacKenzei, D. A., L. C. G. Gendron, D. J. Jeenes, and D. B. Archer. 1994. Physiological optimization of secreted protein production by *Aspergillus niger*. *Enzyme Microbial Technology* 16(4): 276:280.

Maga, E. A., G. B. Anderson, J. S. Cullor, W. Smith, and J. D. Murray. 1998. Antimicrobial properties of human lysozyme transgenic mouse milk. *Journal of Food Protection* 61(1): 52-56.

Mainwaring, D. O., M. G. Wiebe, G. D. Robson, M. Goldrick, D. J. Jeenes, D. B. Archer, A. P. Trinci. 1999. Effect of pH on hen egg white lysozyme production and evolution of a recombinant strain of *Aspergillus niger*. *Journal of Biotechnology* 75(1): 1-10.

Masuda, T., N. Ide, and N. Kitabatake. 2005. Structure–Sweetness Relationship in Egg White Lysozyme: Role of Lysine and Arginine Residues on the Elicitation of Lysozyme Sweetness. *Chemical Senses* 30: 667–681.

Maullu, C., G. Lampis, T. Basile, A. Ingiani, C. M. Rossolini, and R. Pompei. 1999. Production enriched lysozyme biomass from cheese industry by products. *Journal of Applied Microbiology* 86: 182-186.

Martinova, M., D. Hadjieva, S. Vlaev. 2010. Gas–liquid dispersion in a fibrous fixed bed biofilm reactor at growth and non-growth conditions. *Process Biochemistry* 45: 1023-1029.

Merolla, M. and M. Fromer. 2008. Lysozyme. Available at; http://maptest.rutgers.edu/drupal/?q=node/51. Accessed 25 May 2014.

Melo, L. F., R. Oliveira. 2001. Biofilm Reactors. In *Multiphase Bioreactor Design*, 271-309. In J. M. S. Cabral, M. Mota, J. Tramper. New York: NY: Taylor & Francis.

Merico, A., D. Capitanio, I. Vigentini, B. M. Ranzi, and C. Compagno. 2004. How physiological and cultural conditions influence heterologous protein production in *Kluyveromyces lactis*. *Journal of Biotechnology* 109: 139–146.

Meyer, A. S. 2003. Antimicrobial enzymes. In *Food Preservation Techniques*. P. Zeuthen and L. Bogh-Sorensen, ed. CRC press.

Morita, S., M. Kuriyama, M. Nakatsu, M. Suzuki, and K. Kitano. 1995. Secretion of human lysozyme by Acremonium chrysogenum using a Fusarium alkaline protease promoter system. *Journal of Biotechnology* 42: 1-8.

Mörsky P. 1983. Turbidimetric determination of lysozyme with *Micrococcus lysodeikticus* cells: Reexamination of reaction conditions. *Analytical Biochemistry* 128(1): 77-85.

Mu, Y., G. Wang, and Han-Q. Yu. 2006. Kinetic modeling of batch hydrogen production process by mixed anaerobic cultures. *Bioresource Technology* 97: 1302–1307.

Mulvey, R. S., R. J. Gualtieri, and S. Beychok. 1974. Comparative studies of the solution behaviour of hen egg-white and human lysozyme. In *Lysozyme, .* 281-300. E. F. Osserman, R. E. Canfield, and S. Beychok, ed. New York, USA: Academic Press.

Muraki, M., Y. Jigami, H. Tanaka, F. Kishimoto, H. Agui, and S. Ogino. 1985. Short Communication Expression of Synthetic Human Lysozyme Gene in Escherichia coli. *Agricultural and Biological Chemistry* 49 (9): 2829~2831.

Muraki, M., K. Harata, N. Sugita, and K. Sato. 1996. Origin of carbohydrate recognition specificity of human lysozyme revealed by affinity labeling. *Biochemistry* 35(42): 13562-13567.

Mulchandani, A. and H. T. J. Luong. 1988. Biosynthesis of pullulan using immobilized Aureobasidium pullulans cells. *Biotechnology and Bioengineering* 33: 306–312.

Nakashiam, T., F. H. Fukuda, S. Kyotoni, and H. Morikawa. 1988. Culture conditions for intracellular lipases production by *Rhizopus chinensis* and its immobilization within biomass support particles. *Journal of Fermentation Technology* 66: 444–448.

Naudi, A. S. 2000. *Natural Food Antimicrobial Systems*. New York, NY: CRC Press.

Nicolella, C., M. C. M. van Loosdrecht, J. J. Heijnen, B. Nyvad, O. Fejerskov O. 1997. Assessing the stage of caries lesion activity on the basis of clinical and microbiological examination. *Commun Dent Oral Epiderm* 25: 69–75.

Nikolov, L. and D. Karamanev. 2000. Review article Wastewater treatment with particulate biofilm reactors. *The Canadian Journal of Chemical Engineering* 65(2): 214–217.

Norwood, D. E., and A. Gilmour. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *Journal of Applied Microbiology* 88: 512–520.

Nyvad, B. and O. Fejerskov. 1997. Assessing the stage of caries lesion activity on the basis of clinical and microbiological examination. *Community Dentistry and Oral Epidemiology* 25: 69-75.

Ohleyer, E., H. W. Blanch, and C. R. Wilke. 1985. Continuous Production of lactis acid in a cell recycle reactor. *Applied Biochemistry and Biotechnology* 11(4): 317-332.

Ophir, T., and D. L. Gutnick. 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. *Applied Environmental Microbiology* 60: 740–745.

Oriel, P. 1988. Immobilization of recombinant *Eschericia coli* in silicone polymer beads. *Enzyme and Microbial Technol*ogy 10: 518–523.

Osserman, E. F. and D. P. Lawlor. 1966. Serum and Urinary Lysozyme in monocytic and monomyelocytic leukemia. *Journal of Experimental Medicine* 124(5): 921–952.

Osserman, E. F., Canfield, R. E., and Beychock S. 1974. *Lysozyme*. New York, NY: Academic Press.

Ozmihci, S. and F. Kargi. 2009. Fermentation of cheese whey powder solution to ethanol in a packed-column bioreactor: effects of feed sugar concentration. *Journal of Chemical Technology and Biotechnology* 84: 106–111.

Parente, E. and A. Ricciardi. 1999. Production, recovery and purification of bacteriocins from lactic acid bacteria. *Applied Microbiology and Biotechnology* 52(5): 628-638.

Park, Y. H. and D. A. Wallis. 1984. Steady-state performance of a continuous biofilm fermentor system for penicillin production. *The Korean Journal of Chemical Engineering* 1: 119–128.

Park, Y. H., E. Y. Kim,W. T. Seo, K. H. Jung, and Y. J. Yoo. 1989. Production of cephalosporin C in a fluidized-bed bioreactor. *Journal of Fermentation and Bioengineering* 67: 409–414.

Park, Y. S., K. Toda. 1992. Multi-stage biofilm reactor for acetic acid production at high concentration. *Biotechnol Lett* 14: 609–612.

Park,Y. S., K. Kai, S. lijima, and T. Kobayashi. 1992. Enhanced galactosidase product ion by high cell-density culture of recombinant *Bacillus subtilis* with glucose concentration control. *Biotechnology and Bioengineering* 40: 686-696.

Parmar, S. 2012. Biofilms and Human Implants. Available at: http://microbewiki.kenyon.edu/index.php/Biofilms_and_Human_Implants. Accessed 12 July 2013.

Parry, R. M., R. C. Chandan, and K. M. Shahani.. 1965. A Rapid sensitive analysis of muramidase. *Proceedings of the Society of Experimental Biology and Medicine* 119(2): 384.

Pellegrini, A., U. Thomas, N. Bramaz, S. Klauser, P. Hunziker, and R. Von Fellenberg. 1997. Identification and isolation of a bactericidal domain in chicken egg white lysozyme. *Journal of Applied Microbiology* 82:: 372–387.

Pfeffer, J. M., H. Strating, J. T. Weadge, and A. J. Clarke. 2006. Peptidoglycan o acetylation and autolysin profile of *Enterococcus faecalis* in the viable but nonculturable state. *Journal of Bacteriology* 188(3): 902–908.

Pham, T. K. and P. C. Wright. 2008. Proteomic analysis of calcium alginate-immobilized *Saccharomyces cerevisiae* under high-gravity fermentation conditions. *Journal of Proteome Research* 7(2): 515-525.

Pichler, W. J., and P. Campi. 1992. Allergy to lysozyme/egg white-containing vaginal suppositories. *Ann Allergy* 69(6):521-5.

Pigott, D. C. 2008. Foodborne Illness. *Emergency Medicine Clinics of North America* 26: 475–497.

Pometto III, A. L., and A. Demirci. 2006. Chapter 21: Technologies used for microbial production of food ingredients. In *Functional Foods and Biotechnology* 521-533.

K. Shetty, G. Paliyath, A. L. Pometto, R. E. Levin, ed. Boca Raton, FL.: CRC Press Taylor & Francis Group.

Pongtharangkul, T. and A. Demirci. 2006a. Evaluation of culture medium for nisin production in repeated-batch biofilm reactor. *Biotechnology Progress* 22: 217–224.

Pongtharangkul, T. and A. Demirci. 2006b. Effects on pH profiles on nisin production in biofilm reactor. *Applied Microbiology Biotechnology* 71: 804–811.

Pongtharangkul, T. and A. Demirci. 2006c. Effects of fed-batch fermentation and pH profiles on nisin production in suspended-cell and biofilm reactors. *Applied Microbiology Biotechnology* 73: 73–79.

Pongtharangkul, T. and A. Demirci. 2007. Online recovery of nisin during fermentation and its effect on nisin production in biofilm reactor. *Applied Microbiology Biotechnology* 74(3): 555-62.

Porro, D., M. Sauer, P. Branduardi, and D. Mattanovich. 2005. Recombinant Protein Production in Yeasts. *Molecular Biotechnology* 31: 245-259.

Pramod, T. and K. Lingappa. 2008. Immobilization of *Aspergillus niger* in polyurethane foam for citric acid production from carob pod extract. *American Journal of Food Technology* 3: 252–256.

Qureshi, N., B. A. Annous, T. C. Ezeji, P. Karcher, and I. S. Maddox. 2005. Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. *Microbial Cell Factories* 4(24): 1-21.

Qureshi, N. and T. C. Ezeji.2008. Butanol, 'a superior biofuel' production from agricultural residues (renewable biomass): recent progress in technology. *Biofuels, Bioproducts and Biorefining* 2(4): 319–330.

Rahman, M. S. 1999. *Hand Book of Food Preservation*. New York, NY: Marcel Dekker.

Ramaswamy, R., J. Ahn, V. M. Balasubramaniam, L. R. Saona, and A. E. Yousef. 2007. Chapter 3: Food Safety Engineering. In *Handbook of Farm, Dairy, and Food Machinery,* 45-69. M. Kutz ed. Columbus, OH: William Andrew, Inc.

Ray, B. 2004. *Fundamental food microbiology*. 3rd ed. Boca Raton, Florida: CRC Press.

Reid, C. W., N. T. Blackburn, and A. J. Clarke. 2006. Role of Arginine Residues in the Active Site of the Membrane-Bound Lytic Transglycosylase B from *Pseudomonas aeruginosa*. *Biochemistry* 45: 2129-2138.

Richard, M. P., C. C. Ramesh, and M. S. Khem. 1965. A rapid and sensitive assay of muramidase. *Proceedings of The Society for Experimental Biology and Medicine* 119: 384–386.

Rossolini, G. M., M. L. Riccio, E. Gallo, and C. L. Galeotti. 1992. *Kluyveromyces lactis* rDNA as a target for multiple integration by homologous recombination. *Gene* 119: 75–81.

Sakurai, A., H. Iami, and M. Sakakibara. 1999. Citric acid production suing biofilm of *Aspergillus niger*. *Recent Research Developments in Biotechnology and Bioengineering* 2: 1–13.

Sankpal, N. V., A. P. Joshi, and B. D. Kulkarni. 2001. Citric acid production by *Aspergillus niger* immobilized on cellulose microfibrils: influence of morphology and fermenter conditions on productivity. *Process Biochemistry* 36: 1129–1139.

Sanroman, A., J. Pintado, J. M. Lema. 1994. A comparison of two techniques (adsorption and entrapment) for the immobilization of *Aspergillus niger* in polyurethane foam. *Biotechnology Techniques* 6: 389–394.

Salazar, O. and J. A. Asenjo. 2007. Enzymatic lysis of microbial cells. *Biotechnology Letters* 9: 985–994.

Salton, M. R. J. 1958. The lysis of microorganisms by lysozyme and related enzymes. *Journal of General Microbiology* 18: 481-490.

Schaffrath, R., and K. D. Breunig. 2000. Genetics and molecular physiology of the yeast *Kluyveromyces lactis*. *Fungal Genetics and Biology* 30: 173–190.

Schekman, R. 1982. The secretory pathway in yeast. *Elsevier Biomedical Press* 7(7): 243-246.

Schleifer, K. H. and K. Otto. 1972. Peptidoglycan .Types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews* 36(4): 407-477.

Shahbazi, A., M. R. Mims, Y. B. Li, V. Shirley, S. A. Ibrahim, and A. Morris. 2005. Lactic acid production from cheese whey by immobilized bacteria. *Applied Biochemistry and Biotechnology* 121: 529-540.

Shieh, W. K. and J. D. Keenan. 1986. Fluidized bed biofilm reactor for wastewater treatment. *Advances in Biochemical Engineering/Biotechnology* 33: 131-169.

Shin, Youn-O.. 2002. Extraction of lysozyme using surfactants.Department of Chemical Engineering, McGill University, Montréal,Canada.

Shuler, M. L. and F. Kargi. 2002. Chapter 3 Engineering Principles for Bioprocesses. In *Bioprocess Engineering Basic Concepts*, 245-285. Upper Saddle River, NJ: Prentice Hall Inc.

Siegel, R. S. and R. A. Brierley. 1990. Use of a cell recycle reactor to increase production of a proteolysis-susceptible peptide secreted from recombinant *Saccharomyces cerevisiae*. *Biotechnology* 8(7): 639-43.

Srivastava, P. and S. Kundu. 1999. Studies on Cephalosporin-C production in an air lift reactor using different growth modes of Cephalosporium acremonium. *Process Biochemistry* 34: 329–333.

Souza , A. A. U. de, H. L. Brandõ, I. M. Zamporlini,H. M. Soares, and S. M. de A. G. U. de Souza. 2008. Application of a fluidized bed bioreactor for cod reduction in textile industry effluents. *Resources, Conservation and Recycling* 52: 511–521.

Sutherland, I. W. 1972. Bacterial exopolysaccharides. *Advances in Microbial Physiology* 8: 143-212.

Stark, M. J. and A. Boyd. 1986. The killer toxin of *Kluyveromyces lactis*: characterization of the toxin subunits and identification of the genes which encode them. *EMBO Journal* 5: 1995–2002.

Stewart, P. S., R. Murga, and de Beer D Srinivasan. 1995. Biofilm structural heterogeneity visualized by three microscopic methods. *Water Research* 29: 2006--2009.

Swinkles, B. W., A. J. J. van Ooyen, and F. J. Bonekamp. 1993. The yeast *Kluyveromyces lactis* as an efficient host for heterologous gene expression. *Antonie van Leeuwenhoek* 64: 187-201.

Teixeira, P. and R. Oliveira. 1999. Influence of surface characteristics on the adhesion of *Alcaligenes denitrificans* to polymeric substrates. *Journal of Adhesion Science and Technology* 13: 1287–1294.

Terada, A., T. Yamamoto, K. Hibiya, S. Tsuneda , and A. Hirata. 2004. Enhancement of biofilm formation onto surface-modified hollow-fiber membranes and its application to a membrane-aerated biofilm reactor. *Water Science and Technology* 49(11): 263-268.

Urbance, S. E., A. L. III Pometto, A. A. DiSpirito, and A. Demirci. 2003. Medium evaluation and plastic composite support ingredient selection for biofilm formation and succinic acid production by *Actinobacillus succinogenes*. *Food Biotechnology* 17: 53–65.

Urbance, S. E., A. L. III Pometto, A. A. DiSpirito, and Y. Denli. 2004. Evaluation of succinic acid continuous and repeated-batch biofilm fermentation by *Actinobacillus succinogenes* using plastic composite support bioreactors. *Applied Microbiology Biotechnology* 65: 664–670.

van Boekel, M. A. J. S. 2008. Chapter 12: Kinetics of microbial growth. In *Kinetic modeling of reactions in foods, 12_1-12_40.* Boca Raton, Florida: Taylor & Francis Ltd.

Vanderkelen, L., J. M. Van Herreweghe, K. G. A. Vanoirbeek, G. Baggerman, B. Myrnes, P. J. Declerck I. W. Nilsen, C. W. Michiels, and L. Callewaert. 2011. Identification of a bacterial inhibitor against g-type lysozyme. *Cellular and Molecular Life Sciences* 68: 1053–1064.

Vassilev, N. B., M. C. Vassileva, and D. I. Spassova. 1993. Production of gluconic acid by *Aspergillus niger* immobilized on polyurethane foam. *Applied Microbiology Biotechnology* 39: 285-288.

Venkatadri, R., S. P. Tsai, N. Vukanic, and L. B. Hein. 1992. Use of biofilm membrane reactor for the production of lignin peroxidase and treatment of pentachlorophenol by *Phanerochaete chrysosporium. Hazardous Waste & Hazardous Materials* 9(3): 231-243.

Vick Roy, T. B., H. W. Blanch, and C. R. Wilke, 1982. Lactic acid production by *Lactobacillus delbreuckii* in a hollow fibre fermenters. *Biotechnology Letters* 4: 483-488 .

Vocadlo, D. J., G. J. Davies, R. Laine, and S. G. Withers. 2001. Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate. *Nature* 412: 835-838.

Volesky, B. and J. Vortuba. 1992. Modeling and Optimization of Fermentation Processes. Elsevier.

Verduyn, C. 1991. Physiology of yeasts in relation to biomass yields. *Antonie Van Leeuwenhoek* 60: 325–353.

Wang, J. 2000. Production of citric acid by immobilized *Aspergillus niger* using a rotating biological contactor (RBC). *Bioresource Technology* 75: 245–247.

Webb, C., H. Fukuda, and B. Atkinson. 1986. The production of cellulase in a spouted bed fermentor using cells immobilized in biomass support particles. *Biotechnology and Bioengineering* 28: 41–50.

Weuster-Botz, D., A. Aivasidis, and C. Wandrey. 1993. Continuous ethanol production by *Zymomonas mobilis* in a fluidized bed reactor. Part II: Process development for the fermentation of hydrolysed B-starch without sterilization. *Applied Microbiohgy Biotechnology* 39: 685-690.

WHO. 2007. Food safety and foodborne illness. *World Health Organization Fact sheet* 237, revised March 2007. Geneva, Switzerland.

Williams, J. A. 2002. Keys to bioreactor selection. Available at: ww.cepmagazine.org. Accessed 25 October 2011.

Wilken, L. and Z. L. Nikolov. 2006. Factors influencing recombinant human lysozyme extraction and cation exchange adsorption. *Biotechnology Progress* 22: 745-752.

Wilkens, J., H. Goodman, B. J. Mackay, V. J. Iacono, and J. J. Pollock. 1982. Bacteriolysis of *Streptococcus* mutans GS5 by lysozyme, proteases, and sodium thiocyanate. *Infection and immunity* 38(3): 1172-1180.

Wimpenny, J., W. Manz, and U. Szewzyk. 2000. Heterogeneity in biofilms. *FEMS Microbiology Reviews* 24: 661–671.

Xu, H., H. Y. Lee, and J. Ahn. 2011. Characteristics of biofilm formation by selected foodborne pathogens. *Journal of Food Safety* 31: 91–97.

Yang, S. T., Y. M. Lo, and B. D. Min. 1996. Xanthan gum fermentation by Xanthomonas campestris immobilized in a novel centrifugal fibrous-bed bioreactor. *Biotechnology Progress* 12: 630–637.

Yang, B., J. Wang, B. Tang, Y. Liu, C. Guo, P. Yang, T. Yu, R. Li, J. Zhao, L. Zhang, Y. Dai, and N. Li. 2011. Characterization of Bioactive Recombinant Human Lysozyme Expressed in Milk of Cloned Transgenic Cattle. *PLoS ONE* 6(3): 1-10.

Yoshimura, K., A. Toibana, K. Kikuchi, M. Kobayashi, T. Hayakawa, K. Nakahama, M. Kikuchi, and M. Ikehara. 1987. Differences between *Saccharomyces cerevisiae* and *Bacillus subtilis* in secretion of human lysozyme. *Biochemical and Biophysical Research Communications* 145(2): 712-718.

Yu, Z., Q. Meng, H. Yu, B. Fan, S. Yu, J. Fei, L. Wang, Y. Dai, and N. Li. 2006. Expression and bioactivity of recombinant human lysozyme in the milk of transgenic mice. *Journal of Dairy Sci*ence 89: 2911–2918.

Zajsek, K. and A. Gorsek. 2010. Modelling of batch kefir fermentation kinetics for ethanol production by mixed natural microflora. *Food and Bioproducts Processing* 88: 55-60.

Zhao, L., Y. Chen, and D. W. Schaffner. 2001. Comparison of logistic regression and linear regression in modeling percentage data. *Applied and Environmental Microbiology* 67(5): 2129–2135.

Zhang, Y., Y. Ma, F. Yang, and C. Zhang. 2009. Continuous acetone–butanol–ethanol production by corn stalk immobilized cells. *Journal of Industrial Microbiology Biotechnology* 36: 1117–1121.

Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. Van‟t Riet. 1990. Modeling of the bacterial growth curve. *Applied Environmental Microbiology* 56: 1875-1881.

VITA

DUYGU ERCAN

duygueng@yahoo.com

EDUCATION

2010- present   Ph.D., Agricultural and Biological Engineering, The Pennsylvania State University (CGPA: 3.94 / 4.00).

2007-2009   M.Sc., Food Engineering, Izmir Institute of Technology, Turkey (CGPA: 3.93 / 4.00).

2002-2007   B.S., Food Engineering, Ege University, Turkey (CGPA: 87.57 / 100).

PUBLICATIONS

i)   ***Peer-reviewed:***

Ercan, D., F. Korel, Y. Karagül Yüceer, and Ö. Kınık. 2011. Physicochemical, textural, volatile, and sensory profiles of traditional Sepet cheese. Journal of Dairy Science. 94:4300–4312.

Ercan, D., F. Korel, and H. Orsahin. 2013. Microbiological Quality of Traditional Sepet Cheese. *International Journal of Dairy Technology* (Accepted).

Ercan, D. and A. Demirci. 2013. Current and future trends for biofilm reactors for fermentation processes. Critical Reviews in Biotechnology. In-press. Published Online. DOI:10.3109/07388551.2013.793170.4.

Ercan, D. and A. Demirci. 2013. Production of human lysozyme in biofilm reactor and optimization of growth parameters of *Kluyveromyces lactis* K7. *Applied Microbiology and Biotechnology* 97(14): 6211-6221.

Ercan D. and A. Demirci. 2013. Enhanced *Kluyveromyces lactis* K7 human lysozyme production in biofilm reactor by medium optimization. *Biochemical Engineering* (Accepted).

Ercan D. and A. Demirci. 2013. Effects of fed-batch and continuous fermentation on human lysozyme produtiin by Kluyveromyces lactis K7 in biofilm reactor. *Biochemical Engineering* (Submitted).

ii)   ***Book chapters:***

Ercan D., A. Demirci, T. Pongtharangkul, and A. L. Pometto III. 2014. Applications of biofilm reactors for production of value-added products by microbial fermentation: In Biofilms in the Food Environment, 2nd Edition, A. Demirci and A. L. Pometto III, Eds. Wiley-Blackwell. In-press.

Demirci, A., G. Izmirlioglu, and **D. Ercan**. 2012. Fermentation and enzyme technologies in food processing: In Principles and Applications, 2nd Edition, S. Clark, S. Jung, and B. Lamsal, Eds. Wiley-Blackwell, Hoboken, NJ. In-press.

AWARDS

- Second place in graduation among Food Engineering Students at Ege University (2007).
- Second place in poster presentation award at Conference of Food Engineering, Leesburg, VA (2012).
- Research grant by Agricultural Sciences Graduate Student Competitive Grants Program, Pennsylvania State University (2013).
- Third place in biotechnology division competition at IFT conference, Chicago, IL (2013).