OPTIMIZATION OF GROWTH CHARACTERISTICS FOR THE POTENTIAL PROBIOTICS TO BE USED IN FISH AQUACULTURING

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OPTIMIZATION OF GROWTH CHARACTERISTICS FOR THE POTENTIAL PROBIOTICS TO BE USED IN FISH AQUACULTURING

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

OPTIMIZATION OF GROWTH CHARACTERISTICS FOR THE POTENTIAL PROBIOTICS TO BE USED IN FISH AQUACULTURING

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The aquaculturing of marine and fresh water fish is continually increasing in the world and in Turkey. Of the three fish species; sea bass (*Dicentrarchus labrax*), seabream (*Sparus aurata*) and trout (*Oncorhynchus mykiss*), the latter has the highest share in Turkey, with a current production rate of 170000 tons per annum. The use of antibiotics in true feed is being phased out in the world. Probiotics have emerged one way of making up for the lack of antibiotics in the feed. In order to increase the probiotics usage, optimization of the strains' growth conditions, development of sustainable and economic manufacturing process are required. While there are commercial probiotics for the farm animals, there is none so far produced for the aquaculture, in Turkey. *Enterococcus faecium* is one of the widely used probiotic in farm animals sector. Due to the characteristics of *E. faecium* to tolerate acid and bile salts in the gastrointestinal tract, to produce inhibitory compounds and show antagonistic effects towards target fish pathogens, to stimulate immune system and better growth, the microorganism may be evaluated as a strong potential probiotic for aquaculturing.

Aim of this study was to optimize the culture conditions in order to maximize the biomass concentration and the biomass yield of *E. faecium.* In the first part of this study, several growth media, temperature and pH values were tested. In the second part, the microorganism was grown using different fermentation modes in bioreactor. In the last part, a membrane filtration system was incorporated to the fermentor in order to recycle biomass. Results showed that temperature 37 °C and pH 6.5 yielded higher amount of biomass compared to others. The biomass concentration was 6.3 g/L at pH 6.5, 37 °C, 350 rpm when the fresh medium was added continuously and biomass was recycled back to the fermentor using the membrane filter.

Key words: Probiotics, Enterococcus faecium, fermentation, trout

ÖZ

BALIK YETİŞTİRİCİLİĞİNDE KULLANILACAK POTANSİYEL PROBİYOTİKLERİN BÜYÜME KARAKTERİSTİKLERİNİN OPTİMİZASYONU

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Tuzlu ve tatlı sularda kültür balıkçılığı, dünyada ve Türkiye'de, sürekli olarak artmaktadır. Levrek, çupra ve alabalık üçlüsünden alabalık Türkiye'de en çok üretilendir. Bugün, Türkiye'de üretim, yılda 170000 tona ulaşmıştır. Dünyada, balık yetiştiriciliğinde, antibiyotik kullanımı sona erdirilmektedir. Probiyotik kullanımı, yemdeki antibiyotik eksikliğini giderebilmek adına ortaya atılmıştır. Probiyotiklerin kullanımını arttırmak için bunların üretim koşullarının optimize edilmesi, sürdürülebilir, ekonomik üretim süreçlerinin geliştirilmesi gerekir. Çiftlik hayvanları için (özellikle kanatlılar) endüstriyel ürünler mevcutken, kültür balıkları için böylesi probiyotik ürünler bulunmamaktadır. *Enterococcus faecium,* çiftlik hayvanlarında sıkça kullanılan probiyotik suşlardandır ve gastrointestinal asitlik ve yüksek safra oranını tolere edebilmesi, büyümeyi destekleyici özellikleri ve belirli balık patojenlerine karşı antagonistik etkisi başta olmak üzere gösterdiği yararlı etkiler nedeni ile balık yetiştiriciliği için güçlü bir potansiyel probiyotik olarak değerlendirilmektedir.

Bu çalışmanın amacı, *E. faecium*'un biyokütle ve biyokütle verimliliğini arttırmak için kültürün büyütülme koşullarının optimizasyonudur. Çalışmanın ilk kısmında, farklı bileşiklerden veya farklı oranlarda bileşiklerden oluşan ortamlar, sıcaklık ve pH değerleri denenmiştir. İkinci kısımda ise, mikroorganizma, biyoreaktörde farklı fermentasyon yöntemleri denenerek büyütülmüştür. Son aşamada ise, biyokütleyi fermentöre geri döndürmek üzere fermentöre entegre edilmiş membran filtrasyon sistemi denenmiştir. Sonuçlara göre optimum sıcaklık olan 37 °C ve 6.5 pH değerlerinde, en iyi büyüme gözlemlenmiştir. 37 °C, 350 rpm ve 6.5 pH değerlerinde, en yüksek değer olan 6.3 g/L biyokütle, sürekli steril ve taze besi ortamının beslendiği ve biyokütlenin fermentöre geri döndürülüğü membran filtrasyon sistemini içeren deneyde elde edilmiştir.

Anahtar Kelimeler: Probiyotik, Enterococcus faecium, fermentasyon, alabalık

Dedicated to my family...

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CHAPTER 1

INTRODUCTION

Fishery is a significant sector throughout the world and also in Turkey. Fish culturing is improving in recent years and it is expected to become critically important in the future. The world's fish farming industry's % 13 consists of aquaculturing (Fishery Information Data and Statistics, 2007). Rainbow trout production has been continuously increasing since 1950s, especially in Europe, it has been important for the U.S., also. Current researches and developments are on increasing production of the trouts and sales by improved growth, diets, controlling maturation and gender etc. As production continues to be improved, researches are needed to maintain lower costs, by this way the industry can develop (FAO, 2013).

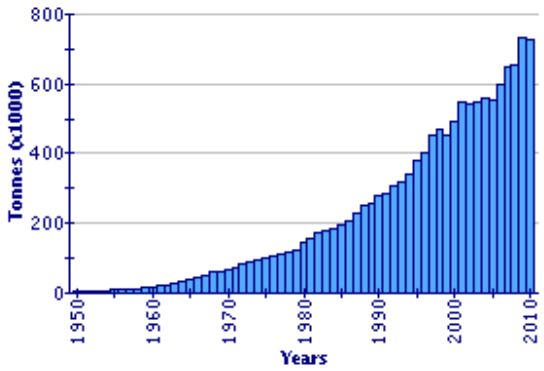

Figure 1.1 Exponential Growth of the Rainbow Trout Farming Throughout the Years, in the World (FAO, 2013)

Turkey is appropiate for fish culturing due to its climate and location. In recent years, fishery products sector and economy of Turkey have improved significantly. Today, there are over 350 fish farms that produce nearly 170000 tons of fish per year.

Rainbow trout growth is one of the first applied farming methods which gained success throughout the years. In Turkey, first trout farming trials were conducted in the 1970's. Trout farming has been improving since then. Rainbow trout is the predominant species raised. In Turkey, trout farming, for 2002, had the capacity of about 40000 tons in over 100 facilities. Today, nearly 55000 tones of trout are annually produced. In the seas, in 13 facilities, about 2500 tons/year trouts are produced. There are facilities which produce over 1000 tons of trout, annually.

1.1 Aquaculturing of Rainbow Trout

For successful trout farming, the trouts should be maintained in plenty of water with a temperature that is below 20 °C and with a pH range between 6.5 and 7.5. The rainbow trout's stomach is acidic (pH ranging between 2 and 4) (Mcdonald, 1996). This should be taken into consideration for live

feeds or probiotic usage. The trout fry eggs do not need to be fed until a period of time such as 18-20 days have passed. For juvenile fish and adult trout feeding, the used feeding powders should be richer in protein. When it is hard and expensive to maintain powder feeds, some growers prepare powder feeds by eliminating from pellet fish feeds. For adult trouts' feeding, three paths may be taken; feeding with pellet feeds, mixed feeds and fresh wet feeds (tar-get, 2013).

Aquaculturing makes significant contribution to many nations' economies but fish diseases are major problem for this sector in many countries. It is the same for rainbow trout. There are a number of diseases and parasites that may be effective on rainbow trout aquaculturing. Some of the most crucial diseases that are effective on rainbow trouts are summarised in the table below.

In some cases, antibiotics and other pharmaceuticals were used in treatments but their inclusion in this table should not propose a Food and Agriculture Organization's recommendation.

Table 1. 1 Some of the Diseases that are Effective on Rainbow Trouts (FAO, 2013)

DISEASE	AGENT	TYPE	SYNDROME	MEASURES
Furunculosis	***Aeromonas salmonicida***	Bacterium	Inflammation of intestine; reddening of fins; boils on body; pectoral fins infected; tissues die back	Antibiotic mixed with food, e.g. oxytetracycline
Similar to furunculosis	*Aeromonas liquefaciens*	Bacterium	Smaller lesions on body that become open sores; fins become reddened and tissues break down	Same treatment as furunculosis
Vibriosis	***Vibrio anguillarum***	Bacterium	Loss of appetite; fins and areas around vent and mouth become reddened; sometimes bleeding around mouth and gills; potential high mortality	Same as furunculosis, plus vaccine for greater protection
Bacterial kidney disease (BKD)	*Corynebacterium*	Bacterium	Whitish lesions in the kidney; bleeding from kidneys and liver; some fish may lose appetite and swim close to surface; appear dark in colour	Same as furunculosis
White spot	*Ichthyophthirius multifilis*	Protozoan	White patches on body; becoming lethargic; attempt to remove parasites by rubbing on side of tank	Formalin bath for surface parasites; copper sulphate for parasites below surface; prevented by fast-flowing water

Vibrio and Aeromonas have been described as two of the most common pathogenic species in shrimp and fish that cause infections in the gastrointestinal tract of the fisheries (Groff & LaPatra, 2000) and cause serious losses in larval and adult fish phases throughout the whole world. The antibiotics were suggested for many salmonoid bacterial diseases and against bacterial infections. The most common salmonoid diseases may be defined as vibriosis, furunculosis and enteric red mouth which are caused

by the referred microorganisms, *Vibrio* (especially, *V. anguillarium*), *Aeromonas* (especially, *A. salmonicida*) and *Yersinia* spp. (especially, *Y. ruckeri*).

Antibiotic usage in fish farming was initially preferred for better utilization of feed, better growth, protection from pathogens, preventing toxins, increasing the absorption of nutrients in the intestine. However, in the mean time, antibiotic usage is evaluated to cause problems in animal farming for human consumption and they are also found to cause allergic reactions in both animals and consumers. It can also eliminate the beneficial bacteria from the intestine and when the antibiotic usage is quited within the process, it may cause restrengthening of the pathogenic bacteria. Antibiotic usage for the stated problem has led to further problems of drug resistance and resulted in trade restrictions. According to European Union Regulation 1831/2003/EC which is on additives used in animal nutrition, antibiotic usage in fish and shrimp culturing as growth promoters has been banished. This has caused researches of alternatives and there came probiotic usage proposal instead of usage of the antimicrobial compounds for improving zootechnical performances such as survival, production, feed conversion and growth rates of cultured aquatic species. Today, in aquaculturing, probiotic usage instead of antibiotic usage is very crucial.

1.2 Lactic Acid Bacteria

The Lactic acid bacteria (LAB) are Gram-positive, catalase-negative bacteria that are cocci or rod shaped. They are prokaryotes. They are mainly non-motile microorganisms and they do not sporulate nor respire. They include Lactobacilli, Cocci (Enterococci) and Carnobacteria (Salminen & von Wright, 1998). Generally, the families of Lactobacillales may be defined, as follows; Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, Streptococcaceae (MetaMicrobe, 2013).

The LAB are widespread in nature (they are in soil, milk, human body, vegetables, meat..). They are referred as Generally Regarded As Safe (GRAS) organisms (Ljungh & Wadström , 2006). The LAB are categorized according to their sugar fermentation pathway. They produce lactic acid as their main product and therefore, they have the ability to inhibit other undesired microorganisms' growth. The LAB are either homofermenters or heterofermenters. This varies due to their end product of the fermentation. For the homofermenters, lactic acid is the main end product, whereas for the heterofermenters, there is not one major end product for the fermentation of glucose. Besides lactic acid, they may produce acetic acid and ethanol or carbon dioxide. These compounds, other than lactic acid are important due to their capability to produce specific tastes and aromas. The heterofermentative lactobacilli also may produce mannitol and some species can produce dextran (Battcock & Azam-Ali, 1998).

The lactate that is produced mainly by LAB and other organic compounds such as organic acids, bacteriocins etc. are inhibitory to undesired bacteria. Some of the lactic acid bacteria, namely, *Lactobacillus*, *Carnobacterium*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, and *Leuconostoc* are known to produce bacteriocins (Nes, Diep, Håvarstein, Brurberg, Eijsink, & Holo, 1996). Bacteriocins are small peptides that are characterized by their ability to inhibit pathogenic bacteria. Their spectrum of activity vary, some may be effective on many bacteria (BIOMIN Holding GmbH, 2013). The bacteriocins, from LAB, have become recognized in pathogen inhibition and control in food-stuffs. The antimicrobial effect of lactic acid bacteria has been appreciated for many years and has extended the shelf life of many foods through fermentation processes. It is very rare that a LAB is evaluated as causing disease for humans.

For the growth of LAB, it is important to mention maintenance energy and overflow metabolism. The maintenance energy is the energy required for functions other than biomass paroduction (Pirt, 1965).

The overflow metabolism is a metabolic situation when the rate of glycolysis exceeds a critical value. This case may be observed in glucose-sensitive yeast types or acetate formation, lactate formation of cell cultures. In this metabolism, high rate of glycolysis cause by-product formation from pyruvate. The overflow metabolism is also known as the Crabtree effect. It is known that at high glycolytic fluxes, a decrease in respiratory capacity may be observed. The accumulation of by-product occurs when the microorgansims grow at a high rate of glucose consumption (Enfors, 2003). This has been observed in cultures of mammalian cells, *Saccharomyces cerevisiae*, in *Escherishia coli* but not in LAB, so far.

There are two pathways for lactic acid production. One is glycolytic (Embden-Meyerhof) pathway that the homofermentors use, the other one is 6-phoshoglucanate/phosphoketolase pathway. Glycolytic pathway is the one that is used by Enterococci, like most of the LAB in the excess sugar.

In the homolactic fermentation, the fermentation of 1 mole of glucose yields to two moles of lactic acid;

$C_6H_{12}O_6$ → 2 $CH_3CHOHCOOH$
glucose lactic acid

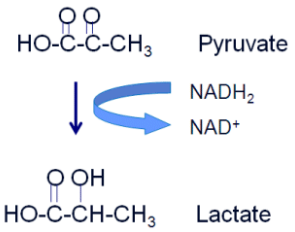

Figure 1.2 Fermentation of Glucose into Lactate (Bisson, 2013**)**

The lactic acid (also, known as 2-hydroxypropionic acid) is a weak organic acid. The asymmetrical carbon in location of the carboxylic function allows two different stereoisometric forms which are L(+) (S-LA) and D(-) (R-LA). They are mirror images of each other. The (+) and (-) signs define the polarized light rotation, that are respectively to the right and to the left. The L and D show the spatial configuration of the molecule with reference to the two glyceraldehyde isomers. Lactic acid has simultaneous carboxylic and hydroxyl groups that are reasons for it to act as an acid or an alcohol. Lactic acid L (+) form is the one that is most frequently occurring in nature, the D (-) form is also present and rarely, a mixture of both may be present in varying proportions. Lactic acid may be found in almost all tissues, physiolocigal fluids and secretions. In the human body, L (+) form dominates. In urine, lactic acid D (-) can be detected (the metabolism is known to occur in the liver). In organisms, lactic acid L (+) is formed by a reduction reaction of pyruvic acid. The hydrogen transfer, seen above, is conducted by a NADH-dependent enzyme, namely, dehydrogenase lactate. The two lactic acid isomers may be produced by the same bacterial species. For *Lactobacillus*, specifically, *Lb. plantarum* and *Lb. helveticus*, pH conditions, like the age of the cultures, can be effective on the L (+)/D (-) ratio (Galactic, 2013).

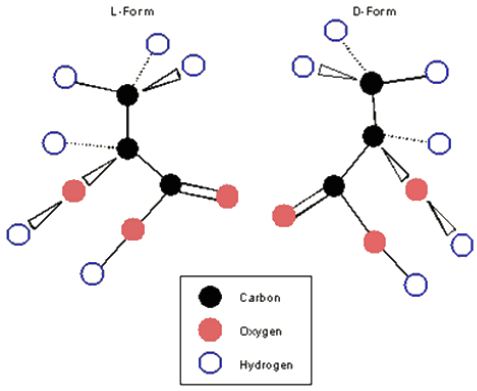

Figure 1.3 L(+) and D(-) Forms of Lactic Acid (Galactic, 2013)

LAB may be found in dairy industry as well as meats, alcoholic beverages, vegetables. Many strains of LAB have been researched over the years for beneficial characteristics and health benefits. In food technology, the following lactic acid bacteria are referred to be used; *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Aerococcus, Streptococcus, Pediococcus, Tetragenococcus, Vagococcus* and *Weisella*.

Streptococcus thermophilus and *Lb. bulgaricus* are required to make yogurt. In Canada, also, many yogurts in markets contain *Lb. acidophilus* and *Bifidobacterium bifidum*. *Lb. casei* is oftenly observed in cheeses. All these referred bacteria are known to be probiotics.

Many studies have underlined LAB existence in fish gut and intestine of the freshwater fish. The lactic acid bacteria that are isolated from microflora of fish intestines are referred to possess probiotic characteristics.

The industrial usage of these microorganisms has gained great interest, now. LAB may be preserved and delivered in liquid, spray dried, frozen or lyophilized form. High viability and cell activity are crucial while maintaining these microorganisms as industrial products, by using these methods. Heat shock increases survival of the cells that are in exponential growth phase during spray-drying but these cells are found to be less resistant to spray-drying than the ones in stationary phase (Teixeira, Castro, & Kirby, 1995). The sugar content in growth media should be firstly checked, to be analysed as protective agents. Other compounds, such as sorbitol, trehalose which were previously claimed as effective in protecting bacteria during drying and subsequent storage may also be used (Carvalho, et. al., 2003).

1.3 Probiotics

1.3.1. Definition of Probiotics

An original definition for probiotics is “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 1989). Generally, probiotics may be defined as non-pathogenic microorganisms that effect positively upon ingestion.

Probiotic has an accepted definition by World Health Organisation such as; “Live microbial food ingredients (bacteria, yeast) that are beneficial to health”. So, being beneficial to the health of the host

is crucial for a probiotic. Pilot-scale in vivo tests need to be conducted in order to gain knowledge on economic advantages of the process, the cost-benefit analysis (Vine, et. al., 2006).

There are certain specialities that a microorganism should possess in order to be referred as probiotic. These qualifications are being able to survive both in the stomach acids, as well as in bile acids and gut, being able to survive in sufficient amounts so that they could be effective, being able to adhere well onto the intestine wall and colonize, showing beneficial effects on human health (like antagonistic effects towards targeted pathogens stimulation of the immune system, maintenance of normal intestinal flora, showing antagonistic effects towards the pathogens by secreting related compounds (such as bacteriocins) production of short-chain fatty acids which then maintain reducing pH of the colon (inhibiting pathogens), providing nutrition, reduction of intestinal permeability, modulation of immune function of gastrointestinal mucosa or by competing for nutrients and adhesion sites, (BC Dairy Association) (MetaMicrobe, 2013). Another important factor is that probiotics should always lack pathogenicity.

Probiotic bacteria's adhesion site, mucous membrane, consists of various bacteria including *E. faecium*. The microflora on this membrane enhances protection towards potential pathogens and regulates production of bacteriocins, formation of short-chain fatty acids, competition for receptors and metabolic substates etc (Herich & Levkut, 2002).

For usage in aquaculturing, in order to say that a microorganism is a probiotic, that microorganism should basically be capable of bacterial attachment onto the fish intestine surface and also compete with target pathogen bacteria for adhesion sites (Sica, et. al., 2012). The efficiency of potential probiotics will require long-term application studies in fish farms to be validated.

Considering a probiotic as safe is crucial and microorganisms' safety are studied under three different concepts; non-pathogenic microorganisms, pathogens and opportunistic pathogens. Every living microorganism possessing ability of surviving and imroving in its host has the opportunity of causing infection. Most of the microorganisms of gut are not pathogenic for healthy individuals (Salminen, et. al., 1999).

Probiotics compromise a large range of microorganism including *Bacillus*, *Lactobacillus* and *Enterococcus* species (Panigrahi, et al., 2007). The representative species as probiotics are as follows; *Lb. acidophilus, Lb. johnsonii, Lb. casei, Lb. gasseri, Lb. plantarum, Lb. rhamnosus, B. longum, B. breve, B. bifidum, B. infants, E. faecalis, E. faecium.*

1.3.2. Probiotics' Beneficial Effects and Usage

Probiotics are well established as important to be used for humans, poultry and cattle. Probiotic usage in poultry is proven to be advantegous. Several researches have shown that the LAB and other specified microorganisms can increase resistance to disease and can be enriched within the intestinal tract. Researches, that are conducted so far, have targeted identifying beneficial microorganisms and substrates along with the conditions under which they are effective. It has been supported with affirmative results that usage of probiotics as growth factor in animal feeding has increased viable mass, benefiting from feed, egg efficiency and has enhanced health.

The usage of probiotics in animal feeding, specifically poultry, has been accepted as successful due to the tendency to avoid antibiotic usage and the problem of antibiotic resistance and bacteriocin production is effective in this success (Gagic, et. al., 2003). Their usage in aquaculture has recently become preferable mainly due to the problem of antibiotic resistance and the tendency to avoid using antibiotics. Most scientific studies propose that selected potential probiotics may protect fish against different pathogens as well as increase the performance of growth and appetite of the cultured fish,

enhance faster, qualified culturing with less loss. In marine product culturing, researches on the usage of probiotics for such purposes have been increased and the usage of probiotics have become popular (Irianto & Austin, 2002) (Korkut, et. al., 2003) (Vine, et. al., 2006).

Lactobacillus, Carnobacterium, Pediococcus, Enterococcus and *Leuconostocs* are referred as bacteriocin producing microorganisms (Nes, et. al., 2000). Bacteriocin production is related with bacterial growth, it is released during the microbial growth of the bacteriocin producing microorganisms, therefore, it is also changed by growth parameters that are effective on microorganism's growth (Hur, et. al., 2000).

Today, specified bacteria are known as water quality improving agents, food sources or seed biofilters. Conversely, most of the microorganisms are unknown or not widely used because of the lack of comprehensive researches, expertise (Olafsen, 2001).

Currently, commercial probiotic products are available in liquid or powder presentations. The interest has been focused on optimizing the fermentation conditions to increase the viability and functionality of probiotics maintained as final industrial products, focusing on improving performance (Lacroix & Yıldırım, 2007).

1.3.3. Probiotics for Marine and Aquaculturing

The probiotic usage in aquaculture is comparatively new. The initial interest was focused on their use as growth promoters and to improve the health of animals, however, new areas have been found, such as their effect on reproduction, nutrient utilization, improvement of water quality or stress tolerance, although this requires a more scientific development. FAO and WHO recognized the need for guidelines for a systematic approach to evaluate probiotic usage in food, in order to define their health claims. "Guide for the Evaluation of Probiotics in Food" was prepared. The working group had stated that no pathogenic or virulent properties were found in lactobacilli, generally. The guide is not focused on aquaculturing sector but it enlightens safety of probiotic usage also in this sector (FAO/WHO, 2006).

For increasing the quality of the water and getting rid of the virus and pathogens, filtration of the water, addition of sodium chloride, ozonation, UV sterilization and artificial antibiotic usage are preliminary methods to be conducted but they are not enough, in long term. For utilitizing long-term healthy and qualified water, after such procedures, addition of beneficial microorganisms will limit pathogenic and virus growth (Maeda, 1999). Probiotic usage for water quality enhancement as well as enhancement of fish microbiota for larva fish has been supported (Nogami & Maeda, 1992).

Probiotics to be used in aquaculturing have the following definition "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health" (Gatesoupe, 1999). Another definition provided includes, the ability of a probiotic to modify the "host-associated or ambient microbial community" and, to improve the quality of its surroundings, both of which can be considered as biocontrol (Verschuere, et. al., 2000).

Up to now, many studies on the efficiency and advantageous usage of probiotics in marine culturing have been conducted but there are few studies focusing the bacterial mode of action (Vine, Leukens, & Kaiser, 2006).

The probiotics that are used for humans and terresteral animals are proven to be potentially beneficial in aquaculturing sector, as well (Das, et. al., 2008; Vine, et. al., 2006). It is known that the first trials

with fish sector as probiotics were the ones prepared for terrestial animals after initial trial of their ability to survive in water environment.

The probiotics that are studied for usage in aquaculturing are both Gram-negative and Gram-positive bacteria, bacteriophages, yeasts and unicellular algae (Irianto & Austin, 2002). Probiotics for usage in marine larviculture are referred to ideally be aerobic or facultative anaerobes (Vine, et. al., 2006). The fish species for which lactic acid bacteria has been studied as probiotics, are as follows; Atlantic salmon (*Salmo salar*) (Gildberg, et. al., 1995), rainbow trout (*O. mykiss*) (Nikoskelainen S., et. al., 2003), turbot *(S. maximus*) (Gatesoupe, 1994). There is a lack of information on beneficiary microorganisms targeting juvenile rainbow trouts.

Like in other sectors, in aquaculturing of the fish, many pathogens have caused huge losses. For a probiotic to be evaluated as effective in aquaculturing, generally the focused issue is production of inhibitory compounds (Kesarcodi-Watson, et. al., 2008). The effect of competition on the microbiota of the fish is also evaluated as antagonism effect. In aquaculturing, the referred probiotics should, generally, possess characteristics such as; enhancing reduction in mortality or increasing survival under gastrointestinal tract conditions including acidity of the stomach, composition of the environment and bile salt concentration (Soccol, et al., 2010), improving immune system against potential diseases (caused by potential pathogens), well adhesion onto the gut and colonization there, production of digestive enzyme activities, phagocytic and lysozyme activities (Irianto & Austin, 2002). These characteristics have been researched for the microorganisms to improve farming of the fish including trouts, focusing on lactic acid bacteria and *Bacillus* strains. Studies increased to a great extend over the years, on the subject. The basis of these studies include these most common modes of actions; immune response stimulation, alteration of microbial metabolism by effecting some enzymes' levels, showing antagonistic effect towards the potential pathogens by competition for adhesion sites, nutrients, oxygen or by the production of inhibitory compounds (Fuller, 1989). The enzymatic support on nutrients and digestion is also important for facilitating digestion as well as enhancing benefiting from the feed (Bairagi, et. al., 2002).

It is concluded that probiotics that are selected/isolated form the fish's intestine or the environment it's grown in might propose better growth, probiotic effects (Koca B., et. al., 2011).

The microorganisms that generally are evaluated as probiotics for fish farming are referred as follows; *Lb. acidophilus, Lb. sporogenes, Lb. rhamnosus, Lb. plantarum, Carnobacterium divergens sp., Lb. lactis ve Pediococcus acidilactici* (Casteks, et al., 2008). Most of the bacteria that are proposed to be biological control aids as probiotics are LAB (*Lactobacillus* and *Carnobacterium*), *Vibrio* (*V. alginolyticus*), *Bacillus* and *Pseudomonas*. *Aeromonas* and *Flavobacterium*'s probiotic effects have been investigated (Balcazar, et. al., 2006).

It had been found that two weeks of feeding trouts with probiotics caused improval in immune system (Irianto & Austin, 2002). It is referred that, in rainbow trout's digestive tract, especially *Aeromonas*, *Pseudomonas* and *Enterobacteriaceae* exist. These bacteria groups are observed to possess wide spreading in fresh water environment and are advantageous for their characteristics to survive under the conditions of the fish's digestive tract (Austin & Austin, 1989).

Enterococcus, together with *Bifidobacterium* are referred as promising probiotics, in various studies.

In rainbow trout growth, *Lb. rhamnosus* was studied and applied with a dose of 10^9 and 10^{12} cells/g of the feed. The application lasted for 51 days and reduced mortalities up to % 33.7, when challenged with *A. salmonicida*. This study also showed that increasing the dose would not mean better growth and survival. In this case, lower dose was much more effective in increasing the mortalities (Nikoskelainen, et. al., 2001).

When a probiotic is found to be effective in vivo in aquaculturing, the next step is to find appropiate delivery method to the fish. Some probiotics may be evaluated with live food. Probiotics have been evaluated within artificial feed, live feed and in water (Irianto & Austin, 2002). It is suggested that maintaining larvae probiotics in higher numbers than it exists in aquaculturing water is better than ingesting the probiotics via livefood or directly (Ringø, et. al., 1996).

The researches show that probiotics isolated from and applied to the same species are more efficient than the strains that are isolated from different origins. A study showed that anaerobic bacteria that are isolated from 3 different fish intestines, are found potential probiotics due to their enzymatic characteristics (Ramirez & Dixon, 2003). However, *Lb. rhamnosus* (a human probiotic) was found to be advantegous for enhancing rainbow trout growth against furunculosis (Nikoskelainen S., Ouwehand, Bylund, Salminen, & Lilius, 2003). For rainbow trouts, the effect of *Lb. rhamnosus JCM 1136* strain usage on immune system and intestinal flora, as feed additive was investigated. 1 x 10^9 to 1 x 10^{10} cfu/g of the viable strains' addition to feeding for 30 days had resulted in increase in the number of probiotic bacteria on fish intestine, digestion of the feed, fagocitic activity of the leucosites (Panigrahi, et. al., 2004).

Probiotics in marine product culturing is promising but much more studies are required on this subject. In Turkey, there is not such a professional application.

1.4. *Enterococcus faecium*

1.4.1. Characteristics of the Bacteria:

E. faecium is from the Enterecocci family. Enterococci may be found in the gastrointestinal tract, oral cavity, upper genital tract of mammals, in birds, reptiles, insects, plants, soil and water. *E. faecium* is kind of bacteria that may be isolated from the healthy gastrointestinal microbial flora of the animals and humans. *E. faecium* is one of the most common lactic acid bacteria that is used in animal nutrition and has been considered advantegous for usage in commercial aquaculturing.

Specific characteristics of the strain may be defined as below;

It is gram positive bacterium that is spherical as cocci. The bacteria can be motile and non-motile (the used strains were motile in the thesis study). The bacteria are non-sporulating, facultative anaerobe and homo-fermentative (the coccus is a lactic acid bacterium). The bacteria are capable of growing at 10ºC as well as at 45ºC, it possesses resistance to 6.5% NaCl. The bacteria can grow in the presence of 40% bile and at pH 9.6. (Deibel, 1964) (Martinez, et. al., 2003). Capability of growing at low temperatures (12 °C) is advantageous due to the fact that the fish is farmed under conditions that contain similar temperature values and also, this ability is referred to maintain better non-specific immune responses (Panigrahi, et al., 2007).

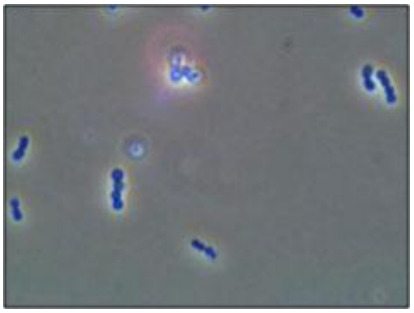

Figure 1.4 Microscopic Picture (1000-fold Magnification) of *E. faecium* (BIOMIN Holding GmbH, 2013)

The bacteria are known to show resistance to antibiotics (clindamycin, oxalillin, trimethoprin-sulphamethoxazole, vancomycin, gentanicin, tetracyclin etc.) and they possess tolerance to basic, acidic, isonic or hypertonic environments. They produce ammonium from arginine & produces acid from mannitol, sucrose. The bacteria hydrolize esculin and they do not hydrolyze gelatin. The expected final pH in glucose broth is 4- 4.4 (decreased from neutral pH).

In one study that investigated *E. faecium* as potential probiotic for humans, the strain was capable of adhering efficiently to mucus from the intestine, stomach and esophagus but much more onto the mucus from gills and skin (Nikoskelainen, et. al., 2001).

It, naturally, comprises a major population in the gut. There are many studies in literature referring and supporting *E. faecium* usage as probiotics in livestock. It is also known to be used as probiotic for human consumption for over a decade. *Enterobacteriae, together with Aeromonas*, *Plesimonas* families, are known to be dominant in fresh water fish (Sakata, 1990).

Specific strains of the microorganism are also evaluated as cost-effective, healthy and non-risky replacing the use of antibiotics, approved by European Union, the United States, FDA, AAFCO and some other recognized institutions (Morandi, et. al., 2005).

Another study showed the direct l-lactic acid production of *E. faecium* (Shibata, 2007). *E. faecium* strain was tested by sugar fermentative test and 16S rDNA sequence analysis. Direct l-lactic acid fermentation was carried out with various starches, among which sago starch fermentation was found to be similar to glucose fermentation. The strain showed direct lactic acid fermentation with starches and produced lactic acid of high optical purity (98.6%).Optical purity of the produced lactic acid was determined by the F-kit D/l-lactate assay with d-lactate dehydrogenase (LDH) and l-LDH activity. The *E. faecium* was found to be capable of producing l-lactic acid from all the starches tested, which resulted in different production (Shibata, 2007).

In a study that was conducted with *E. faecium* which was isolated from chicken intestinal tract, lactic acid and bacteriocin production, alone and in mixed culture with *Salmonella* serotypes, were observed. The production of lactic acid, together with bacteriocin maintained antibacterial action against pathogens. (Audisio, et. al., 1999).

Enterococci have been evaluated to inhibit pathogens in the intestine, stimulate the immune system and reduce blood cholesterol levels. *E. faecium* SF68 has been used as an alternative solution to antibiotic therapy, for treatment of diarrhoea (Frigerio & Moroni, 1979).

In another study that investigated antagonistic effect of *E. faecium* on *E. coli K88*, and *E. faecium* was found to have the ability to survive gastrointestinal tract and show antagonistic effect up to 90 %, therefore it was referred as advantageous probiotic. It was found crucial to continue the studies with

probiotic characterization of the strain, such as stimulation of immune system, molecular identification (Garcia-Galaz, et. al., 2004).

Although some of the *E. faecium* strains are proven probiotics without virulence factors that are used in animal feeding sector (Başyiğit, 2010), on some occasions, they may be referred as pathogens that may cause serious hospital borne infections. They are referred as low grade pathogens that are resistant to specified antibiotics. They are referred to cause diseases like meningitis, endocarditis, bacteraemia and particularly urinary infections, central nervous system infections, intraabdominal abscesses, and infections in newborn infants (Klein, 2003) (Cermak, et. al., 2009; Moellering, 1991).

The pathogenity is related to multi-drug resistance and virulence factors. The virulence of a microorganism is regulated with virulence coding genes on the genome in regions that are termed as pathogenicity islands (PAI) (Hacker & Kaper, 2000). The PAI of *Enterococcus* was firstly found in *E. faecalis*, in 1980 (Huycke, et. al., 1991). The findings have leaded to the fact that more research is needed to be conducted on Enterococci and their interactions with the host in order to understand pathogenicity (Giridhara Upadhyaya, et. al., 2009). Enterococci that are referred as probiotics are not infectious, no infection has been reforted for veterinary medicine, there appears to be no serious risk (Rinkinen, et. al., 2003). *E. faecium* strains were found to be generally free of virulence determinants (Eaton & Gasson, 2001).

A protein called the enterococcal surface (Esp) is referred to contribute to the virulence of *E. faecium* (in clinical isolates, they do not exist in the strains which colonize guts) by forming biofilms. These biofilms cause colonization of tubing that is used in hospitals and this can lead to infections of the blood and urinary tract infections. The regulation of this Esp gene allows *E. faecium* to change its response when it enters a host (Van Wamel, et. al., 2007). Additional virulence factors may be defined as follows; aggregation substance (AS), cytosolin, and gelatinase. AS allows the microbe to get attached to target cells and facilitate the transfer of genetic material between cells. Cytosolin is a protein found in the cytosol and lyses erythrocytes. Gelatinase can hydrolyze peptides. The presence of virulence factors differ among strains and usually are specific for the host the strain colonizes (Gülhan, et. al., 2006). Vancomycin-resistant enterococci (VRE) have been referred as causing hospital outbreaks worldwide. This lineage is characterized by ampicillin resistance, a pathogenicity island, and an association with hospital outbreaks. *E. faecium* has become an important hospital-origined pathogen, especially in immunocompromised patients, that effects treatment options because of cumulative resistance to antimicrobial agents (Phage Therapy Center, 2013).

Their antibiotic resistance is referred to be gained by mobile genetic elements' horizontal transfer and has been referred to be spread throughout the enterococci, mostly caused by conjugative plasmids of the pheromone-responsive and broad host-range incompatibility group 18 type. This transferring occurrence has been evaluated to be related with hospital pathogenity. Up to now, pheromone-responsive plasmids' transfers have been proposed for *E. faecalis* but not for *E. faecium*. There are plasmids called as Inc-18-type plasmids which are observed in *E. faecium* to a big extent and which maintain persistence of the plasmid even in the absence of direct antibiotic selection due to toxin-antitoxin system. pRE25 which is a plasmid known to be transferred by *Listeria innocula* and *Lc. lactis* is capable of host range transmissions of resistance and other genes. This plasmid (12 kb) has been found in the fish pathogen *Lc. garvieae*. For the popular vancomycin resistance in *E. faecalis* and *E. faecium*, a plasmid called pHTβ and its derivatives are known to be effective. They are known to be effectively transferred among the strain, *E. faecalis*. The issue should further be studied. Also, there is Tn1546 that has been found to reside on broad range plasmids of Inc18 and firstly, the Tn1546 transposon *vanA* has been isolated from *E. faecium*. Conjugative transfer of chromosomal determinants has been reported among *E. faecium* and from *E. faecium* to *E. faecalis*. Plasmids are known to mediate the transfer of antibiotic resistance, virulence, other adaptative possible traits throughout genus or between other genuses. This is evaluated as source of hospital adaptation of the strains. Further studies with *E. faecium* should be conducted and it should be enlightened if a similar mechanism may contribute to *E. faecium* genome diversity (Palmer, Kos, & Gilmore, 2010).

The pathogenicity of the strain is referred as relatively low under normal conditions. Enterococci are considered as threat due to their great tolerance towards negative environmental conditions and antibiotics (Votava, et. al., 2003). They may be evaluated as conditionally pathogenic microorganisms.

1.4.2. Antagonistic Effects of *E. faecium* on Specific Fish Pathogens

E. faecium is referred to show antagonistic effect on *A. salmonicida* (Panigrahi, et al., 2007) which causes furunculosis disease in fish. One other common fish disease is referred as vibriosis caused by *V. anguillarum*. Preventing diseases, esp. in juvenile fish is very important due to economic manners (small fish have high mortality rates and are too small for vaccination) (Nikoskelainen, et. al., 2001).

Another finding was *on E. faecium*'s effect to reduce the *E. coli*, *Staphylococcus aureus* and *Clostridium spp.* in the intestinal microbiota of the juvenile rainbow trout (Merrifield, et. al., 2010).

One reference proposes *E. faecium*'s industrial product's usage (as added to the feed) as biological control agent/antagonistic effector on *Anguilla anguilla* (increasing mortality to a great extend) (Chang & Liu, 2002).

In one study, inhibition of *A. hydrophila* by *E. faecium* in *Cyprinus carpio* was investigated and the strain was found to show effective antagonistic characteristics. The used *Enterococcus* strains were isolated from *Mugil cephalus* intestine. Agar spot method was used to detect the antagonistic effects towards *A. hydrophila* (Gopalakannan & Venkatesan, 2011).

In the studies that are conducted by *E. faecium*, antagonistic effects against *A. hydrophila* were observed by cross-streaking and the agar spot method. The strain's probiotic effects were confirmed and it was checked for nonpathogenicity to fish (Gopalakannan & Venkatesan, 2011). It may still be declared that few reports exist on controlling Aeromonas by usage of the probiotics.

The studies that are held in vitro and included agar spot method claimed that *E. faecium* (strain IMB 52) has antagonistic activity against a wide range of aquatic pathogens such as, *Y.ruckeri, V. harveyi, S. agalactiae and A. veronii* (Rosskopf, 2010).

E. faecium (strain IMB 52) is found to have the capability to populate the intestine. By this way, it could inhibit Vibrio species' growth. *E. faecium* was detected in the fish gut and faeces, even after 10 days from intake of the probiotic. It was observed to enhance immune response, to improve the growth of the fish and boost the survival rate of the shrimps that are infected by Vibrio. Therefore, *E. faecium* (strain IMB 52) is considered as safe and advantegous potential probiotic for aquatic species (BIOMIN Holding GmbH, 2013).

1.4.2.1. Evaluation of *E. faecium* as a Probiotic

The selection criteria for probiotics to be used within fish aquaculturing are defined as, good intestinal colonization, competition enhancement against pathogen (for adhesion, nutrients etc.), bile resistance, capability to survive at low pH's, and maintain stimulation of growth, (Gatesoupe, 1999). These searched criteria also point out *E. faecium* as a highly advantageous potential probiotic with the ability of high survival against such harsh conditions and potential of showing antagonistic effects on target fish pathogens.

In rainbow trouts, Enterococci content includes *E. faecium*, as well, therefore, it is suggested that *E. faecium* would better adapt to the environmental conditions of aquacultured rainbow trout (Merrifield, et. al., 2010). The reason for high content in the intestinal tract of the microorganism is due to; *E.*

faecium's ability to grow advantegously well under the conditions that the rainbow trout is cultured (temperature) and the rainbow trouts' intestinal conditions (bile concentration, acidity), its capability to antagonisticly effect the potential pathogens that may cause damage in rainbow trouts and having good adhesive properties on the intestinal mucus of the fish (Merrifield, et. al., 2010).

In a study, it was observed that freeze-dried *Lb. rhamnosus, E. faecium or Bacillus subtilis* (1×10^9 cfu/g) improved rainbow trouts' growth, after 45 days of feeding. The fish fed with *E. faecium* was found to show better performance, this finding was related with the growth temperature advantage. Temperature is a significant parameter of the microbial growth and *E. faecium* because of its psychrotolerant characteristic growth was well at temperatures down to 12 °C, suiting rainbow trout growth conditions (Panigrahi, et al., 2007).

E. faecium is referred as maintaining better growth (11%) when tried for sheat fish, after 58 days feeding with a dose of 2×10^8 bacteria g^{-1} of feed (Bogut, et. al., 2000).

Lactiferm could be a good example of *E. faecium*'s industrial application as probiotic. It is an approved feed additive that contains viable *E. faecium* strains for using in chickens. Its intended usage is within the dose range of 2×10^8 and 8×10^9 cfu/L of water. It is intended to be used in powder form (4×10^{11} cfu/g product), in coated form and in drinking water of the targeted animals (2×20^8 and 8×10^9). Due to the fact that *E. faecium* is one of the gut microbiota's natural components, the approach is that there would not be any extra harm to the environment. It is a product that is proved to be effective on piglets and calves.

For the Lactiferm, cells were incubated within a typical lactic acid bacteria growth medium and then the medium was centrifugated and the cells are gained. The cell was freeze-dried after the addition of cryoprotectants (maltodextrin, sorbitol). The product was prepared either in powder form or in water soluble form. During storage, the microorganism cell numbers remain constant when refrigerated or frozen (at about 5×10^{10} cfu/g) (EFSA, 2012) (EFSA, 2012).

1.5. LAB and *E. faecium* Fermentation

In one study, different pH's (5.0, 5.5, 6.0, 6.5) and temperatures (25 °C and 37 °C) were tried and optimum values were investigated for *E. faecium*. In this study, the generation time (GT), the conductimetric methods and the detection time (DT) were evaluated. The data were studied, obeying general linear model. The optimum pH was found to be 6.5 whereas, the optimum temperature is found out to be 37 °C, and it is found that metabolic activity is affected by these parameters (Morandi S., et. al., 2005).

Another study supported 37 °C as the optimum value for the strain's fermentation (Rosskopf, 2010).

In one study, it was observed that for the microorganism's growth, the optimum MRS composition was proposed to include peptone (40.0 g/L), meat extract (30.0 g/L), yeast extract (40.0 g/L), lactose (24.0 g/L), glycerol (5.8 g/L), Tween 80 (3.0 g/L), triammonium citrate (1.0 g/L), K_2HPO_4 (2.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.10 g/L), $MnSO_4 \cdot 7H_2O$ (0.05 g/L) and dipotassium PO_4 (2.0 g/L). The defined medium maintained growth with higher bacteriocin activity and higher viable cell production (14.22 log CFU/mL) (Kanmani, et al., 2010).

In another study that investigated role of environmental factors and medium composition on bacteriocin production of *E. faecium*, peptone, yeast extract and lactose were found out to be significant compounds for growth and bacteriocin production of the targeted microorganisms. In the experiments, the temperatures of 25 °C, 30 °C, 35 °C, 40 °C, 45 °C and pH values of 5.5, 6.0, 6.5 and 7.0 were tried. For modeling part, all experiments were held at 6.5 pH and 35 °C as found favorable conditions, for 16 hours of fermentation. ANOVA (analysis of variance) was used to estimate the statistical parameters of the optimization in the selected medium, MRS. *E. faecium* that is isolated

from fish intestine was used, within the study. The strain was found to have the potential to be used against vibriosis, in seafood biopreservation (Kanmani P. , et. al., 2011).

The study of Parente, et. al., (1997) investigated growth and bacteriocin production by *E. faecium DPC1146* both in batch and continuous fermentation processes. It was found that growth was strongly inhibited by lactic acid. For the batch fermentations of the microorganism, the best condition that gave the maximum microorganism activity included 20 g/L glucose. Increasing the glucose content did not lead to extra bacterial activity. Five batch fermentations (with initial glucose concentrations of 5, 10, 20, 25 and 30 g/L) were conducted at 37 °C, pH 5.5, at 200 rpm agitation speed in a stirring tank with 2.5 L working volume. The continuous experiments were held with 1 L working volume, at the same temperature and pH conditions, agitated at 500 rpm. The basal medium containing 20 g/L glucose was used for all of the fermentation processes. The volume, in the continuous fermentation, was kept constant at 0.8 L by a peristaltic pump and the fermentation was batch for the first 8 hours, then the continuous feeding of the substrate was started. The optical density was measured at 600 nm with spectrophotometer. For dry weight calculations, culture samples were centrifuged, in preweighted tubes, the pellets were washed with distilled water and recentrifuged. Lastly, the pellets were dried at 105 °C. It was found for the batch fermentations that, with the high amounts of substrate (glucose), the biomass was lower and the growth was slower. Bacteriocin production was also slower at the highest substrate amount and no decrease in bacteriocin activity occurred. The experimental data for cell dry weight, lactic acid and substrate concentration were smoothed via the usage of a generalized logistic model;

$$Ci = K/[1 + \exp(a_0+(a_1)t+(a_2)t_2+(a_3)t_3+(a_4)t_4)]$$

In the equation above, *C*i stands for the dry cell weight (X), lactic acid (P) or substrate (S) concentration, K is a constant that is set arbitrarily to 1.2 times of the *C*i value, and αi are the parameters estimated due to non-linear regression. The calculated data varied with a 1.4% percent from the experimental data.

The cell growth rate (r_x), lactic acid production rate (r_p), substrate consumption rate (r_s) were calculated by taking the derivative of the above equation;

$$-dCi/dt = -Ci(1-Ci/K)(a_1+2\cdot(a_2)\cdot t+3\cdot(a_3)\cdot t_2+4\cdot(a_4)\cdot t_3)$$

Whereas, specific growth rate (μ), specific lactic acid production rate (π) and specific glucose consumption rate (ν) were calculated from the division of the rates obtained from the second equation by the smooted values of calculated dry cell weight (from the first equation).

The relationship between the specific growth rate, instantenous glucose content (S) and lactic acid concentration (P) was explained as follows;

$$\mu = \mu_{max}\cdot[S/(K_{S\mu} +S)][K_{P\mu}/(K_{P\mu} +P)]$$

where μ_{max} is maximum specific growth rate, $K_{S\mu}$ is the substrate saturation constant, $K_{P\mu}$ is the non-competitive inhibition constant. In batch cultures where S is much higher than the $K_{S\mu}$ (values of $K_{S\mu}$ have been calculated for Enterococci) (Ohara, Hiyama, & Yoshida, 1992), the equation mentioned may be simplified as follows;

$$\mu = \mu_{max}(K\cdot P_\mu/(K\cdot P_\mu + P)) \quad => \quad 1/\mu = 1/\mu_{max} + (1/(K\cdot P_\mu(\mu_{max})))\cdot P$$

So, a graphic showing 1/μ versus P was maintained, in the study. Lower specific growth rates were found to be related with higher P and S concentrations.

Also, according to Luedeking-Piret model (Luedeking R. and E. Piret, 1959);

$$\pi = (Y_{P/X})\cdot\mu + m_P$$

Where $Y_{P/X}$ and m_P are defined constant for growth-associated and non-growth associated lactic acid production. The estimated constants were as follows; 3.8 +/- 0.2 g/g as $Y_{P/X,}$ 0.5 +/- 0.1 h^{-1} as m_P. A linear relationship was observed between π and μ. According to observations for continuous fermentation, increase in dilution rate caused decrease in biomass production. Residual glucose concentration was never below 1.9 g/L and a maximum lactic acid concentration of 18.2 g/L was obtained at the lowest dilution rate. For the continuous fermentations, the estimations are as follows;

π (lactic acid production rate, per hour) = $P \cdot (D/X)$

ν (specific glucose consumption rate, per hour) = $(S_F - S) \cdot (D/X)$

where D is the dilution rate (h^{-1}). P, X, S are steady state concentrations (g/L). X is the biomass concentration and S_F is the glucose concentration in the feeding medium.

Growth was observed to be inhibited strongly by lactic acid produced and possibly by glucose.

In another study, the batch fermentations of *E. faecium* were held at pH; 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5, in 250 mL Erlenmeyer flasks with 50 mL working volume, at 200 rpm, 30 °C for 20 hours. In addition to this, the realkalized fed-batch cultures were fermented at the same temperature, in a 6 L bench top fermentor at 200 rpm, an aeration of 0.5 L/h and continuous recording of the pH with a neutral initial pH and working volume of 4 L. In the first fed-batch fermentation experiment, 400 g/L sterile concentrated glucose solution weas fed into the fermentor and in the second fermentation, the feeding substrate consisted of a mixture of a 400 g/L concentrated lactose and the medium of the fermentation. For the batch and realkalized fed-batch fermentations, the initial inoculation to the fermentation medium was a 2% (v/v) culture that was fermented for 12 hours, in the same fermentation medium. The pH control was stabilized by feeding 5 M NaOH into the medium. For analytical assays of this study; the fermentation biomass' dry cell weight was found with a standard curve and the cells were centrifugated at 12000 rpm for 15 minutes, at 4 °C, washed twice with saline (0.8% NaCl). The culture supernatants were used to find out the sugar content, lactic acid, acetic acid, ethanol butane-2,3-diol,phosphorous and nitrogen. The total viable counts were done with pour plating (with three parallels), after an incubation of 30 °C for 48 hours. The final biomass (0.24 g/L) was 9 x 10^8 cfu/mL, at the end of batch fermentations. The same trend was seen in batch and fed-batch fermentations with different strains and culture media (Guerra, et al., 2010).

Within a study, the incubations with *E. faecium* were held at 30 °C for 24 hours, harvested by centrifugation at 16500 g for 10 minutes and washes three times with peptone water (0.85% NaCl, 0.1% polypeptone), then the microorganisms were freeze-dried and stored at – 20 °C. The daily rations for feeding were kept at 4 °C. The turbidities for growth at different temperatures were observed at 600 nm (Panigrahi, et al., 2007).

In one study, fermentations were performed in a 15 L fermentor with a working volume of 10 L. Sterilization was performed at 121 °C for 20 min. Citrate and glucose solutions were autoclaved separately (for, again, 121 °C and 20 min) and added to the fermentation under sterile conditions. The inoculation was done with 1.0% (v/v) and initial cell load was 1.5 x 10^4 cfu/ml. The agitation was performed at 100 rpm. The temperature, pH, and agitation were computer controlled and monitored online. Microaerophilic conditions were maintained during the fermentation. The pH of the medium was kept by pumping 10 N NaOH and 2 N HCl (Vaningelgem F., et. al., 2006).

BHI (Brain Heart Infusion) broth was referred as growth medium for *E. faecium* at 37 °C, incubated for 24 hours (Hasman H., et. al., 2006).

Glucose, peptone and yeast extract are examined to be the basic required medium components, during the previous studies that were held at Food Engineering Biotechnology Laboratory. Fermentation conditions maintain online analysis, and sampling.

1.5.1. Biokinetic analysis and modeling

There are various models for describing different microorganisms' growth models in batch conditions. The generally used ones can be defined as Logistic and Modified Gompertz equations and the Monod model. The Luedeking and Piret expression may be used for describing the time-course of the production of growth-associated (primary), nongrowth-associated (secondary), or mixed products. In realkalized fed-batch cultures, these mentioned models are not sufficient to describe the biomass and product formation. A valid model should include the effects of the parameters, initial pH, substrate limitation, substrate inhibition, product inhibition on the fermentation process. The significance of the estimated parameters and sensitivity of the fitted model are evaluated by using statistical methods. Lastly, the scope of the model should be explained (the systems, conditions that the model could be appropriate to use). Calculating the best-fit values and figure out the best fitting model would be reasonable. In a study, the models were maintained by using the nonlinear curve-fitting software of SigmaPlot. The lactic acid production in the fed-batch fermentations were well described with the Luedeking and Piret model. A paired-samples t-test was conducted to figure out whether the differences between the mean concentrations of biomass and antimicrobial substances produced are significant ($P<0.05$). It was suggested that the usage of the right models may develop better strategies for optimum fermentation and economic favors (Guerra, et al., 2010).

According to Monod Kinetics, which represents the nutrient effect for growth, specific growth rate (μ) is related with substrate concentration. Each specific growth rate is determined for a different initial substrate concentration.

$$\mu = (\mu_{max})\cdot(S)/(K_S+S_0)$$

where S_0 is initial substrate concentration and K_S is affinity of the cell to the substrate. The slope of the graph, $1/\mu$ vs. $1/S$ gives the K_S/μ_{max} and the intersection gives $1/\mu_{max}$.

For batch fermentation of microorganisms, there are two models; lumped model (including perfect mixing, single independent variable and ordinary differential equations) and distributed model (including imperfect mixing, additional independent variable and partial differential equations).

General formula of mass or species balance is as follows;

In – Out + Generation – Consumption = Accumulation

For the fermentation in batch reactor (unsteady-state operation);

-Consumption (reaction) = Accumulation

$-(-r_A)\cdot(V) = d(C_A\cdot V)/dt$ (V (volume) is constant)

Where r_A is substrate consumption rate, C_A is substrate concentration (g/L).

$t = -\int dC_A/(-r_A)$

For continuous stirred tank reactor (CSTR),

In – Out + Generation – Consumption = 0 (at steady-state) (V is constant)

$C_{A0}\cdot(Q) - C_A\cdot(Q) - (-r_A)\cdot V = 0$

$\Gamma = (C_{A0} - C_A)/(-r_A)$ where Γ is time required to process one reactor volume.

For chemostat,

At steady-state operation,

Cell balance: In – Out + Generation = 0

$X_0 \cdot (Q) - X \cdot (Q) + (r_x) \cdot V = 0$

Where Q is flow rate, X_0 is initial cell concentration and r_x is cell growth rate.

The dilution rate (amount of reactor volume that process per unit time),

$D = 1/\Gamma = Q/V$ (1/sec)

If $\mu = (\mu max \cdot S)/(K_S+S)$ ans sterile feed (X_0=0), $S = (K_S \cdot D/(\mu_{max} - D))$

where S is the glucose concentration.

At steady-state operation,

Substrate balance:

In – Out – Consumption = 0

$S_0 \cdot (Q) - S \cdot Q - (-r_s) \cdot V = 0$

$S_0 \cdot (Q) - S \cdot (Q) - (\mu \cdot (X)/(Y_{X/S})) = 0$

The dilution rate,

$D = 1/\Gamma = Q/V$

$X = Y_{X/S} \cdot (S_0 - S)$ where X = f(S) and S = f(D), therefore, X = f(D)

At steady-state operation,

Product balance:

$0 - P \cdot Q + (r_p) \cdot V = 0$

$P \cdot D = r_p$

Where r_p is product formation rate, P is lactic acid concentration.

$r_p = \alpha \cdot r_x + \beta \cdot X = \alpha \cdot D \cdot X + \beta \cdot X = (\alpha \cdot D + \beta) \cdot X$ according to Luedeking-Piret equation (Luedeking R., Piret E. L., 1959).

$P = (\alpha + (\beta/D)) \cdot X$

For the case of retaining biomass in chemostat (with a filter allowing liquid outflow but no biomass outflow occurs in the system),

For the practical biomass retention with biomass feedback (CSTR + recycle);

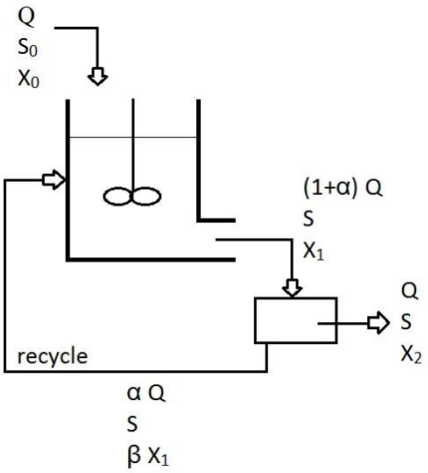

Figure 1.5 CSTR for the System with Biomass Retention with Biomass Feed-back

The feed was sterile ($X_0 = 0$), but in the recycle stream, the biomass concentration was βX_1,

X_1, X_2, and βX_1 are different from each other but substrate concentrations are the same.

Cell balance: (control volume is the whole system)

In – Out + Generation = 0

$-Q\cdot(X_2) + \mu\cdot(V)\cdot X_1 = 0$

$\mu\cdot(X_1) = (Q/V)\cdot X_2$

$\mu\cdot(X_1) = (D)\cdot X_2$

$X_2 = (\mu/D)\cdot X_1$

Cell balance: (control volume is the fermentor)

In – Out + Generation = 0

$\alpha\cdot(Q)\cdot\beta\cdot X_1 - (1+\alpha)\cdot Q\cdot X_1 + \mu\cdot X_1\cdot V = 0$

$\alpha\cdot(D)\cdot\beta\cdot X_1 - (1+\alpha)\cdot D\cdot X_1 + \mu\cdot X_1 = 0$

$\alpha\cdot(D)\cdot\beta - (1+\alpha)\cdot D + \mu = 0$

$\mu = (1+\alpha-\alpha\cdot\beta)\cdot D$

Since, β is greater than 1, the chemostat can be operated at dilution rates that are higher than the specific growth rate when cell recycle is used.

Substrate balance:

In – Out – Consumption = 0

$(Q\cdot S_0 + \alpha\cdot Q\cdot S) - (1+\alpha)\cdot Q\cdot S - (\mu\cdot X_1/Y_{X/S})\cdot V = 0$

Where $S = (\mu \cdot K_S)/(\mu_{max} - \mu) = (K_S \cdot (1 + \alpha - \alpha \cdot \beta) \cdot D)/(\mu_{max} - D \cdot (1 + \alpha - \alpha \cdot \beta))$

$D \cdot S_0 + \alpha \cdot Q \cdot S - (1 + \alpha) \cdot Q \cdot S - (\mu \cdot X_1/Y_{X/S}) \cdot V = 0$

$D \cdot (S_0 + \alpha \cdot S - ((1 + \alpha) \cdot S) = \mu \cdot X_1/Y_{X/S}$

$((D \cdot (Y_{X/S}))/\mu)(S_0 - S) = X_1$

$X_1 = ((D \cdot (Y_{X/S}))/\mu)(S_0 - ((\mu \cdot K_S)/(\mu_{max} - \mu)))$

$X_1 = ((D \cdot (Y_{X/S}))/(D \cdot (1+ \alpha - \alpha\beta)) 2(S_0 - ((D \cdot (1+ \alpha - \alpha \cdot \beta)K_S)/(\mu_{max} - D(1+ \alpha - \alpha \cdot \beta))))$

$X_1 = Y_{X/S}/(1 + \alpha - \alpha \cdot \beta) (S_0 - ((K_S(1 + \alpha - \alpha \cdot \beta) \cdot D)/(\mu_{max} - D \cdot (1 + \alpha - \alpha \cdot \beta)))$

For fed-batch fermentation,

Cell balance:

$\mu \cdot X \cdot V = d(X \cdot V)/dt$

$X \cdot (dV/dt) + V \cdot (dX/dt) = X \cdot Q + V \cdot (dX/dt)$

$dX/dt + X \cdot Q/V = \mu \cdot X$

$dX/dt = \mu \cdot X - X \cdot Q/V$ where Q/V is D

When the volume changes occur in fed-batch studies, the equations would be as follows;

$r(x) \cdot V = dC_xV/dt \Rightarrow r(x) \cdot V = V(dC_x/dt) + C_x(dV/dt)$

where $V = V_0 + (Q_0) \cdot t$

Substrate balance:

$S_0 \cdot Q - 0 - (\mu \cdot X/Y_{X/S}) \cdot V = d(SV)/dt = S(dV/dt) + V(dS/dt) = S \cdot Q + V \cdot (dS/dt)$

$dS/dt = (S_0 - S) \cdot D - (\mu \cdot X/Y_{X/S})$ (Bayındırlı, 2008)

When $\mu \cdot X/ Y_{X/S}$ is reffered as B

$dS/dt = (S_0 - S) \cdot D - B$

$dS/dt = -D \cdot S + S_0 \cdot D - B$

When $S_0 \cdot D - B$ is referred as C_1

$dS/dt = -D \cdot (S - (C_1/D))$

$(dS/dt)/(S - (C_1/D)) = -D$

$((dS/dt)/(S - (C_1/D)))dt = -Ddt$

The integral of the equation is as follows;

$\int dS/(S-(C_1/D)) = -Dt + C_2$

$\ln (S-(C_1/D)) + C_3 = -Dt + C_2$

$\ln (S-(C_1/D)) = - Dt + C_4$

Where $C_4 = C_2 – C_3$

$e^{\ln(S-(C1/D))} = e^{-Dt + C4}$

$e^{-Dt + C4} = S – (C_1/D)$

$S = e^{-Dt + C_4} + (C_1/D)$

$S = e^{-Dt} \cdot e^{C_4} + (C_1/D)$

When e^{-Dt} is referred as C_5

$S = C_5 \cdot (e^{-Dt}) + (C_1/D)$ (C_2 and C_3, therefore C_4 may be taken as 0. So, C_5 may be taken as 1)

$S = (e^{-Dt}) + S_0 – (\mu \cdot X/((D) \cdot Y_{X/S}))$ Equation 1

$dX/dt = Q \cdot (X_0 – X) + (\mu \cdot X) \cdot V$

$dS/dt = (S_0 – S) \cdot D – (\mu \cdot X/Y_{X/S})$

$dX/dt + Q \cdot X - (\mu \cdot X) \cdot V = Q \cdot X_0 = 0$

where $V/Q = 1/D$

$(dX/dt) \cdot (1/Q) + X \cdot (1-(\mu/D)) = 0$

Call $1-\mu/D$ as A

$(dX/dt) \cdot (1/Q) = – X \cdot (A) = A \cdot (–X)$

$dX/dt = Q \cdot (A) \cdot (–X)$

$dX/(–X) = Q \cdot (A)\, dt$

the equation is multiplied by -1 and the integral of this equation is as follows;

$\ln (X) = -Q \cdot (A) \cdot t$

$e^{\ln (X)} = e^{-Q \cdot (A) \cdot t}$

$X = e^{-Q \cdot (A) \cdot t}$ Equation 2

The result of equation 2 as X value is evaluated in the equation 2 for the calculations.

In one study on *Lb. casei* growth, conducted by Aguirre et. al. (1959), the biomass and lactose concentration profiles in batch experiments were analyzed in order to obtain the experimental biomass production rate (d[X]/t) and the lactose consumption rate (d[S]/dt). A first order growth kinetics was assumed;

$r(x) = d[X]/dt = \mu \cdot [X]$

In order to calculate μ for each time interval, the biomass concentrations were fitted in an equation as follows;

$[X] = \alpha + \beta \cdot t \cdot \gamma \cdot t^2 + \sigma \cdot t^3$

Differentiating:

$r_x \cdot d[X]/dt = d[\alpha + \beta \cdot t + \gamma \cdot t^2 + \sigma \cdot t^3]/dt \cdot \beta + 2\gamma \cdot t + 3 \cdot \sigma \cdot t^2$

Therefore, μ can be calculated from this formula;

$\mu = r_x \cdot [X] = d[X]/dt/[X] = (\beta + 2 \cdot \gamma \cdot t + 3 \cdot \sigma \cdot t^2)/[X]$

(similar calculations were preferred for substrate profiles)

Product concentrations as low as 1 g/L were found to severely affect the specific growth rate (μ). Their results also enlighted that non-diluted whey with no suplemention can enhance high probiotic biomass growth. The fed-batch growth was observed as cost-effective with good growth performance.

In the study of Guerra et. al. (2010), it was underlined that different models, such as Logistic and modified Gompertz Equations, as well as the Monod model have been generally used to describe different microorganisms' growth in batch cultures. On the other hand, the classical Luedeking and Piret expression (Luedeking & Piret, 1959) has been used to describe the time course of the production of growth-associate (primary), nongrowth-associate (secondary), or mixed products.

However, these referred models have been found to be inadequate for describing the production of biomass and antimicrobial products in realkalized fed-batch fermentations. Other models were developed.

Fed-batch fermentations were carried at 30 °C in a 6 L bench top fermentor, at 200 rpm, an aeration flow rate of 0.5 L/h. The fermentations were initially batch fermentations, after 12 hours, when lower pH value is reached, the batch fermentations were converted into repeated fed-batch and realkalized mode by rapidly withdrawing a volume of 100 mL of the culture from the fermentor.

After determination of the total sugar concentration in the sample withdrawn, the medium pH was arranged to 7.0 with 5M NaOH. In the first realkalized fed-batch culture (fed-batch fermentation I), the fermentor was fed with a 400 g/L concentrated lactose and sterile distilled water (if needed). In the second fed-batch culture (fed-batch fermentation II), the feeding substrate consisted in a mixture of a 400 g/L concentrated lactose and CW (concentrated whey) medium. Both batch and realkalized fed-batch cultures were started with a 2% (volume/volume) inoculum of overnight fermented cultures.

The culture supernatants were analyzed in order to determine total sugars, phosphorous, nitrogen, lactic acid, acetic acid, ethanol, and butane-2,3-diol.

A paired-samples t-test was used in order to figure out whether significant differences ($P < .05$) existed between the mean concentrations of biomass and antimicrobial substances produced in the batch and the realkalized fed-batch cultures. The same statistical test was used to compare the values of the parameters obtained after modelling separately the first and the second growth pulses observed in the realkalized fed-batch fermentations. The higher concentrations of biomass (0.24 g/L) and the highest final viable cell concentrations (9×10^8 cfu/mL) were produced at an initial pH value of 7.0 after 12 h of incubation. Afterwards, the biomass and lactic acid production and nutrient consumption rates were found to decline. An alternative way for increasing the productions of biomass and antimicrobial products of *E. faecium* in whey was preferred as to increase the period in which the cells are active. For this sake, after 12 hours of fermentation, a 400 g/L lactose solution was fed into the fermentor. Compared with the batch cultures, this fed-batch fermentation technique had proposed an increase in the metabolically active period of the cells.

The maximum specific growth rate and the specific death rate were determined by linear regression from the plots of ln OD_{600} versus time. The Gompertz equation as previously described were used to calculate lag phase duration, exponential growth rate, generation time and maximum population density (ICMSF, 1996).

1.6. Fermentation in a Membrane Bioreactor

There are a number of studies for lactic acid bacteria fermentation by using membrane bioreactors focusing on biomass growth or product formation. In the study of Puzanov (1999) with *Lb. rhamnosus*, the contents of the reaction vessel were continuously pumped through membrane module and recycled back to the vessel and the total volume of the system was maintained as constant by equalizing the incoming feed flow rate to the product flow rate. The expected result from the system was pointing out high dilution rates, and high productivity. A microfiltration membrane module was connected to fermentor. There were two peristaltic pumps for the feed and permeate flow control. Cell evacuation was enhanced with another peristalthic pump.

After the inoculation was done, the system was left in a batch mode for 7 hours and then, in continuous mode for 17 hours at a dilution rate of 0.3 h^{-1}. The three process variables were chosen to be dilution rate, initial substrate concentration and agitation rate in the system. The samples were taken every 2-3 hours and were analyzed for biomass concentration, residual substrate concentration and lactic acid concentration. At the end of the fermentation, permeate flux was measured to examine membrane fouling.

It was shown that initial glucose concentration was crucially affecting biomass production, lactic acid concentrations and glucose conversion. It was also observed that, in the presence of excess nutrients, the biomass was lower (16.5 - 29.8 g/L vs. 29.0 - 35.6 g/L respectively). Higher lactic acid content inhibited microbial growth to a greater extent. The dilution rate's effect on biomass growth was weaker than the initial glucose content but, statistically, it was found to be still significant.

The study of Jung and Lovitt (2010) was held at pilot-scale, for the growth of lactic acid bacteria. Firstly, batch fermentation was held within 3 L medium in a 5 L stirred tank reactor. The analyzed values were maximum growth rate, biomass growth rate and volumetric biomass production rate. The perfusion culture of lactic acid bacteria was performed in membrane cell recycling reactor, externally connected to ceramic filtration membranes; one for feed sterilization, another one for perfusion of spent medium and another for cell retention.

Four types of LAB were used in the experiments. One of the microorganisms, *O. oeni*, that was inoculated by 10% (v/v) of the main culture and the other ones were inoculated by 5% (v/v) in STR and MBR. When the fed glucose amount was increased, the biomass was found to be inhibited due to produced lactic acid's effect of inhibition and the biomass yield decreased. In STR, for *Lb. bucheri*, μ_{max} was 0.2 h^{-1} and the biomass was found to be 1.83 g/L, the consumed glucose was 55 g/L in 30 hours with $Y_{x/s}$ (yield) = 0.007. For the *B. longum*, μ_{max} was found to be 0.28 h^{-1}, biomass was 4.04 g/L after 11 hours of fermentation. In MBR, for *Lb. brucheri* growth, 16.2 DCW g/L, a growth rate of 0.074 h^{-1} were found and the dilution rate was slowly increased from 0.05 h^{-1} to 0.83 h^{-1} (the concentrations of acid (all the inhibitory compounds) was kept below 150 mmole/L). Within the *B. longum* fermentation, the biomass was 22.2 g DCW/L for 11 hours of fermentation, the dilution rate could not be increased, over 0.57 h^{-1}, due to membrane fouling by extracellular products at the product membrane. The ceramic membranes were referred to be used. The fouling of the ceramic membranes had increased with increased stirring rate. The μ_{max} decreased greatly from STR to MBR, however, in MBR, the final biomass concentration (X) and volumetric biomass production rates ($P_{x/t}$) were greatly improved when compared to STR system. In MBR, *Lb. bucheri*'s μ_{max} decreased to 65 %, whereas for *B. longum*, μ_{max} decreased to 54 %. Within these fermentations, concentrations of organic acid and ethanol in MBR were still high allowing growth inhibition. The inhibitory organic acids and ethanol

end products inhibition were continuously removed through the product membrane. Volumetric biomass production, increased to a very great content in the MBR from an average value of 5.46 to 28.86 times and the total biomass increased 5.5 times to 23.7 times.

The MBR was designed using a 26 mL main vessel, 10 L pipe line of stainless steel, heat-exchanger, 2 centrifugal coupled magnetic pumps to control flowrate, 2 membrane filters, product membrane of 1 m^2 effective area and a feed membrane of 0.1 m^2 effective area.

In the study of Ramchandran et. al. (2012), fermentation by a submerged membrane was investigated and a whey-based medium with yeast-extract (5 g/L), skim milk powder (5 g/L), lactose (16.7 g/L) and whey powder (16.7 g/L) was suggested. The medium which is used for the backwashing of the membrane module was a solution of yeast extract and lactose possessing the same concentrations as whey-based medium. Firstly, % 0.071 (v/v) inoculation was made and fermentation was conducted for 30 hours. While the cell counts in the batch fermentation decreased towards the end of the fermentation process, for fermentations held with the membrane system, there was no difference in the counts.

The backwash was found enormously advantageous as well as using fresh medium for backwash instead of membrane permeate, preventing the return of the inhibitory products into fermentor, and also feeding yeast extract lactose medium which increased the counts 1.6 times. Therefore, this method strengthened the system by turning to continuous medium removal followed by replacement also with whey-based medium until 12 h fermentation. This process had increased the counts more, by 2.2 times. Using yeast extract with higher lactose (160 g/L) in backwashing step had increased to a much greater extent of 2.4 times.

It was estimated that a submerged membrane system must at least maintain 5 folds of increase in viable cells. Alkali consumption was a direct indicator of acid production which indirectly indicated metabolic activity in the fermentor.

Within the fermentor, at the first step, membrane only removed 1/2 - 1/3 of the lactic acid produced. At the later step (after 18-24 hours), membranes were observed to remove more lactic acid than produced by cells. Achieving the right balance between nutrient concentration in the replacement medium and removal of the membrane permeate was critical.

1.7. Quorum-Sensing

For the growth of microorganisms, one issue to mention could be quorum-sensing which is defined as communicating of the bacteria with a signaling system that causes controlling of the behavior of the total bacteria. This control mechanism is maintained by usage of the secreted signaling molecules (Henke & Bassler, 2004). There are studies pointing out that some bacteria, which block the quorum sensing of bacteria competitors by producing enzymes, inactivate the signaling system (Defoirdt, et. al., 2004). Such microorganisms may have great potential to block pathogenic diseases in aquaculturing by excreting antagonistic compounds. For example, for *A. hydrophila* and *A. salmonicida* species, a connection has been found between virulence factor and quorum sensing (Swift, et al., 1997). Quorum-sensing may also effect a probiotic's capability to produce compounds that are beneficial to host.

1.8. Aim of the Study

In Turkey, intensive culturing of rainbow trout (*O. mykiss*) has been practiced. Therefore, the sector's economical value is recognized and probiotic usage in trout farming sector will contribute to healthy

fish production to be proposed to human consumption. In probiotic usage, the most important phases are: isolation of the potential probiotic strains, feeding the target fish with the condense probiotic and observing that they do not harm the healthy fish, their safety should also be proven by in vitro methods. Afterwards these microorganisms will, economically, be grown and dried to be ready for regular usage in fish farms. As future study, the probiotics will be tried in the fish farms.

This project aims to figure out the favorable and economical growth conditions for isolated *E. faecium*, as a potential probiotic for rainbow trout aquaculturing. In this study, the effect of varying mediums, parameters and continuous filtration are conducted and the best yields, growth rates and most importantly, biomass amounts are calculated.

CHAPTER 2

MATERIALS AND METHOD

2.1. Chemicals

All the experiments were made by using the chemicals of analytical grade and commercially provided from Sigma and Merck. Whey was kindly provided by ACKAR Dairy Company at Kazan, Ankara.

2.2. Organism

Two *E. faecium* strains were used in this study. One was isolated from the industrial product, Biotexin-L and the other strain was isolated from turkey droppings. The first one was used as the model organism and was only used in the initial studies, where growth media and the optimum temperature and pH values were tested. Fermentor runs, except for initial studies on optimum temperature and pH, were conducted using the second strain. It was activated on GPY (2% Glucose, 0.5% Peptone, 0.5% Yeast Extract) agar that was incubated at 37 °C overnight. Later, the plate was kept at 4 °C and also the isolated microorganisms were stored in 15% glycerol at -20 °C to be later activated and used in GPY broths and in other referred media.

2.3. Culture Conditions

E. faecium was cultivated in shake flasks and in bioreactor. In bioreactor, batch and fed-batch fermentations were conducted by inoculation of microorganism, approximately at a concentration of 1 x 10^6 cfu/L. For the last experiments, the bioreactor was integrated with a membrane filtration unit and fresh medium was continuously fed. Also, a backwashing step's effect was experimented for this membrane filtrated system. Constant volume was maintained in the system.

2.4. Preculture of Fermentor

E. faecium was grown in the same medium, glucose peptone yeast extract (pH=6-6.5). The inoculation level was 2% (volume/volume). The cultures were inoculated at 37 °C. The batch fermentation medium was composed of glucose (2%, 5%), yeast extract (0.5%), peptone (0.5%). The fermentation was done in 3 L fermentor with a working volume of 2 L. Temperature, pH and stirring speed control was done throughout fermentations and air was supplied only for one set of experiments.

2.5. Preparation of the Fermentor

As mentioned above, the working volume was 2 L, in 3 L fermentor (Biyotronik, Turkey). Temperature, pH, dissolved oxygen level and stirring rate may be controlled during fermentation in this bioreactor. Throughout the study, pH was kept at 6.5 via adding 5 N NaOH by a peristaltic pump that was syncronized with the pH controller. pH probe was calibrated with commercial standard buffer solutions at pH 9, 7 and 4. The temperature was kept at 37 °C during the whole process. For the batch experiments with membrane filtering step at the end, % 88-100 oxygen level within the fermentor was maintained. Fermentor was sterilized by autoclaving at 121 °C for 15 minutes. The samples were taken periodically and kept under adequate conditions for analysis after the OD value was measured at 600 nm using a spectrophotometer. Also, plate counting, dry cell weight calculation and counting via Thoma chamber were conducted.

2.6. Batch Fermentation

Batch fermentations were performed either in shake flasks or in the 3 L fermentor. Glucose was initially added with yeast extract and peptone, then, the consumption of the glucose was measured. The initial experiments conducted in shaken Erlenmeyer flasks and the batch experiments for figuring out the optimum growth temperature and pH were conducted by using the isolate from the industrial product, Biotexin-L, whereas, all the rest of the batch, fed-batch and continuously membrane-filtrated experiments were conducted by the other strain isolated from the turkey droppings.

For one set of experiments, at the end of the fermentation, the product was filtrated (via ceramic membrane filter, pore size 1 micrometer, effective area 0.566 m^2) and pumped back into the fermentor to serve as the inoculation material for the next fermentation that was held under the same conditions and within the same medium of the previous fermentation.

2.7. Fed-Batch Fermentation

After 2% inoculation (v/v) of the preculture into the fermentor, feed media that consisted of glucose at varying amounts were continuously added for required times (until the growth comes to the stationary phase). The total lactic acid produced and the glucose consumed were calculated. Results were used to calculate the product yields, glucose consumption yields.

Fed-Batch Fermentation with Continuous Feeding of Fresh Medium and Membrane Filtration with Maintained Constant Volume

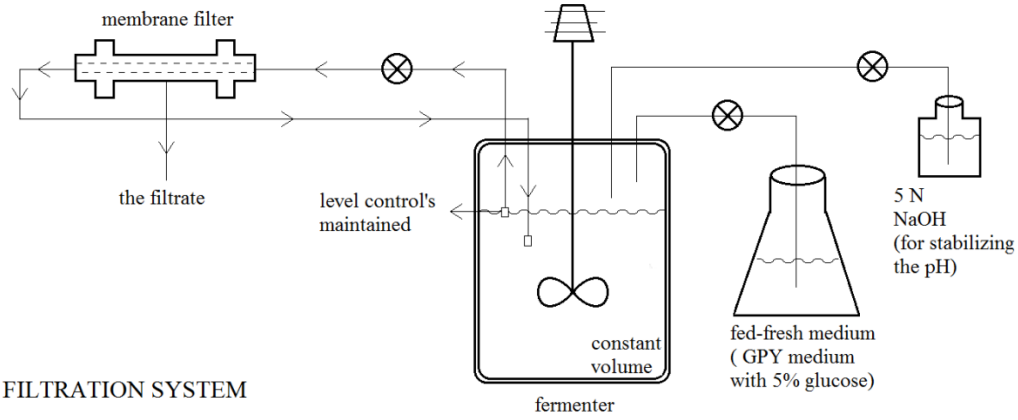

Figure 2.1 The System with Membrane Filtration Unit and Feeding of the Fresh Medium (GPY with 5% glucose content)

In this system, the fermentation medium and conditions were the same as the previous fermentation processes, The filtration unit is externally attached to the fermentor on two sides, one for feeding the condensed solution back into the fermentor (containing the microorganisms that are separated from the medium taken away to be filtered) and one side for taking the medium to be filtered via a peristaltic pump (Watson Marlow pump, 101U-R, with max 32 rpm, speed: 1200 ml/hour, during the fermentation process, to maintain constant volume for changing filtration performance, increased to 1800 ml/hour). This medium taking side is arranged to a level that is nearly 1 L (actually, nearly 1.2 L, the equipment was not suitable for arrangement at exactly 1 L). By this, level control was maintained for filtering the fermentation medium. The fresh GPY (Glucose 5%, Peptone 0.5%, Yeast Extract 0.5%) medium was arranged to be fed at a rate of 90 ml/hour via a peristaltic pump (Masterflex, Model 7013-21). The inoculation was done by 2 % (v/v) as before and batch fermentation was initiated. To increase the fermentation rate, during the fermentation process, after a time, the diameter of the pipe line that goes through the pump ejected to the filtration unit was increased, the filtration unit's position was changed from vertical to horizontal line, the diameter of the the pipe line that was just at the enterance of the filter line, to the fermentor, which was feeding the condense solution with microorganisms back into the fermentor was decreased so the pressure was increased and therefore, the flow rate at that point, was changed and the rpm was increased to 350.

All the fermentation results were evaluated for the yield differences, maximum biomass and specific growth rates. Product yield was the lactic acid produced per total glucose consumed (g product per g glucose). The biomass yield was the difference of the biomass that is measured at the end and at the start of the continuous culture per total glucose consumed. The dry biomasses were determined from a standard curve of dry cell weights vs. OD values, and the OD values were observed at 600 nm with spectrophotometer.The specific growth rates were calculated and compared.

Fed-Batch Study of with Continuous Feeding of Fresh Fresh Medium and Level-Controlled Membrane Filtration, Together with Backwashing with Fresh Medium

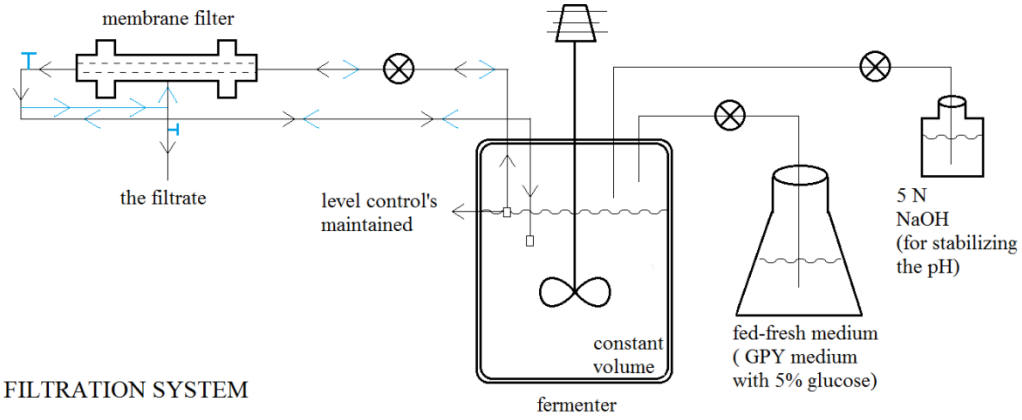

Figure2.2 The System with Mebrane Filtration Unit, Feeding of the Fresh Medium (GPYwith 5% glucose content) and Backwashing Pathway

In this system, there is an additional pipe line maintaining backwashing of the membrane filter, into the fermentor with reverse pulse of the peristaltic pump (Watson Marlow pump, 101U-R, with max 32 rpm, speed: 1800 ml/hour) that the filtering system is attached to. The fresh medium is fed via a peristaltic pump (Masterflex, Model 7013-21) with a speed of 134 ml/hour. The fermentation process was conducted under the same conditions of temperature, pH. The rpm was initially set to 200 but after 6 hours of fermentation, when the filtration and fresh medium feeding have initiated, the stirring speed was raised to 350 rpm. The membrane filtration and fresh medium feeding continued together, for another 6 hours. At the end of around 12 (between 12 and 12 and a half hours) hours of fermentation, the backwashing of the membrane was firstly done for 10 minutes after every two hours. The backwashing system was excluded from the system after additional 14 hours. The outlet pipeline of the filtration unit was constricted periodically according to the loss of filtration performance, in order to cause pressure (therefore, flow rate) difference and affect filtration.

2.8. Analytical Methods

The fermentation media taken periodically were centrifuged at 12000 g for 10 minutes. Pellets were discarded and the supernatants were kept within HPLC solution at -18 °C for further HPLC studies. Samples were thawed at -4 °C, diluted with HPLC eluent and analyzed for lactic acid and glucose content by HPLC (LKB, USA). An organic acid analysis column was used. The column was kept at 50-65 °C and was eluted with 5 mM H_2SO_4. For the last experiments with filtration unit attachment, Varian Metacarb 87C column was used and the elution solution was dilute water.

The detector cell was kept at 35 °C. The known concentrations of glucose and lactic acid were used as standard solutions in the preparation of the standard curves.

2.9. Dry Weight Determination

The dry weight of the *E. faecium* was determined as follows; 1 mL of the sample enhanced from fermentation was centrifugated and the supernatant was removed and the remained pellet was washed once with 0.85% NaCl solution. The washed pellets are, then, placed in an oven at 100 °C and dried for 24 hours (until the weight remained constant).
The fermentation biomass' dry cell weight was found using a standard curve and the cells are centrifugated at 12000 rpm for 15 minutes, at 4 °C, washed twice with saline (0.8% NaCl).

2.10. D-lactic Acid Analysis

The lactic acid analysis was done with Megazyme D-lactic acid assay kit. In the light of Megazyme D-lactic acid (D-lactate) Assay Procedure manual book, (Megazyme K-Date 12/12, 2012), manual assay procedure was conducted.

The quantification of D-lactic acid is reached by two enzyme reactions.

$$\text{D-Lactate} + NAD^+ \underset{\longleftarrow}{\overset{\text{(D-LDH)}}{\longrightarrow}} \text{pyruvate} + NADH + H^+$$

Where D-LDH is D-lactate dehydrogenase. D-lactic acid is oxidized in the existence of NAD^+. Next, a second reaction is required to "trap" the pyruvate product.

$$\text{Pyruvate} + \text{D-glutamate} \xrightarrow{\text{(D-GPT)}} \text{D-alanine} + \text{2-oxoglutarate}$$

Where the D-GPT is D-glutamate-pyruvate transaminase. With this enzyme, the pyruvate is conversed into D-alanine and 2-oxoglutarate, in the presence of excess D-glutamate.

The amount of formed NADH is stoichiometric with the D-lactic acid amount present. Therefore, NADH is measured by the OD difference at 340 nm.

The analysis was done at 340 nm wavelength, room temperature, 1 cm light path. Three solutions are used including buffer, NAD^+ and D-GPT and the absorbance (A_1) is read. Then, another suspension is mixed, containing D-LDH and the absorbance (A_2) is read.

$A_2 - A_1$ is determined for blank and the sample. The sample's absorbance difference with the blank absorbance gives the absorbance difference showing D-lactic acid ($A_{\text{D-lactic acid}}$).

$$C = [(V \cdot MW)/((\varepsilon) \cdot (d) \cdot (v))] \cdot \Delta A_{\text{D-lactic acid}}$$

Where C is the concentration of D-lactic acid, V is final volume (mL), MW is molecular weight of D-lactic acid (g/mol), ε is extinction coefficient of NADH at 340 nm (6300 (lx(1/mol)x(1/cm))), d is the light path (cm) and v is sample volume (mL). When all the known values are put in the equation, the concentration calculation is as follows;

$$C = 0.3204 \cdot \Delta A_{\text{D-lactic acid}}$$

All the samples for growth curves were taken in triplicate and the average values were shown in the graphs, except for the last two experiments with membrane filtration.

All the HPLC analysis was done in duplicate and the average values were shown in the graphs.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Fermentation Process

In the previous studies conducted at METU Department of Food Engineering Biotechnology Laboratory, glucose yeast extract peptone (GPY) medium (2% Glucose, 0.5% Peptone, 0.5% Yeast Extract) was used with an increased pH of 8.3 and *Enterococcus faecium* strains were isolated from the industrial product Protexin-L which is a commercial powdered multi-strain probiotic that is used in animal feeding industry. The stated benefits of the Protexin is establishment of good microflora, improvement of digestibility of feed, treatment and control of scouring and diarrhea, reducing stress, stimulating appetite, suppression of *E. coli*, *Salmonella* and *Aeromonas spp*. Within Protexin-L, the following microorganisms are present *E. feacium* (that is isolated from animal and human intestines), *Lb. acidophilus* (isolated from milk, cheese, other dairy products and human intestines), *S. thermophillus* and *Lb. delbrueckii spp. bulgaricus* (isolated from milk, cheese and other starter cultures), *Lb. plantarum* (isolated from dairy products, fermenting plants, silag pickled vegetables and fermented carbohydrates), *Lb. rhamnosus* (isolated from intestinal tracts of animals, birds and humans), *B. bifidum* (isolated from alimentary tract of breast fed and bottle fed infants), *Aspergillus oryzae* (isolated from japanese soy sauce, Sake, enzyme mixture), *Candida pintolepesii* (isolated from cattle caecum, intestinal content of chinchilla and pigeons). The parameter that leads to isolation of *E. faecium* from the others is high pH due to the fact that no other microorganism in the medium can tolerate such basic pH's rather than *E. faecium* that tolerates pH values up to 9.6. The strains were isolated in Biotechnology Laboratory (METU).

Two types of isolates are used; one is the isolated strain explained above, the other is one is isolated from turkey droppings.

3.2. Effect of Media on Batch Growth of *E. faecium*

The GPY medium was chosen to be the basic growth medium containing 2% glucose, 0.5% peptone, 0.5% yeast extract that possesses main necessities of the microorganism to grow under the appropriate conditions. The *E. faecium* strain was grown in the GPY medium with varying glucose, yeast extract, peptone amounts and also with whey addition instead of glucose as carbohydrate source. The growth conditions of the strains were chosen as 37 °C, 100 rpm. This study was conducted with the isolate from the industrial multi-probiotic product, Biotexin-L.

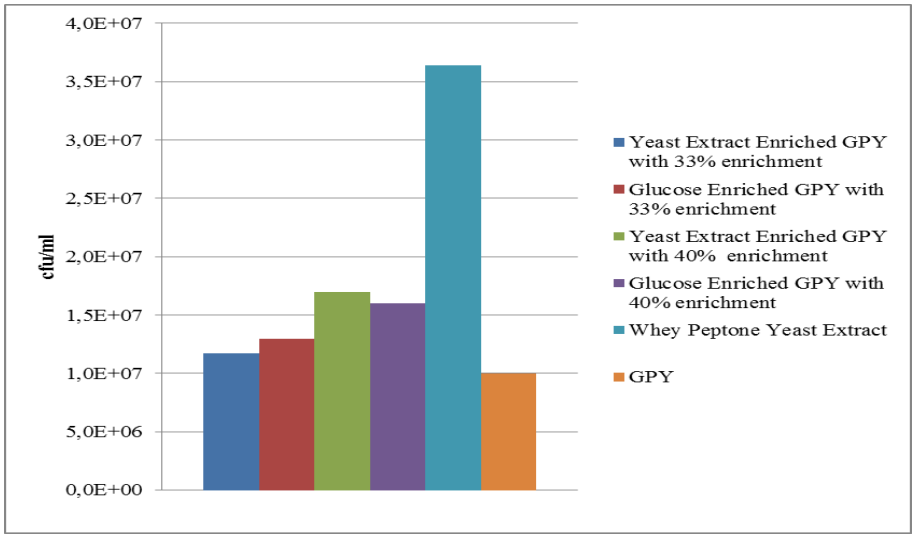

Figure 3.1 The Effect of Growth Media on the Growth of *E. faecium* Under the Same Incubation Conditions

According to the data above, it was observed that whey was a promising nutrient source for the fermentation of *E. faecium*. Especially, for the growth studies in the pilot facility, this would be taken into account. Several trials with whey were conducted but the samples' analysis was not that easy with whey, therefore its usage needs to be improved. The following data are the results of experiments with varying glucose amounts in GPY mediums. The data were obtained by using the *E. faecium* that was isolated from the industrial multi-probiotic product, Biotexin-L.

Batch fermentations in Erlenmeyer flasks in GPY media were conducted with addition of calcium carbonate in order to maintain pH near to neutral level. The results of the experiment is as follows; (This study is also conducted with the isolate from the industrial multi-probiotic product, Biotexin-L)

Table 3. 1 The Microbial Counts and pH Values of the Fermentations with $CaCO_3$ (flasks were shaken at 50 rpm, 37 C, for 20 hours)

Medium	**Number of m/o's**	**pH of the fermentation at the end**
Glucose peptone yeast ext. Broth (GPY)	1×10^7	4.46
GPY (with $CaCO_3$ in the ratio of 1/4 with glucose of the environment)	4.2×10^7	4.76
GPY (with $CaCO_3$ in the ratio of 1/2 with glucose of the environment)	1.14×10^8	5.19
GPY (with $CaCO_3$ in the ratio of 1/1 with glucose of the environment)	4.5×10^7	5.37

3.3. Temperature and pH Effect on Batch Growth of *E. faecium*

The following fermentations were held within the Fermentor (Protem, Turkey). 5 N KOH is used in order to stabilize the decreasing pH of the system at referred pH value. The inoculations were done with 1 x 10^8 strains by 2% (v/v). This study was conducted with the isolate from the industrial multi-probiotic product, Biotexin-L.

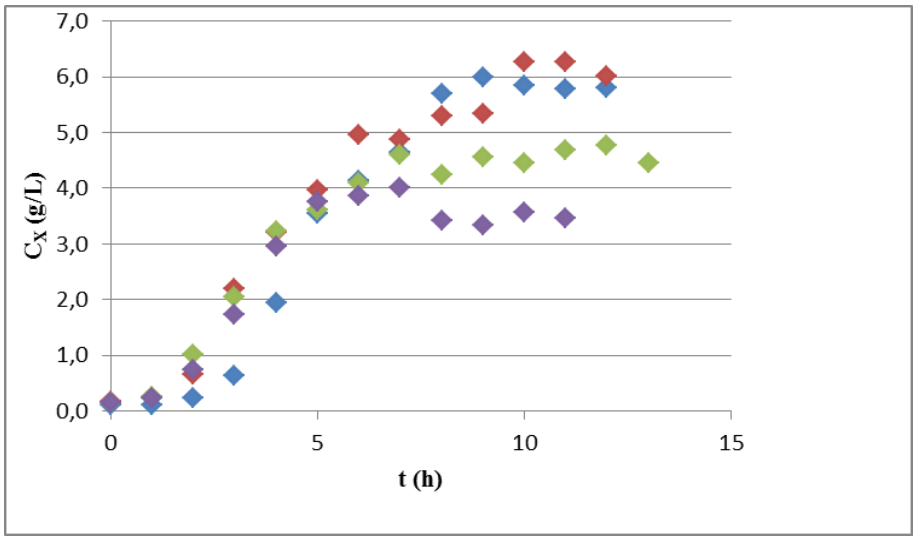

Figure 3.2 Effect of pH on the Growth of *E. faecium*. Time Course of Batch Culture at 37 ºC and 100 rpm in 1.5 L GPY Medium Adjusted to pH Values of 7(♦), 6.5 (♦), 6 (♦) pH 7.5 (♦). C_X, Biomass Concentration.

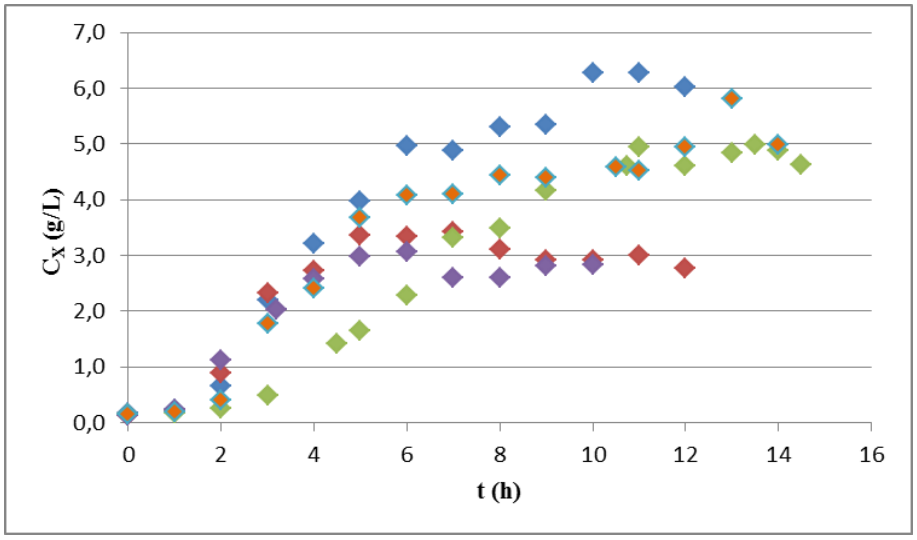

Figure 3.3 Effect of Temperature on the Growth of *E. faecium*. Time Course of Batch Culture at pH = 6.5, 100 rpm in 1.5 L GPY Adjusted to T Values of 30 °C (♦), 35 °C (♦), 37 °C (♦), 40 °C (♦), 45 °C (♦). C_X, Biomass Concentration.

As seen in the two graphics above, the best growth conditions for the strain were found to be 37 °C and pH 6.5 at 150 rpm. The counts reached up to 1 x 10^8 cfu/mL, at pH 6.5 and at 37 °C. The data were parallel to referred values in the literature. The data were obtained by using the *E. faecium* that is isolated from the industrial multi-probiotic product, Biotexin-L.

All of the following data were obtained by using *E. faecium* that was isolated from turkey waste.

In the pilot-scale production trial, DCW was observed to be 3.2 g/L in basic GPY medium with temprature and pH control, at 37 °C and pH 6.5.

3.4. Batch Study of Two Defined Mediums

The next approach was to increase the glucose content of the GPY medium from 2 % to 5 % (from now on, GPY referred contained %5 Glucose) and by this way, observe an enhanced growth. One batch study was conducted by controlling the pH at the optimum value, 6.5, whereas the other fermentation was conducted without pH control. All the studies including this one and the ones explained below, are conducted with the strain that was isolated from the turkey droppings. As it is seen in the graph below and all other studies, the experiments with the isolate from the industrial product, Biotexin-L ended up with higher growth. Due to the fact that, it is already a chosen and studied strain that turned into an industrial product for animal feeding, rest of the studies of the thesis were conducted with the new strain isolated from turkey droppings.

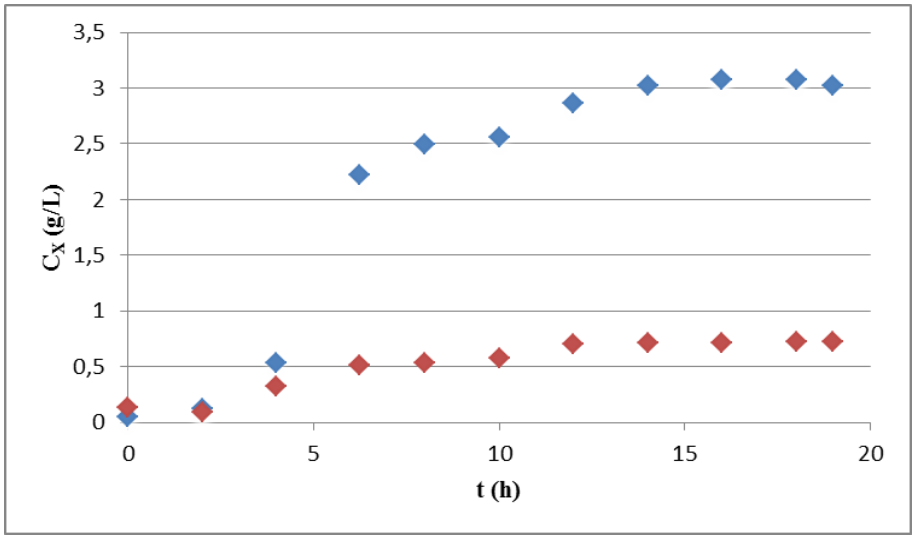

Figure3.4 Growth for 5% Glucose, 0.5% Peptone, 0.5% Yeast Extract Medium for pH Control (♦) and Uncontrolled pH (♦). C_X, Biomass Concentration.

The medium was 5% Glucose, 0.5% Peptone and 0.5% Yeast Extract under 37 °C and 150 rpm. For the pH controlled fermentation, the pH was 6.5.

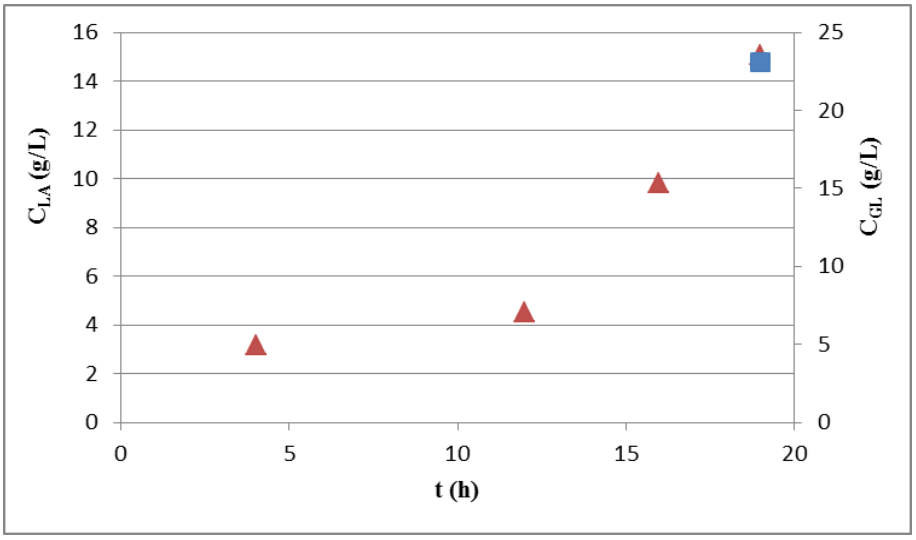

Figure3.5 Lactic Acid Production (■) and Glucose Consumption (♦) for 5% Glucose, 0.5% Peptone, 0.5% Yeast Extract Medium for the Fermentation with pH Control. C_{LA}, Lactic Acid Formation; C_{GL}, Glucose Level.

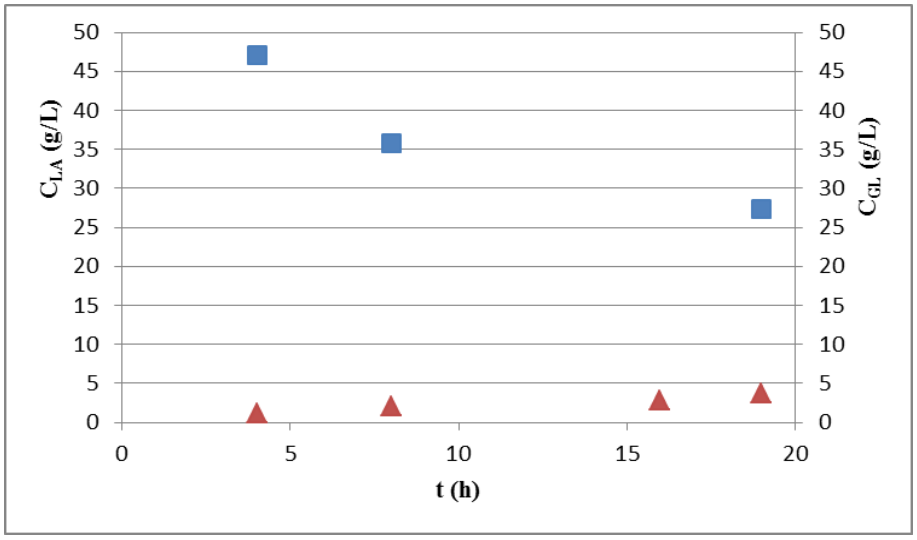

Figure 3.6 Lactic Acid Production (▲) and Glucose Consumption (■) for 5% Glucose, 0.5% Peptone, 0.5% Yeast Extract Medium for the Fermentation without pH Control. C_{LA}, Lactic Acid Formation; C_{GL}, Glucose Level.

The lactic acid produced by homofermentative *E. faecium* was referred as L-lactic acid in the literature. As a result of the analysis conducted in Ankara University, with the D-lactic acid kit (Megazyme D-LA (D-Lactate)), this information was confirmed. The analysis showed that no D-lactic acid existed in the last sample of 24 hours of fermentation process. It was concluded that all lactic acid produced was L-lactic acid.

It was obvious that the growth was much more favored with pH control as observed before. The growth was 4 times higher than the fermentation without pH control. For the experiment under the same conditions but without pH control, the biomass yield (g biomass/g consumed glucose) was found to be 0.032 and the lactic acid yield was found to be 0.65 whereas for the pH controlled experiment under the same conditions, the biomass yield was found to be 0.11 and the product yield was found to be 0.52**.** The DCW (g/L) for the batch fermentation with pH control was 3.4 g/L.

3.5. Fed-Batch Study of Media with Varying Fed Substrate Concentrations

One of the main reasons inhibiting the growth of the strain was high carbohydrate concentration within the fermentation medium. This would cause, by the time, accumulation of inhibitory compounds that are produced (in the case of *E. faecium*, lactic acid). The production of inhibitory products, instead of biomass would be favored (overflow metabolism). The next fermentations were conducted, in the light of this idea. The target was to increase amount of the substrate consumed for biomass production, instead of lactic acid formation. In these fermentations, the initial fermentation media were the same and composed of 5 % glucose and 5 % peptone. From the beginning of the fermentations, at the same time of the inoculation, glucose solutions with varying concentrations were fed continuously to the fermenting media, very slowly.

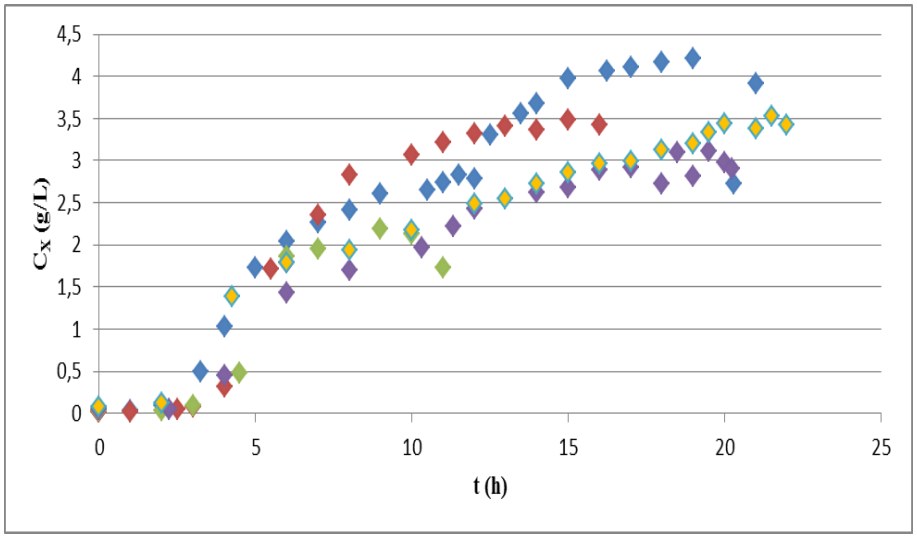

Figure 3.7 Effect of Growth Medium Composition on Growth. C_x: Biomass Concentration. (Fermentations were conducted in the fermentor with a 2 L working volume at 150 rpm, at 37 °C and pH 6.5)

(♦): 1. GPY Medium, pH: 6.5, rpm: 150, 2 L Fermentation, T: 37 ◦C, Batch,
(♦): 2. Peptone Yeast Extract Medium, Continuous 60 g/L Glucose Feeding with a Rate of 8 mL/h,
(♦): 3. Peptone Yeast Extract Medium, Continuous 50 g/L Glucose Feeding with a Rate of 10 mL/h,
(♦): 4. Peptone Yeast Extract Medium, Continuous 67 g/L Glucose Feeding with a Rate of 5 mL/h,
(♦): 5. Peptone Yeast Extract Medium, Continuous 100 g/L Glucose Feeding with a Rate of 5 mL/h.

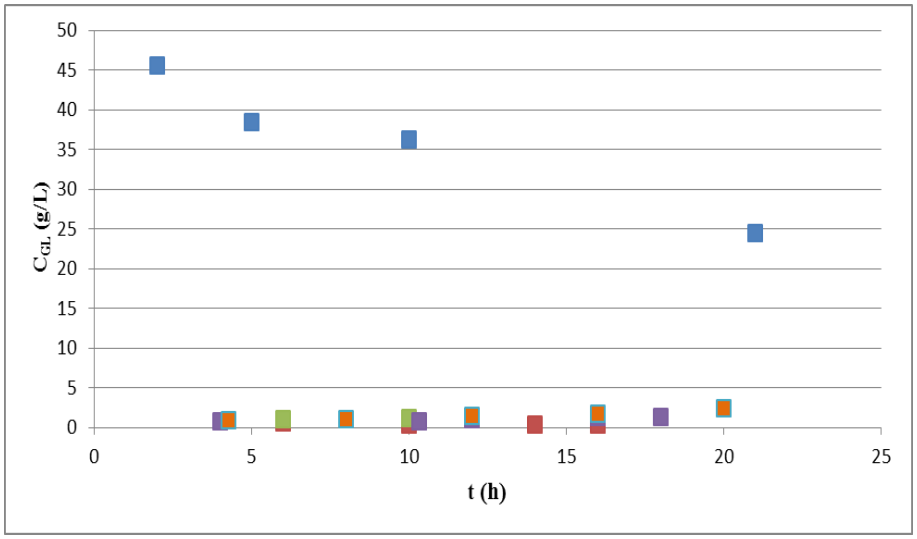

Figure 3.8 Glucose Level in the Defined Media 1 (■), 2 (■), 3 (■), 4 (■), 5 (■). C_{GL}, Glucose Level.

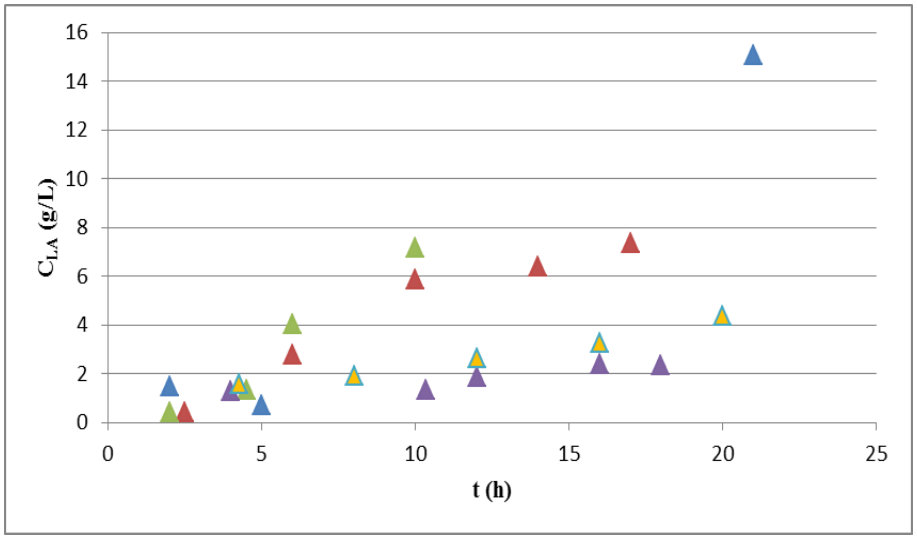

Figure 3.9 Lactic Acid Production in the Defined Media 1 (▲), 2 (▲), 3 (▲), 4 (▲), 5 (▲). C_{LA}, Lactic Acid Formation.

The volume changes caused by the feeding were considered in the calculations. The comparison of biomass yield for these media with varying amounts of glucose fed into the medium was done. The results were as follows; for the experiment 1, the biomass yield (g/g) is found to be 0.15 whereas for the experiment 2, it was found to be 0.53, for the experiment 3, it was 0.34, for the experiment 4 it was 0.35 and lastly, for experiment 5 the biomass yield was found to be 0.39. Also, the product yields (g/g) were compared; for the experiment 1; 0.59, for the experiment 2; 1.16, for the experiment 3, it is 1.46, for the experiment 4 it was 0.32 and for the experiment 5 it was 0.55. The maximum biomass was reached within the experiment 5 and 2 (Dry cell weight: 3.42 g/L), among the fed-bacth fermentatşons. This alternative semi-batch system may be representing a good economical solution for bigger pilot-scale production and later for the industrial production. The maximum biomass yield was from experiment two, an optimization would be necessary, in the future studies, between glucose consumption and biomass production. When the specific growth rates were compared, the values were as follows;

Table 3. 2 Specific Growth Rates in the Defined Fermentations 1, 2, 3, 4, 5

	1	2	3	4	5
$\mu(h^{-1})$	0.12	0.26	0.16	0.15	0.11

As it can be seen, the specific growth rate was the lowest in experiment 5 which maintained maximum biomass amount among the fed-batch fermentations.

All the OD values were maintained in triplicate whereas the HPLC analyses were done in duplicate.

3.6. Batch Study with Membrane Filtration at the End of the Experiment and Effect of Inoculation Amount on *E. faecium* Growth

The *E. faecium* is known to be facultative anaerobic microorganism. Although, from the very beginning, the approach was that the oxygen would not favor the growth of the microorganisms, oxygen effect on the microorganism was also observed. This set of experiments were conducted by maintaining continuous oxygen and keeping the oxygen level in the fermentation medium average values of dissolved oxygen 93-103 % by continuously feeding oxygen through 0.2 µm filter. The stirring rate was raised to 300 rpm for better oxygen mass transfer rate, under the same conditions as 37 °C and pH 6.5.

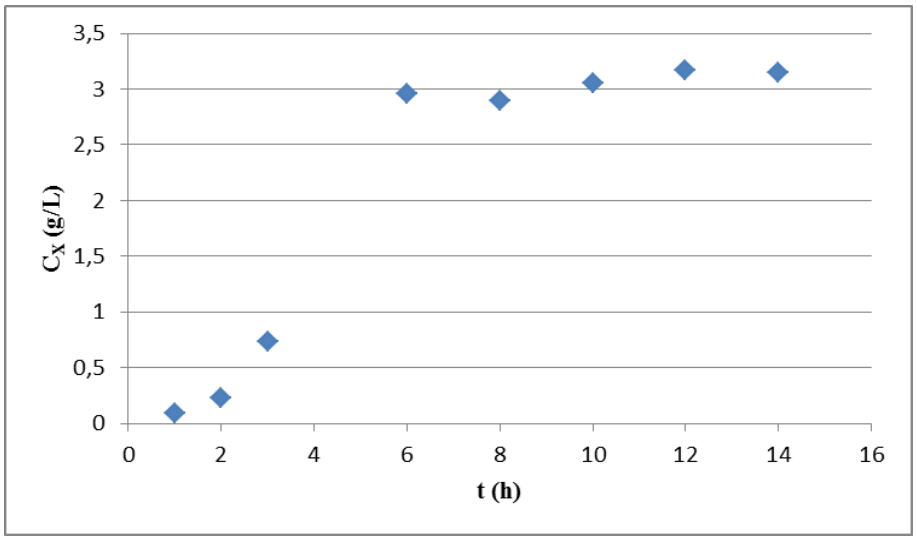

Figure 3.10 First Batch Fermentation with Continuous Oxygen Feeding. C_X, Biomass Concentration.

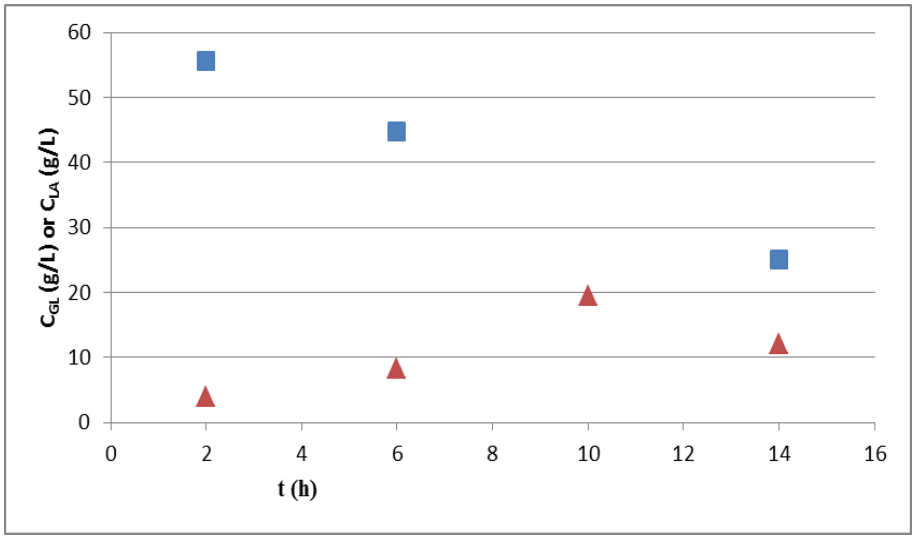

Figure 3.11 Total Glucose Level (■) and Lactic Acid Production (▲) of the Initial Batch Fermentation with Continuous Oxygen Feeding. C_{LA}, Lactic Acid Formation; C_{GL}, Glucose Level in the Fermentor.

When compared with the fermentation under the same conditions but without aeration, high oxygen level and oxygen feeding were found to be insignificant. After the fermentation was finished, the medium with *E. faecium* was filtered with an externally connected ceramic filtration membrane which maintained filtration of the medium hence, condensing the microorganism about ten times. This new condensate was evaluated as the inoculation of the next fermentation under the exactly same conditions.

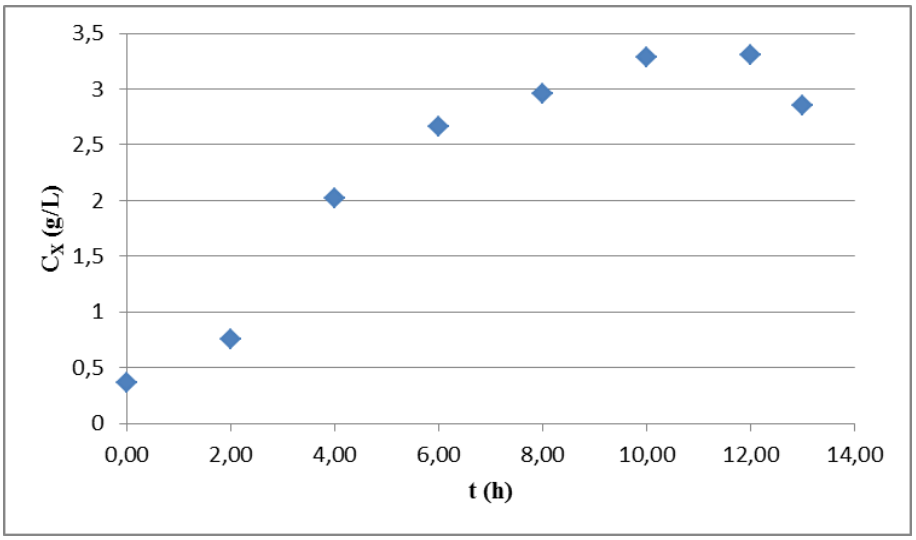

Figure 3.12 Second Batch Fermentation with Continuous Oxygen Feeding. C_x, Biomass Concentration.

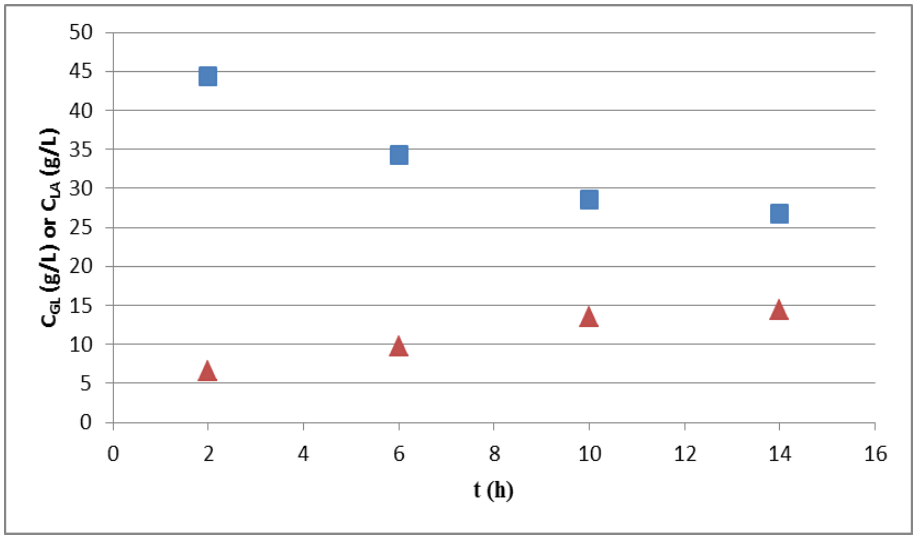

Figure 3.13 Total Glucose Consumption (■) and Lactic Acid Production (▲) of the 2. Batch Fermentation with Continuous Oxygen Feeding. C_{LA}, Lactic Acid Formation; C_{GL}, Glucose Level.

The high inoculation to the fermentation medium was expected to end up with higher biomass growth. At the end of the experiment, it was observed that the biomass gained was slightly different than the previous experiment. The fact that the inhibitory organic compound produced by *E. faecium* and that remained in the fermentor was limiting the growth and this trial was not enough to enhance the growth. Still, the cyle was continued with a third step of fermentation after, again, the medium with *E. faecium* was filtered with an externally connected ceramic filtration membrane which maintained filtration of the medium, condensing the microorganism ten times and letting this condensate to be the next fermentation's inoculate.

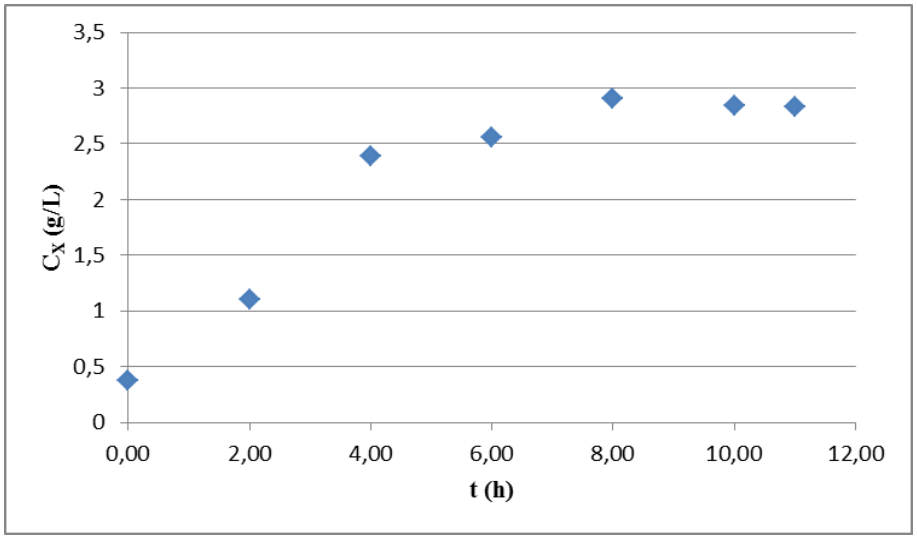

Figure3.14 Third Batch Fermentation with Continuous Oxygen Feeding. C_X, Biomass Concentration.

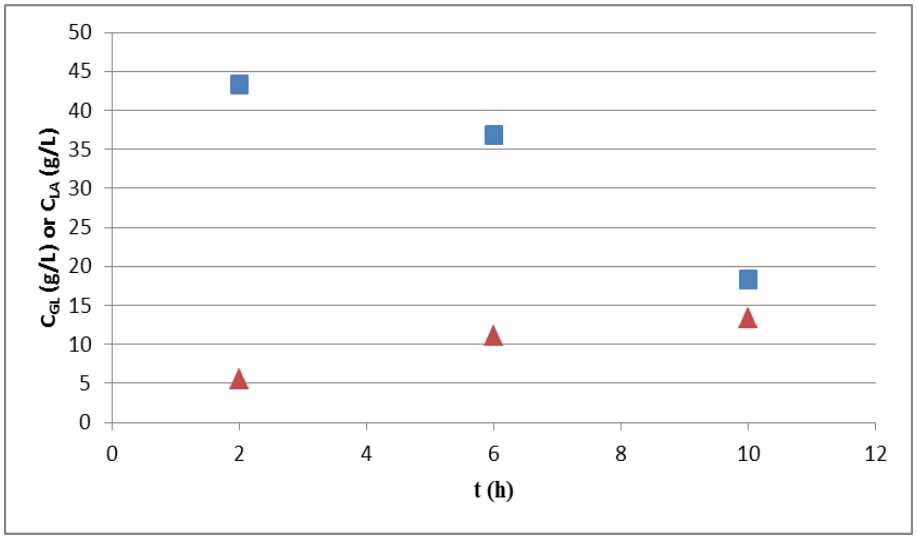

Figure 3.15 Total Glucose Consumption (■) and Lactic Acid Production (▲) of the 3. Batch Fermentation with Continuous Oxygen Feeding. C_{LA}, Lactic Acid Formation; C_{GL}, Glucose Level.

Table 3. 3 Biomass, Biomass Yield, LA Yield and Specific Growth Rate for the Three Consequtive Batch Fermentation Steps

	Biomass (X, g/L)	**Biomass Yield ($Y_{X/S}$) (g/g)**	**Lactic Acid Yield ($Y_{L/S}$) (g/g)**	**Specific Growth Rate (h^{-1})**
1. Fermentation	3.2	0.13	0.48	0.10
2. Fermentation	2.9	0.12	0.62	0.12
3. Fermentation	2.8	0.09	0.42	0.10

At the end of the third experiment, it was concluded that, removal of the inhibiting compounds away from the environment was crucial in order to gain desired amounts of biomass. Changing the inoculation amount by itself was observed to be uneffective on growth. Oxygen supply was also proved not to be preferred, in the fermentations. Also, it was observed that the fermentation periods were continually decreasing, from 14 hours to 11 hours, for the three trials. The maximum biomass was reached in a shorter period, each time.

3.7. Fed-Batch Study of with Continuous Feeding of Fresh Fresh Medium and Level-Controlled Membrane Filtration

It was concluded that the fermentation system should include a filtration system that is capable of continuously filtrating the medium and getting rid of the lactic acid that is produced by the strain and that inhibits the growth of the strain. The continuous fermentation of the microorganisms' effectiveness has been referred in literature many times. As an addition to this idea, feeding fresh medium into the fermentor was decided, in order to keep the volume constant. The filtration unit was

externally attached to the fermentor on two sides, one side for feeding the condensed solution back into the fermentor (containing the microorganisms that were separated with the taken away medium to be filtered) and one side for taking the medium to be filtered, via a pump (Watson Marlow pump, 101U-R). Level control in the fermentor was maintained at the side where the medium in the fermentor was taken to be filtered, arranged to a level that was nearly 1 L (actually, nearly 1.2 L, the equipment was not suitable for arrangement at exactly 1 L). The fresh GPY (Glucose 5%, Peptone 0.5%, Yeast Extract 0.5%) medium was arranged to be fed at a rate of 90 ml/hour by a peristaltic pump (Masterflex, Model 7013-21). All the parameters were the same (pH=6.5, T=37 °C, rpm=150) at the beginning of the fermentation. The inoculation was made 2 % (v/v) as before and batch fermentation was initiated.

After 5 hours of fermentation the rpm was increased to 200 rpm. At the end of 6 hours of fermentation, considering arrival to the active phase of growth, parallel to the literature studies, the feeding of the fresh medium was started. The consideration was to start this process together with filtration. The increased stirring rate was preferred for better mass transfer rate of continuously fed fresh medium.

Figure 3.16 Fermentation Unit with Continuous Fresh Medium Feeding and Continuous Membrane Filtration (at the beginning of the experiment)

The fermentation unit set up can be seen in the picture, above. The filtration unit was considered in a vertical position due to the expectation that the gravitational force might enhance the pressure difference required between the inlet and outlet to the filtration unit but it was a wrong approach. The filtration capacity was low this way and, also, due to a level of approximately 200 ml of volume that has to be reached for 1 L fermentor for the filtration to start, two hours had to pass. The filtration started, after eight hours of fermentation. After a few hours, when it was considered that the biomass growth had been stopped at a value (OD around 3.0-3.5) even lower than the batch fermentation values, the seperated filtered medium's waste was also analysed. The turbidity of the filter waste was 0.171 at 600 nm, at that time which represented a great amount of microorganisms loss from the fermentation. The OD of the filtrate increased up to 0.2-0.27 after 24 hours of fermentation. Then, a move to increase the performance of the filtration unit had to be conducted. Firstly, the diameter of the pipe line that goes through the pump ejected to the filtration unit, taking the medium to be filtrated from the fermentor, was increased. The next decision was to change the filtration unit's position from vertical to horizontal line and therefore, maintaining better pressure between the outlet and inlet on the filter (lowering the level same as the fermentation level). The diameter of the the pipe line that was just before the enterance of the filter line, to the fermentor, which was feeding the condense solution

back into the fermentor, was decreased so the pressure there was changed. The rpm was increased to 350. By this revision of the system, after approximately 30 hours of fermentation, the filtrate's turbidity at 600 nm was decreased to 0.03 and then, to lower values as seen in the picture below. But this time, it was observed that, as expected and as it exists in literature, the filtration rate decreased by time. There was also a technical problem with the feeding rate into the system, therefore the efficiency could be much better. At the end of the experiment, the biomass that accumulated inside the membrane filter was also transferred to the fermentor by washing with 200 ml fresh medium.

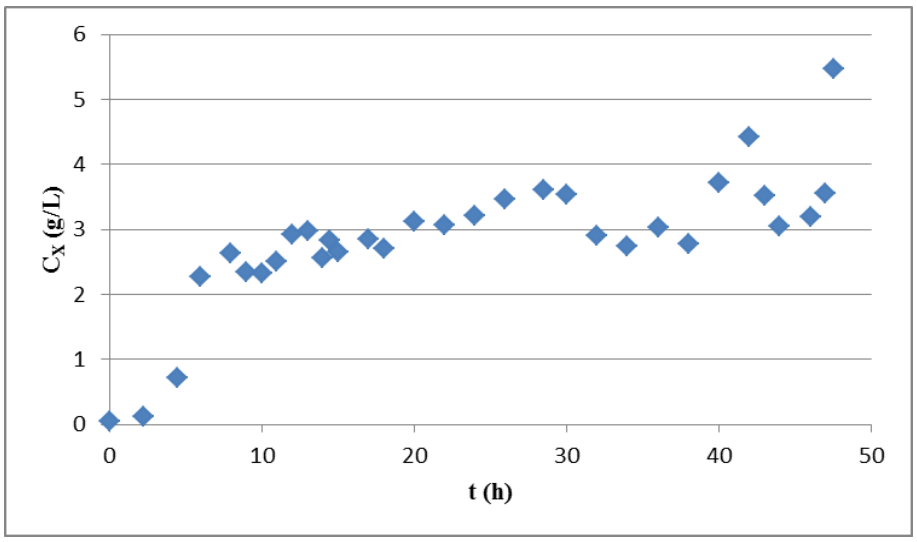

Figure 3.17 Fermentation with Continuous Fresh Medium Feed and Continuous Membrane Filtration. C_X, Biomass Concentration.

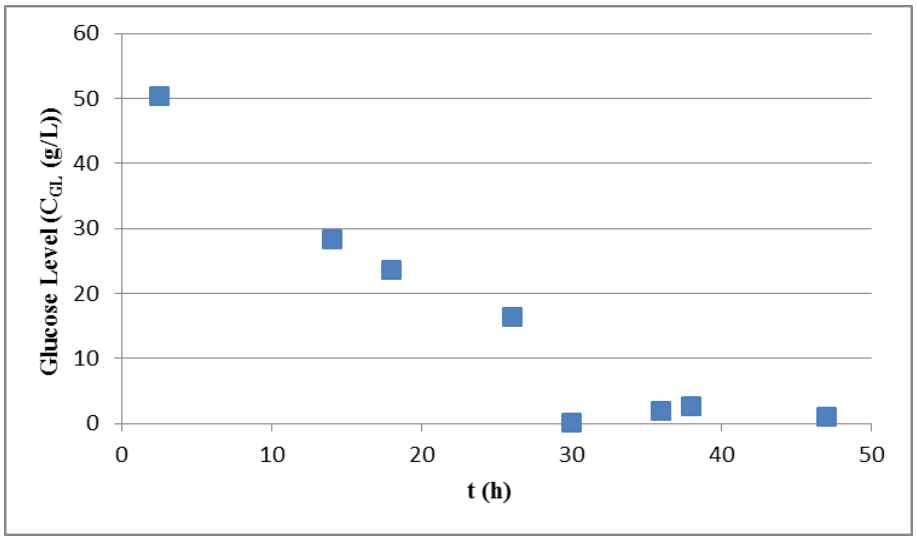

Figure 3.18 Glucose Level in the Fermentor with Continuous Fresh Medium Feed and Continuous Membrane Filtration. C_{GL}, Glucose Level.

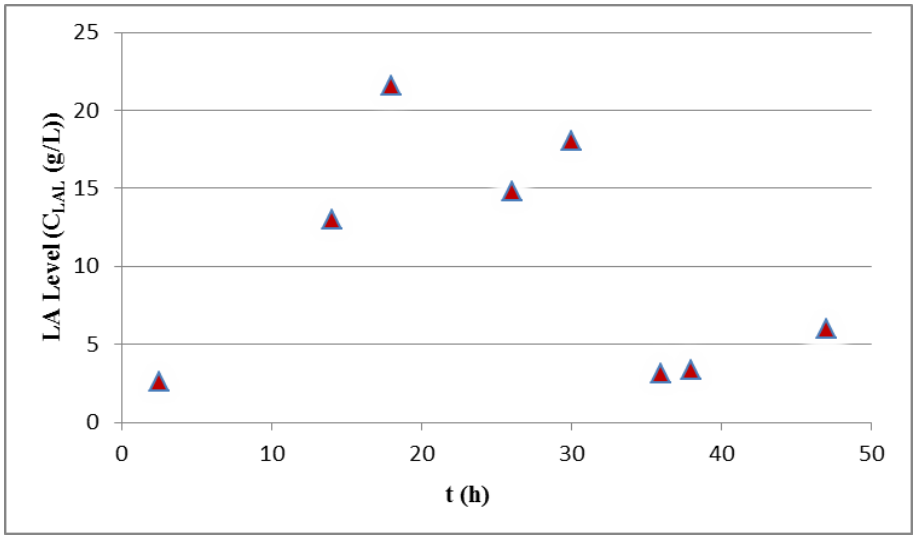

Figure 3.19 Lactic Acid Level in the Fermentor with Continuous Fresh Medium Feed and Continuous Membrane Filtration. C_{LAL}, Lactic Acid Level.

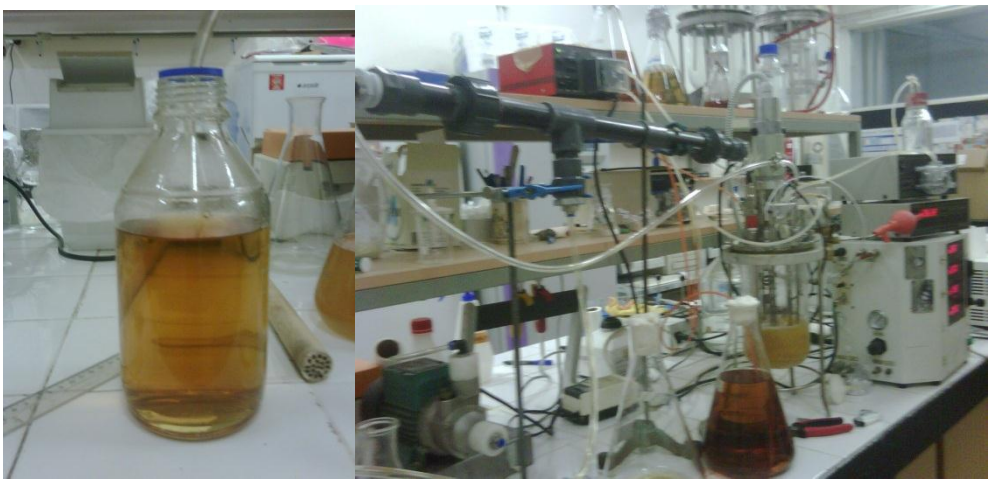

Figure 3.20 Fermentation Unit with Continuous Fresh Medium Feeding and Continuous Membrane Filtration (revised, right) and the Pure Waste Medium (left)

3.8. Fed-Batch Study of with Continuous Feeding of Fresh Fresh Medium and Level-Controlled Membrane Filtration, Together with Backwashing

The addition of a backwashing system was decided as a next step in the fermentation process due to the fact that biomass accumulation on the membrane surface that did not return to the fermentor should be transferred back into the fermentor during the fermentation process. By this way, much better biomass production would be maintained and also, the filtration performance would be increased. The fermentation process was conducted under the same conditions of temperature, pH.

The rpm was initially set to 200 but after 6 hours of fermentation, when the filtration and fresh medium feeding was started, this rpm value was raised to 350 (initial rpm was decided to be set to 350 but it seemed unreasonable due to the data, it had the potential to slow down the growth of the microorganisms at the first start). The fresh medium's feeding speed was increased to 134 ml/hour whereas the filtration rate was set to the pump's maximum level of 1800 ml/hour. The membrane filtration and fresh medium feeding continued together, for another 6 hours. At the end of around 12 (exactly, between 12 and 12 and a half hours) hours of fermentation, the backwashing of the membrane was firstly done for 10 minutes and from then on, every two hours 10 minutes of the backwashing was done. In the figure below the system may be observed;

Figure 3.21 Membrane Bioreactor System Integrated to Fermentation Unit with Continuous Fresh Medium Feeding and Continuous Membrane Filtration, Backwashing System Incorporated

The backwashing of the system was maintained by closing the exit of the membrane filter (returning the condense medium with microorganism to the fermentor) and the channel where the filtrate comes out of the membrane filter. Also, the pathway that was formed with t tubes was opened between that closure and the filtrate's path, as seen in the figure above. The pump was set as reverse mode. So the medium fluid was pumped from the filtrate's exit way, into the membrane filter and came out of the entrance, returning back to the fermentor. Filtration and fresh medium feeding processes, together with 10 minutes of backwashing every two hours continued for 14 hours. The OD values were checked before and after 10 minutes of the backwashing periods. It was observed that there were no significant differences between these OD values, even if the backwashing period was raised to 15 minutes for the last two times. Therefore, it was concluded that this process did not help gaining the biomass which remained on the membrane filter. So, after that conclusion, the experiment was continued without the backwashing step.

In the second day of the fermentation, it was observed that the filter's performance varied, over time, due to the biomass accumulation inside. For small periods, the system was unable to filter at all. At these times, for periods of one hour, the medium only washed inside of the membrane. This unsatisfactory performance of the membrane filtration caused, unintentionally, transferring some of the biomass that was stuck on the membrane, into the fermentor due to this mentioned forward washing for longer period**.** Therefore, it contributed to the target of high biomass maintenance, at the

end of the fermentation. This time, periodically, by squeezing the exit of the membrane filter (increasing pressure difference, there), filtration performance was increased to a preferred level.

At the end of the fermentation process, the biomass accumulated inside the membrane filter was totally recovered by washing with 250 ml fresh medium. Together with this biomass, the total DCW reached up to 6.3 g/L.

The backwashing system was considered to have the potential to be much more effective when the back pulsing was done with fresh medium as washing medium. The future work to improve the system should include this.

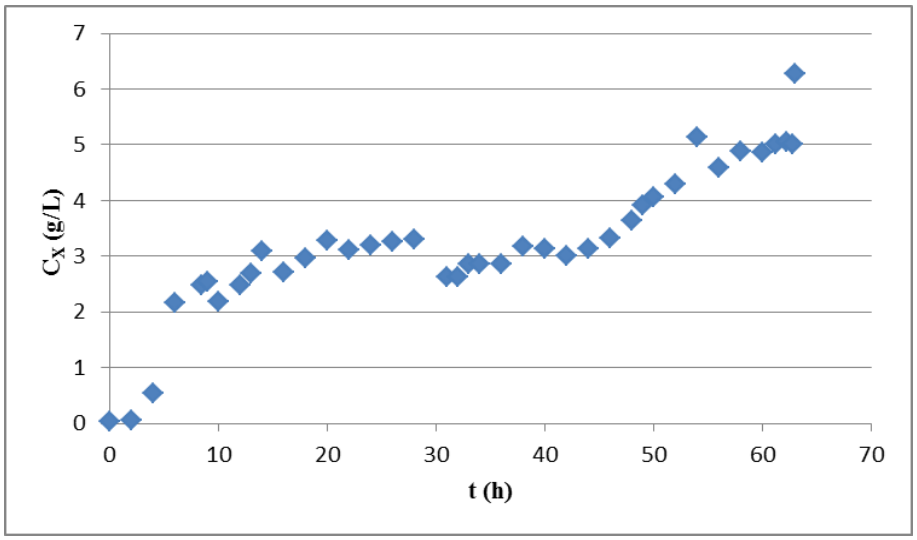

Figure 3.22 Fermentation with Continuous Fresh Medium Feed and Continuous Membrane Filtration, with an Addition of Backwashing Step. C_X, Biomass Concentration.

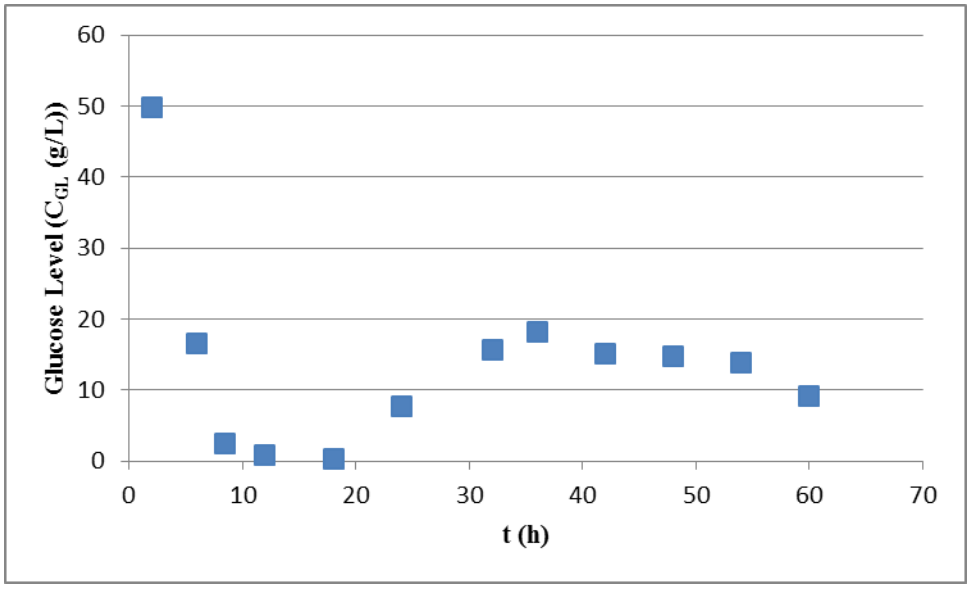

Figure 3.23 Glucose Level in the Fermentor with Continuous Fresh Medium Feed and Continuous Membrane Filtration, with an Addition of Backwashing Step. C_{GL}, Glucose Level.

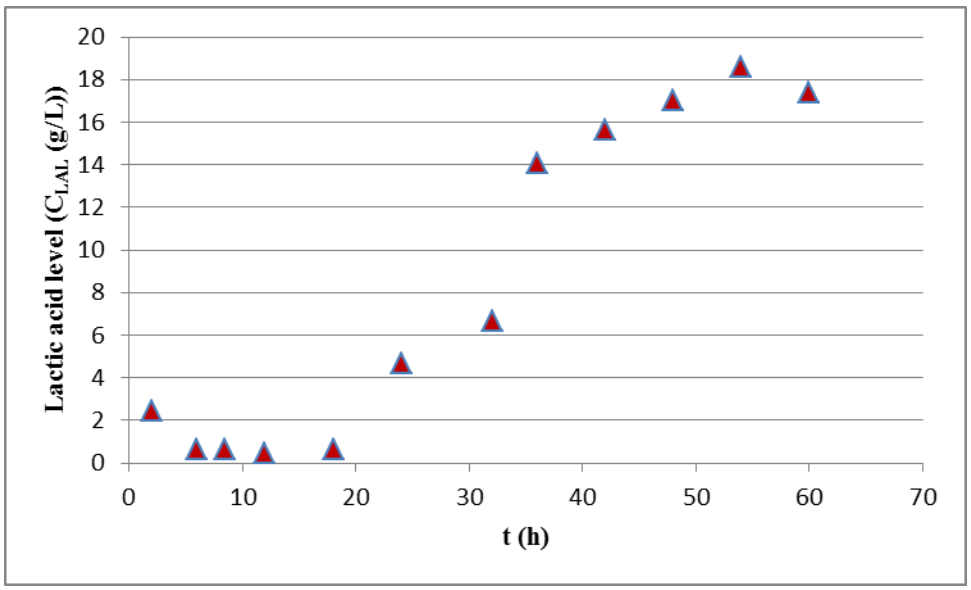

Figure 3.24 Lactic Acid Level in the Fermentor with Continuous Fresh Medium Feed and Continuous Membrane Filtration, with an Addition of Backwashing Step. C_{LAL}, Lactic Acid Level.

The fermentation with continuous fresh medium feed and continuous membrane filtration, the DCW was found to be 5.5 g/L, in the end. The lactic acid level was at an average of 10.3 g/L and for the last 18 hours, it was between 3.1 – 6.0 g/L. As it can be seen on the graphics above, for the filtrated fermentation process, the glucose content was consistently decreasing and after 30 hours, no excess glucose was observed pointing out effective filtration but in the following experiment, due to the insufficiency of filtration after some time, the glucose content was decreasing but not to the expected levels. The observations in the lactic acid formation curves underline the unefficient filtration for both experiments. In the fermentation with continuous fresh medium feeding and membrane filtration, the curve shows that after 30 hours of fermentation the filtration was enhanced to a desirable degree when compared with literature studies. But, in the last experiment with the additional backwashing step, the lactic acid production was expected to be high due to lack of efficient filtration but still, it should not be that high. In the fermentation with continuous fresh medium feed and continuous membrane filtration, with and addition of backwashing step, the DCW was found to be 6.3 g/L. The lactic acid amount was kept at an average value of 8.2 g/L. In the beginning of the experiment the filtration performance was perfect and the level was above 4.6 g/L but afterwards, the efficiency of the filtration began to fall down and that level increased up to 18.5 g/L when the 60^{th} hour of the fermentation was reached. When the specific growth rates were compared; μ for the first fermentation without backwashing was found to be 0.035 h^{-1} and for the last experiment it was found to be 0.02, even worse. This finding is parallel to literature studies in which such membrane systems cause lower specific growth rate but ended up with highest biomass concentration.

As it can be seen the last experiment ended up with a promising biomass value. It may be concluded that membrane filtering with the efficient membrane choice (right effective area, size and type) is enhancing the growth up to more than two times when compared with batch fermentation under the same conditions. Although backwashing step was found uneffective in this experiment, backwashing the membrane with fresh medium is expected to be much more effective, for the future work.

CHAPTER 4

CONCLUSION

Rainbow trout aquaculturing is continuously increasing in Europe and the USA, as well as, in Turkey. Today, nearly 55000 tones of trout are annually produced, in our country. The lactic acid bacteria used in the study, *E. faecium*, may be a strong probiotic alternative for this aquaculturing due to its strong probiotic characteristics and its isolation source may be related to nonpathogenicity of the microorganism. Still, a non-pathogenicity test should be conducted and it should be tried in fish farms and observing the effects on growth. The conjugative transfer of chromosomal determinants has been reported among *E. faecium* and *E. faecalis*. Since plasmids are known to mediate the transfer of antibiotic resistance, virulence, other adaptative possible traits throughout genus or between other genuses, genotyping and phenotyping antibiotic susceptibility tests should also be done as a further research.

In this research, the growth characteristics of the strain were investigated. The studies that are relevant to literature, pointed out that the values, pH 6.5 and T of 37 °C were the optimum values for the growth of the microorganism. The stirring rate values were chosen according to literature. When the varying media's effect on the growth was investigated, it was found that enrichment of the components of GPY medium was effective on growth to a degree but the main difference was observed by whey addition to the standard medium as carbohydrate source. For the pilot-scale studies, the usage of whey should be taken into consideration.

The fed-batch system with condense glucose solution feeding was an economical way to gain promising biomass yields, this is a valuable information for scale-up studies. The maximum biomass production, 6.3 g DCW/h was reached with the membrane filter system, including backwashing, periodically. The next step, as future work, should be to maximize the filtration performance and revise the backwashing step by backwashing with fresh medium to enhance better biomass growth and apply the system in pilot-scale studies.

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APPENDIX A

DEFINITIONS OF YIELD COEFFICIENTS

Yield on Substate:

Lactic Acid Yield: Y_{SL}=lactic acid produced (g/L) / substrate (glucose) consumed (g/L)

Biomass Yield: Y_{XS} = biomass produced (g/L) / substrate (glucose) consumed (g/L)

Specific Growth Rate Calculation (μ):

$\mu\ (h^{-1}) = (\ln X_2 - \ln X_1) / t_2 - t_1$

where the time range is taken as exponential phases for all the experiments

APPENDIX B

SAMPLE CALCULATİONS FOR FED-BATCH FERMENTATİON

Cell balance:

In – Out + Generation = Accumulation

$0 – 0 + \mu \cdot X \cdot V = d(XV)/dt = X \cdot (dV/dt) + V \cdot (dX/dt) = X \cdot Q + V \cdot (dX/dt)$

$dX/dt = (\mu - D) \cdot X$

$\ln (X_2 – X_1) = (\mu - D) (t_2 – t_1)$

In fed-batch fermentation chapter, for experiment 5, the initial glucose amount was zero and the medium is peptone (0.5%) yeast extract medium (0.5%), the glucose solution of 100 g/L was fed at ,approximately, a rate of 0.0047 L/h.

In this experiment, the log phase was assumed to begin at 4 h and 25 minutes and the DCW at this point was found to be 1.56 g/L (X_1). The DCW after 18 h when the log phase was assumed to end, was 3.52 g/L (X_2). The volume increase in this period was 0.065 L.

The dilution rate (D) is Q/V where Q is dV/dt. D is calculated to be 0.061 h^{-1}.

$\ln (X_2 – X_1) = (\mu - D) (t_2 – t_1)$

The specific growth rate found to be 0.11 h^{-1}.

Substrate balance:

In – Out - Consumption = Accumulation

$S_0 \cdot Q – 0 – (\mu \cdot X/Y_{X/S}) \cdot V = d(SV)/dt = S \cdot (dV/dt) + V \cdot (dS/dt) = S \cdot Q + V \cdot (dS/dt)$

$(dS/dt) = (S_0 – S) \cdot (D) – (\mu \cdot X/Y_{X/S})$ where $D = Q/V$

0.0047 (L/h)(100 g/L) = 0.47 g/h is glucose feeding rate.

$Y_{X/S}$ was calculated to be 0.39 according to the equation in Appendix A.

S_0 is zero. The integral of this equation is shown in Introduction part.

$S = (e^{-Dt}) + S_0 – (\mu \cdot X/((D) \cdot Y_{X/S}))$

Where

$X = e^{Q \cdot (A) \cdot t}$ where A is $1 - (\mu/D)$

From the formula above for X value is calculated at a specific time for the fermentation.

When all the known values are used for S calculation, S which is the glucose level in the fermentor, is found.

The empirical data found in this study were evaluated in these equations.

Glucose levels (g/L) and DCW (g/L) checked at specific intervals (by HPLC)

The observations from the analysis did not match and the results of the integral analysis were not the expected values. This may be due to the constant flow rate assumption, constant μ and volume assumption. The fermentation periods consisted of long scales of time and in those periods, such values may change in the experiments.

Formulas for the Experiments with Continuous Membrane Filtration and Fresh Medium Feeding, Together with Feed-Back of Cells into the Fermentor Calculations

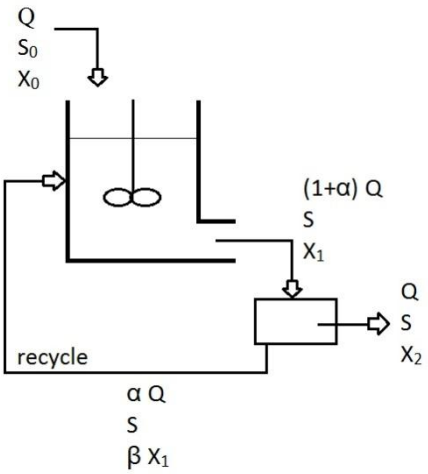

Figure B 1 CSTR for the System with Biomass Retention with Biomass Feed-back

The feed was sterile ($X_0 = 0$), but in the recycle stream, biomass concentration was βX_1.

X_1, X_2, and βX_1 are different from each other but substrate concentrations are the same.

Cell balance: (control volume is the whole system)

In – Out + Generation = 0

$-Q \cdot (X_2) + \mu \cdot (V) \cdot X_1 = 0$

$\mu \cdot (X_1) = (Q/V) \cdot X_2$

$\mu \cdot (X_1) = (D) \cdot X_2$

$X_2 = (\mu/D) \cdot X_1$

Cell balance: (control volume is the fermentor)

In – Out + Generation = 0

$\alpha \cdot (Q) \cdot \beta \cdot X_1 - (1+ \alpha) \cdot Q \cdot X_1 + \mu \cdot X_1 \cdot V = 0$

where $\mu = (1+ \alpha - \alpha \cdot \beta) \cdot D$

α is the recycling ratio of the cells back to the fermentor after filtration. α was set as the same with the fresh medium feeding rate so that constant volume in the fermentor was maintained. Unfortunately, due to the changes in filtration rates at time periods, the volumes filtrated and taken away differed each time and were not checked. Therefore, although X_1 is known, it is not possible to define a constant β value.

Substrate balance:

$(Q \cdot S_0 + \alpha \cdot Q \cdot S) - (1 + \alpha) \cdot Q \cdot S - (\mu \cdot X_1 / Y_{X/S}) \cdot V = 0$

$S = (\mu \cdot K_S)/(\mu_{max} - \mu) = (K_S \cdot (1 + \alpha - \alpha \cdot \beta) \cdot D)/(\mu_{max} - D \cdot (1 + \alpha - \alpha \cdot \beta))$

$X_1 = Y_{X/S}/(1 + \alpha - \alpha \cdot \beta) \, (S_0 - ((K_S \cdot (1 + \alpha - \alpha \cdot \beta) \cdot D)/(\mu_{max} - D \cdot (1 + \alpha - \alpha \cdot \beta)))$

APPENDIX C

CALIBRATION CURVES FOR GLUCOSE CONCENTRATIONS AND LACTIC ACID LEVELS FOR HPLC ANALYSIS

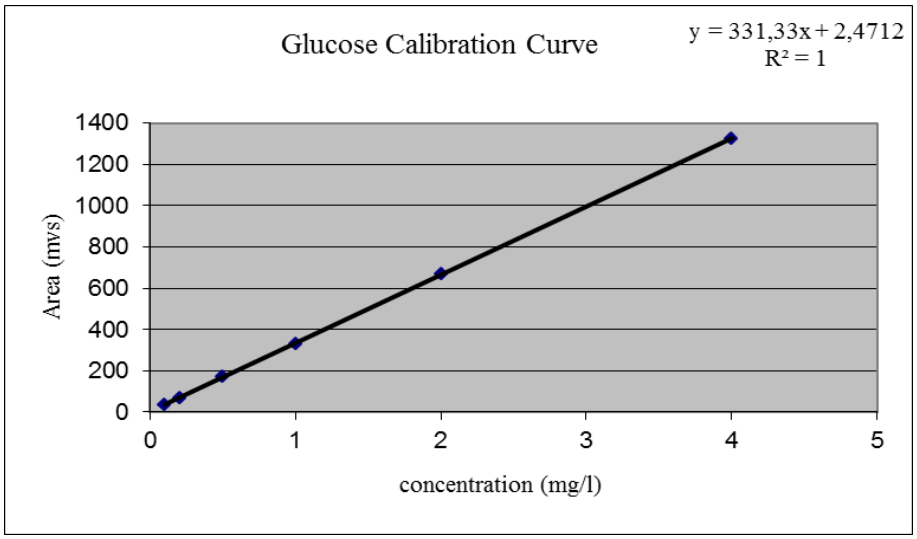

Figure C 1 One Calibration Curve for Glucose Concentrations for HPLC Analysis

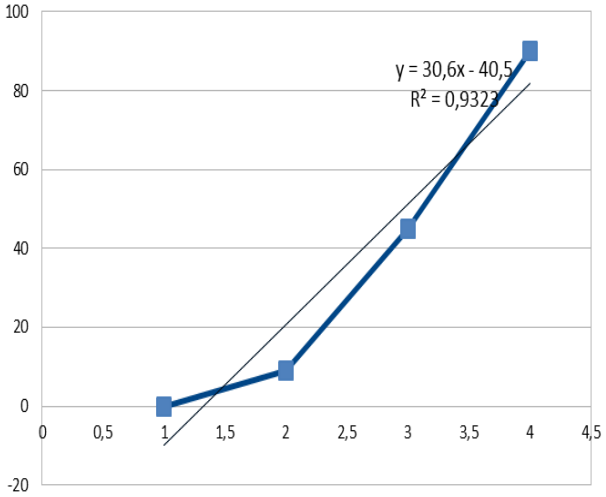

Figure C 2 One Calibration Curve for Lactic Acid Concentrations for HPLC Analysis

APPENDIX D

FREEZE-DRYING EXPERIMENTS

Freeze-drying is widely used to improve the shelf-life of lactic acid bacteria, as starters for fermented products, - 40 °C, under vacuum pressure (P < 0.08 – 0.1 mbar), until the moisture is constant.

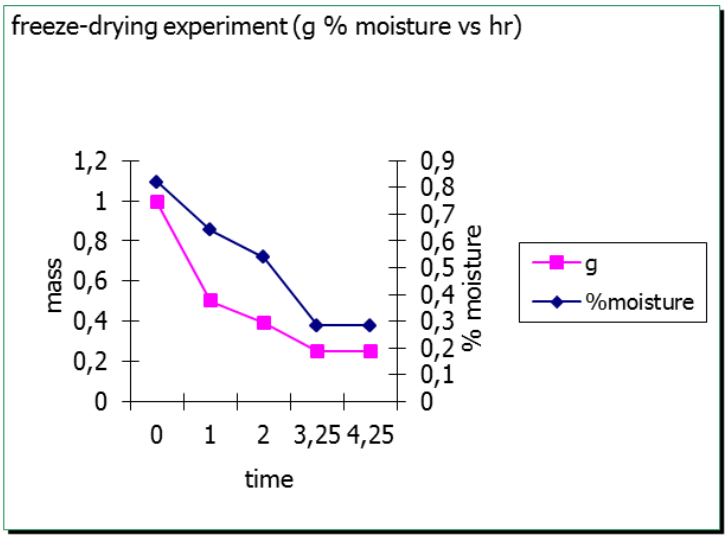

Figure D 1 Freeze-drying Experiment Observations on Mass (■) and Moisture Content (♦) Decreases

At the graphic above, one of the freeze-drying experiments' results are observed. After the fermentations, for 6 times, the microorganisms were freeze-dried under the same conditions. The samples' initial moisture contents varied from 65.6 % to 82 % and at the end on the freeze-drying step. From the freeze-drying experiments, for the final product, a moisture range of 3.2-1% was found. These last moisture contents are parallel to literature.
Also, 10^{13} cfu/g microorganism was gained alive from viability tests.

APPENDIX E

OBSERVATION ABOUT *E. Faecium*'S EFFECT ON SELECTED PATHOGENS

The *E. faecium*'s existence's effect on selected fish pathogens, *Lactococcus garviae*, *Y. ruckeri.* and *V. anguillarium.* was searched by basically, replicate plating them on GPYA (%2 Glucose, 0.5% Peptone, 0.5% Yeast Extract). The strains were plated alone and *E. faecium* was plated with each other strain, as mixed culture. The inoculations were done at all the same initial dilutions (10^{-6} – 10^{-7} cfu/ml).

E. faecium is found to decrease the Lactococ growth 10 folds, whereas it is found to be uneffective on Yersinia growth. Its existance's effect on Vibrio could not be observed.

APPENDIX F

AN EXPERIMENT TO REACH MINIMUM REQUIRED GLUCOSE CONCENTRATION

The *E. faecium* strains were incubated, overnight, at 37 C, 150 rpm within autoclaved (105 C, 20 min) medium that was composed of Yeast Extract (natural and freshly enhanced) and Glucose (20 g/L) and CaCO3 (2 g/L). The final microbial load was counted via thoma chamber under microscobe as 7*(10^7) [not so reliable].

Then, 1 L was santrifuged at 10000 rpm for 6 min. The pellets were dissolved within 40 mL of yeast extract-glucose-CaCO3 sln. 1 mL is taken away and remained 39 mL was inoculated into the fermentor.

1 mL is diluted 100 folds and at 600 nm, this diluted sample's absorbance was found as 0,772. Then it was centrifugated and the supernatant was removed. The wet basis was found to be 0,05 g/900 µL. According to this data, the initial load was found to be 2,166 g (into 1,5 L).

The fermentor was arranged at 250 rpm, 37 C, the pH was kept btw 6-6,5. The pH arrangement was done manually by observing pH trend and glucose addition into the fermentor was done again manually, due to this pH decreasing was observed. Total added glucose was approximately 4 g and the OD reached with this very little amount of glucose is quite high, as seen in the graph, below.

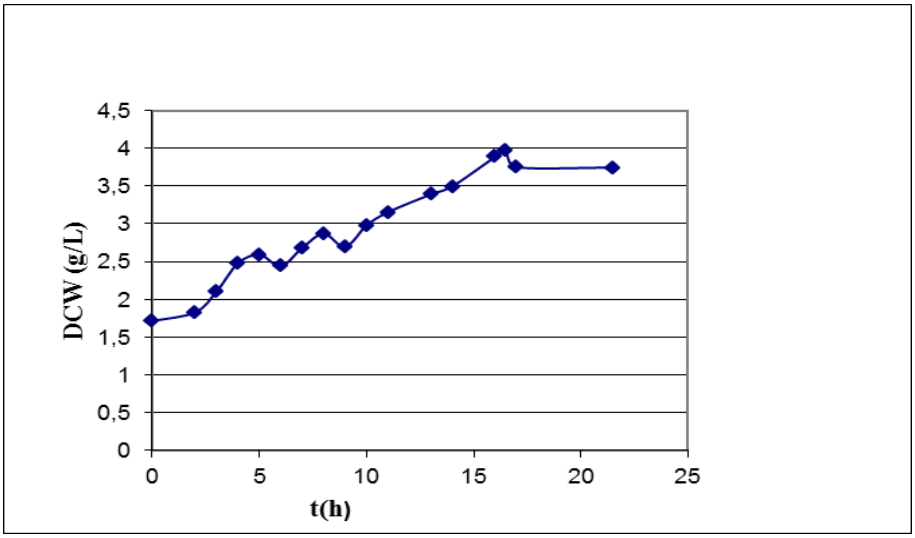

Figure F 1 Time Course of DCW (g/L)

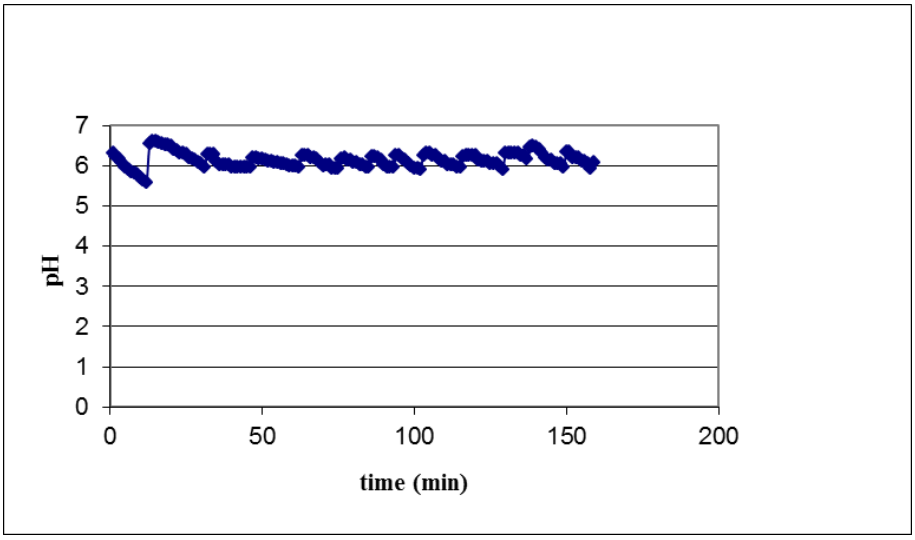

Figure F 2 pH Graph

APPENDIX G

VARYING OTHER MEDIA'S EFFECTS ON GROWTH OF *E. FAECIUM*

G. 1. Growth within Tryptic Soy Broth (TSB) Trials (with varying peptone amounts from meat extract source, at neutral pH's)

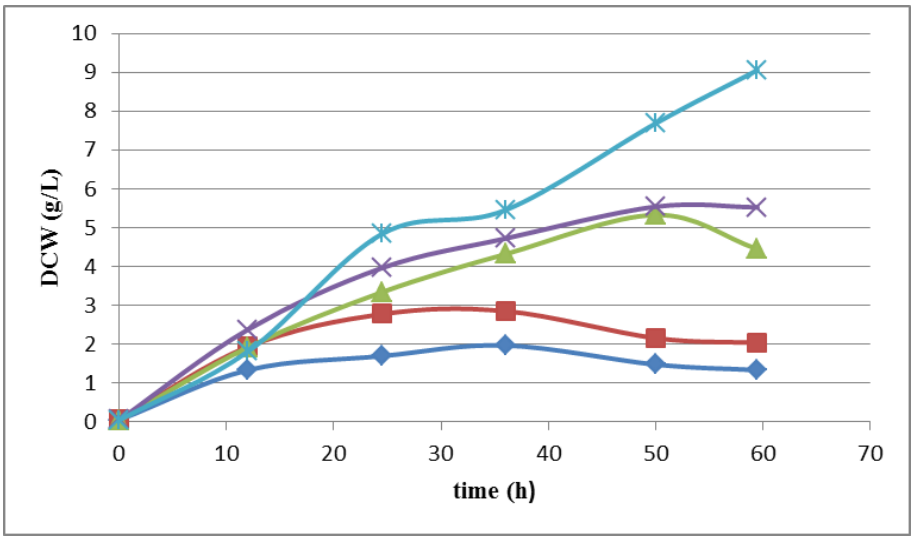

Figure G 1 Time Course of Batch Cultures of *E. faecium*

Gen: Maintained from the Genetic Lab.

G. 2. Growth Media Effects for Some Other Media

Table G. 1 Varying Growth Media

1 **2% Yeast Extract** **5% Glucose** **(0.01% v/v) Visniac Solution**	**2** **2% Yeast Extract** **5% Glucose**	**3** **2% Yeast Extract** **5% Glucose** **5% Peptone**
4 **2% Yeast Extract** **5% Glucose** **5% Peptone** **(0.01% v/v) Visniac Solution**	**5** **5% Glucose** **5% Peptone**	**6** **5% Glucose** **5% Peptone** **(0.01% v/v) Visniac Solution**

Average DCW (g/L) of all the Compared Media over Time:

t(hr)	TSB	NB	NB(+5%G)	1	2	3	4	5	6
12	2.5	1.3	1.6	1.3	1.2	1.0	0.60	0.52	0.41
25	5.2	1.9	1.9	2.6	2.6	0.60	0.64	0.40	0.42
36	6.8	1.9	1.7	2.6	2.7	0.61	0.72	0.81	0.51
48	8.3	1.8	1.9	3.9	3.9	0.64	0.73	0.40	0.54

Where Visniac Solution is 5 g EDTA, 2.2 g $ZnSO_4.7H_2o$, 0.5 g $MnCl_2.4H_2O$, 0.16 g $CoCl_2$, 0.16 g $CuSO_4.5H_2O$, 0.11 g $(NH_4)_6MO_7O_{24}.4H_2O$, 0.74 g $CaCl_2.2H_2O$ and 0.5 g $FeSO_4.7H_2O$

APPENDIX H

ENCAPSULATION OF *E. FAECIUM*

The pellets that are gained by centrifuging the fermentation products for 25 minutes at 10 °C and 6000 rpm, are added into 2% alginate solution. Next, the bacteria are spouted into 0.2 M 3% $CaCl_2$ solution anda re waited for half an hour. The bacteria are, then, rinsed twice with 0.02 M $CaCl_2$. 50 ml free cells and 50 ml encapsulated cells are inoculated into TSB medium by 2% v/v, each.

The observation was as below;

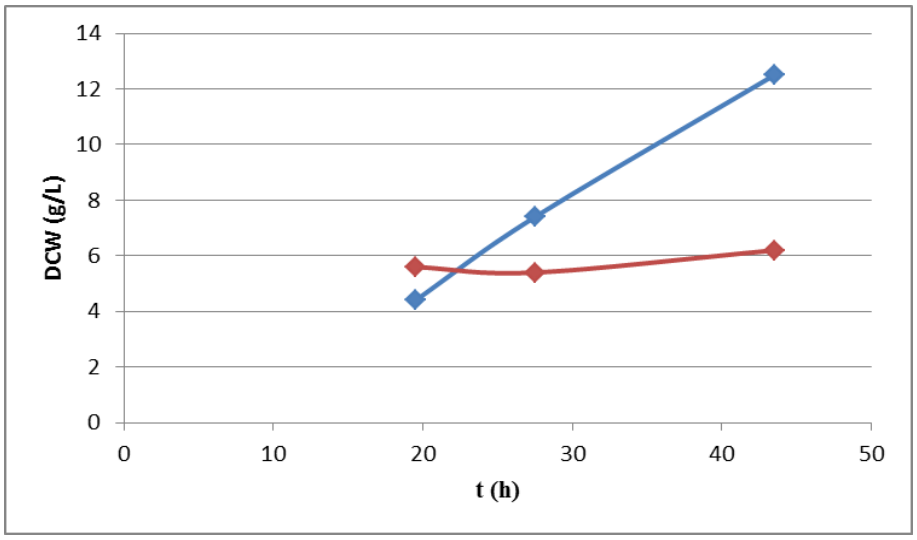

Figure H 1 Encapsulation's Effect on *E. faecium* Growth. Free Cells, (♦); Encapsulated Cells (♦).

The DCW (g/L) of the encapsulated microorganisms, after 43.5 hours of fermentation, was found twice of the free cells.