INTEGRATED APPROACH TO WHEY UTILIZATION THROUGH NATURAL ZEOLITE ADSORPTION/DESORPTION AND FERMENTATION

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in Chemical Engineering

by
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November 2009
İZMİR

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ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Semra ÜLKÜ and to my co-supervisor, Dr. Şebnem HARSA for their guidance, advices, critisizm and encouragement through all the stages of this thesis. I am grateful to my thesis committee members; Dr. Fazilet VARDAR SÜKAN, Dr. Muhsin ÇİFTÇİOĞLU, Dr. Canan TARI and Dr. Oğuz BAYRAKTAR for their valuable contributions and suggestions.

I would like to thank to Scientific Research Commission for supporting this thesis as scientific research project (2006 IZTECH 22).

I would like to express my indebtedness and offer my special thanks to my father Rasim POLAT, my mother Şaziye POLAT, my brother Bora POLAT, my aunt Makbule POLAT, my sister Şermin ELALDI and my brother in-law Muharrem ELALDI. Their support, endless patience, encouragement and understandings were unique in all my life.

I would like to thank to all my friends Seda GENÇ, Naz GÜLTEKİN, Tuğba KIRER, Berna ÜLKÜ, Elif KAYRAN, Çisem BULUT ALBAYRAK, Seyhun GEMİLİ, Sevdiye SAVRIK, Leyla DOĞAN, Gülay DURGUN, Başak ÖZER, Yelda AKDENİZ, Güler NARİN, Özlem DUVARCI, Filiz ÖMÜRLÜ, Berna TOPUZ, Deniz ŞİMŞEK and A.Emrah ÇETİN. I also thank to all members of Biochemical Engineering Research Laboratories and Zeolite Research Group. Special thanks to Rukiye ÇİFTÇİOĞLU for her support.

ABSTRACT

INTEGRATED APPROACH TO WHEY UTILIZATION THROUGH NATURAL ZEOLITE ADSORPTION/DESORPTION AND FERMENTATION

In this thesis, the usage of clinoptilolite rich natural zeolites in the recovery of whey proteins by adsorption/desorption, and in the bioconversion of whey, lactose source, to lactic acid were investigated. The possibility for the utilization of natural zeolite deposits of Turkey; and whey, by-product of cheese manufacturing, in the production of high value added chemicals is important from both economical and environmental concerns.

Natural zeolites with a particle size range of 75-150 μm were prepared and characterized. Aqueous protein solution, whey powder solution and whey were treated with the natural zeolites and dealuminated zeolites. Batch adsorption studies were performed and uptake data were collected. Equilibrium adsorption isotherms were analysed by Langmuir and Freundlich isotherms. Langmuir isotherm fits the experimental data better. Furthermore, experiments were conducted to see the effects of temperature, initial adsorbate concentration, solid/liquid ratio, agitation speed, pH, particle size and dealumination in adsorption process. The adsorption mechanism was analysed with adsorption kinetics models; and considering the effects of particle size, agitation speed and temperature external film diffusion, surface diffusion and surface reaction were found as effective.

The effects of the natural zeolite on the pH, elemental composition and the adsorption tendency of the lactose-lactic acid in the fermentation media were also investigated; and the results were compared with the sythetic ones. Natural zeolites were found as an alternative inexpensive mineral source for the fermentation.

ÖZET

TÜMLEŞİK BİR YAKLAŞIMLA PEYNİRALTI SUYUNUN DOĞAL ZEOLİTLE ADSORPSİYON/DESORPSİYON VE FERMANTASYON YÖNTEMLERİ İLE DEĞERLENDİRİLMESİ

Bu çalışmada, klinoptilolitçe zengin doğal zeolitlerin peynir suyu proteinlerinin adsorpsiyonunda ve desorpsiyonunda ve laktoz kaynağı olan peynir suyunun laktik asite biyolojik dönüşümü sırasında kullanımı incelenmiştir. Türkiye`nin doğal zeolit rezervlerinin ve peynir üretiminin yan ürünü olan peynir suyunun katma değeri yüksek bir kimyasalın eldesinde kullanılma ihtimali, ekonomik ve çevresel açıdan önemlidir.

75-150 μm parçacık aralığında doğal zeolitler hazırlanmış ve karakterize edilmiştir. Sulu protein çözeltisi, peynir suyu tozu çözeltisi ve peynir suyu, doğal zeolitler ve alüminyumu uzaklaştırılmış zeolitlerle işleme sokulmuştur. Kesikli adsorpsiyon çalışmaları gerçekleştirilmiş ve tutma verileri toplanmıştır. Denge adsorpsiyon izotermleri Langmuir ve Freundlich izotermleri açısından analiz edilmiştir. Langmuir izotermi deneysel verilere daha iyi uymuştur. Dahası adsorpsiyon çalışmasında; sıcaklık, başlangıç tutunan madde konsantrasyonu, katı/sıvı oranı, karıştırma hızı, pH, parçacık boyutu ve dealümimasyon etkileri çalışılmıştır. Adsorpsiyon mekanizması, adsorpsiyon kinetik modelleri ile analiz edilmiş, partikül boyutu ve karıştırma hızına bağlı olarak dış film difüzyonu, yüzey difüzyonu ve yüzey reaksiyonu, mekanizma belirleyici basamaklar olarak bulunmuştur.

Doğal zeolitlerin pH, fermantasyon ortamı element içeriği ve laktoz-laktik asit`e yönelik adsorpsiyon eğilimi üzerine etkileri incelenmiş ve sonuçlar sentetik olanlarla karşılaştırılmıştır. Doğal zeolitlerin fermantasyon için hesaplı bir alternatif element kaynağı olduğu bulunmuştur.

Dedicated
to
my family

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CHAPTER 1

INTRODUCTION

Whey is the main by-product of dairy industry. During cheese processing, on average for each kilogram of a final product, 30 kilograms of whey, with the content of high nutritional value compounds, is obtained. This milk fraction composed of the milk sugar lactose, whey proteins which are highly beneficial for living organisms, vitamins, minerals, and fats.

In a macroscopic viewpoint, whey presents excellent growth media for the microorganisms which is a great threat if discarded into environment. The idea that lies beneath this study is the valorisation of this valuable by-product with the help of clinoptilolite rich natural zeolite mineral, from Gördes/MANİSA. This research was handled in two parts. The first part is; the adsorption of the whey proteins onto natural zeolite from whey/whey powder solutions. After removing the solid part, the supernatant that was containing high amount of lactose and many elements are subsequently sent to lactic acid fermentation. The source of the elements are both natural zeolite and whey/whey powder. They Thus, large quantity of the whey/whey powder is transformed into important marketable products which are whey proteins and lactic acid with the aid of natural zeolites` adsorption and release properties.

Lactic acid has a growing world market. The level of production is around 68,000 tons/year and the worldwide growth is expected to be 12–15 % per year. Additionally polylactic acid has a potential to provide a new product platform to compete with hydrocarbon-based thermoplastics. Therefore, the biotechnological production of lactic acid has received a significant amount of interest since it offers an alternative to environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources (Wee et al., 2006). Biotechnological processes are successfully replacing chemical productions, due to lower costs and improved ecoefficiency.

Lactic acid is produced by chemical synthesis or by microbial fermentation. During the chemical synthesis method, racemic (DL) mixture of lactic acid is produced. On the other hand, by microbial production method L(+) and D(-)-lactic acid can be

produced in relatively pure form with respect to the type of microorganism utilized. During lactic acid fermentation process, operating conditions are very important. The fundamental parameters are the type of microorganism, the best working temperature, pH, agitation speed, the type and the amount of initial carbon source, nitrogen source and some additional salts. The carbon sources can be supplied in pure form or as a constituent of crude feedstock. Whey is a crude feedstock used as a carbon source. Lactose in whey is not only a good source to produce lactic acid but also an inexpensive raw material for the bioprocess as well. High cost of lactic acid purification process limits the production of this chemical. In large scale applications, economical raw materials and a system with few unit operations for purification are needed.

Lactic acid is the most widely occurring carboxylic acid in nature. It has been utilized in leather, textile, pharmaceutical and cosmetic industries for many years. Moreover, it is used in food industry as acidulant, preservative and antimicrobial agent. The L-(+) form of lactic acid is biodegradable and can be metabolized by the body and this property leads to the application of lactic acid in biomaterial applications.

The important properties of natural zeolites such as, cation exchange property, adsorption properties, buffering effect, and molecular sieving properties brought this material into biotechnological applications in recent years. Furthermore, utilizing the wide and attainable natural zeolite deposits of our country is important from economical perspective. Initially, the suitability of natural zeolites for the recovery of whey proteins was studied. There are only few studies about adsorption of proteins by synthetic zeolites but not any related with whey proteins and natural zeolites adsorption in literature, recently.

The effect of natural zeolites on pH and lactic acid production during fermentation has not been studied yet. Its effect on fermentation pH and on lactic acid production was both investigated. The addition of natural zeolite amounts into fermentation media did not prevent lactose consumption and lactic acid production. In addition to the main goals, the adsorption of lactose and lactic acid onto natural zeolites were investigated in aqueous solutions, during fermentation and in cell free fermentation broth. The applicability of natural zeolites for the recovery of lactic acid from the fermentation media was investigated.

The main objectives of this study were to investigate the applicability of clinoptilolite rich natural zeolites in the recovery of whey proteins by adsorption and to

investigate their role in lactic acid fermentation by enriching the elemental composition of the fermentation media with the cations released from their structure.

CHAPTER 2

WHEY - WHEY PROTEINS

2.1. Characteristics of Whey

The dairy industry generates significant liquid waste, whose disposal requires a large amount of capital investment. Large quantity of whey is discarded during cheese manufacturing. Most milk plants do not have proper treatment systems for the disposal of whey and the dumping of whey constitutes a significant loss of potential food and energy source, as whey retains about 55% of total milk nutrients (Panesar et al., 2007).

Whey, a by-product of cheese manufacturing, is a milk fraction composed of lactose, proteins, vitamins, minerals, and fats (Table 2.1.). Whey is now generally regarded as a functional food, which has measurable effects on health, and the bioactive properties of whey proteins and whey protein fractions are becoming increasingly recognized. The major bovine whey proteins are boine serum albumin (BSA), β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), and several of the predominant minor proteins, such as lactoferrin and lactoperoxidase (Fong et al., 2008).

The chemical composition of whey is dependent upon chemical composition of the milk, which varies with stage of lactation, feeding, breeding, individual animal differences, and climate. Limited studies have shown that the whey protein composition of these milks follow the general lactational pattern of the cow. In addition, whey composition varies according to slight changes in milk processing parameters (Casper et al., 1998).

Table 2.1. Gross composition of liquid and dried whey.

Component	Fluid whey	Dried whey
Total solids, %	6.35-7.00	96.3-96.5
Protein, %	0.8-0.9	13.0-13.1
Lactose, %	4.85-5.1	68-75
Fat, %	0.3-0.5	0.8-1.0
Lactic acid, %	0.05	0.2
Ash, %	0.5-0.6	7.3-9.6

Whey may be defined broadly as the serum or watery part of milk remaining after separation of the curd, which results from the coagulation of milk proteins by acid or proteolytic enzymes. The type and composition of whey at dairy plants mainly depends upon the processing techniques used for casein removal from liquid milk. The most often encountered type of whey originates from manufacture of cheese or certain casein cheese products, where processing is based on coagulating the casein by rennet, an industrial casein-clotting preparation containing chymosin or other casein-coagulating enzymes. Rennet-induced coagulation of casein occurs at approximately pH 6.5; this type of whey is referred to as sweet whey. The second type of whey, acid whey (pH < 5), results from processes using fermentation or addition of organic or mineral acids to coagulate the casein, as in the manufacture of fresh cheese or most industrial casein. In general, whey produced from rennet-coagulated cheeses is low in acidity, while the production of fresh acid cheeses such as ricotta or cottage cheese yields medium acid or acid whey. The main components of both sweet and acid wheys, besides water, are lactose (approximately 70–72% of the total solids), whey proteins (approximately 8–10%) and minerals (approximately 12–15%). The main differences between the two whey types are in the mineral content, acidity and composition of the whey protein fraction. The acid coagulation approach results in substantially increased acidity (final pH approximately 4.5), necessary for casein precipitation. At this low pH, the colloidal calcium contained in the casein micelles in normal milk is solubilised and partitioned into the whey. Rennet clotting produces a fragment κ-casein molecule, termed glycomacropeptide (GMP), which ends up in whey. GMP constitutes approximately 20% of the whey protein fraction of sweet, rennet-based wheys but is not present in acid wheys, unless renneting is included in the fresh cheese manufacturing

process. Other technological steps used in the pretreatment of milk before the main processes may also influence the composition of whey (Panesar et al., 2007).

Since lactose is the major component of whey solids, in addition to water-soluble vitamins, minerals and proteins, numerous biotechnological processes have been developed to utilise whey to make useful products of industrial importance (Panesar et al., 2007). To convert this waste into an asset many researchers have analyzed a large number of potential utilizations of either whole cheese whey or its major components, lactose and proteins. Among them, the biological conversion of its lactose content into lactic acid using appropriate species of *Lactobacillus* has the double advantage of alleviating a pollution problem and at the same time, producing a marketable product (Joglekar et al., 2006; Wee et al., 2006; Hofvendahl et al., 2000).

Although several possibilities of cheese whey utilization have been explored, a major portion of the world cheese whey production is discarded as effluent. Its disposal as waste poses serious pollution problems for the surrounding environment, since it affects the physical and chemical structure of soil, resulting in a decrease in crop yield and when released into water bodies, reduces aquatic life by depleting the dissolved oxygen. Thus, whey poses a major threat to environmental and human health, for which an effective and permanent solution is urgently needed. Most of the industrially developed countries have stringent legislation governing the disposal of effluents. Biological wastewater treatment technologies can assist in safer disposal of whey within environmental specifications, but these are expensive. To overcome this problem, a better alternative is subjecting whey to processes through which value added products can be manufactured, which may contribute wholly or partially to the disposal costs. Availability of the lactose carbohydrate reservoir in whey and the presence of other essential nutrients for the growth of microorganisms make whey a potent raw material for the production of different bio-products through biotechnological means (Panesar et al., 2007).

Whey is an excellent growth medium for various types of microorganisms. However, economical problems in transporting the whey have been posed as obstacles to adopting any process for utilisation of whey. Clearly this is because of its high water content and storage problems due to being readily subjected to bacterial and fungal spoilage. These problems have been solved to a great extent with the development of reverse osmosis and ultrafiltration techniques used for the concentration of whey. The use of immobilisation technology in utilisation of whey is of significant importance to

improve further the economics of the process. Immobilisation has been the convenient method to allow reutilisation of cells, higher cell densities in bioreactors and easier purification of the final product. Moreover, continuous operation is more easily and efficiently controlled using this technology, which has an advantage over the free cell system in the bioconversion of whey. Innovative uses of whey through microbial fermentation, along with recent biotechnological techniques, and bioreactor design, will certainly remain topics of great interest when trying to solve the major environmental problem faced by the dairy industry (Panesar et al., 2007). The constituents of whey and whey powder are presented in Table 2.1.

Considerable efforts have been made over the past years to find new outlets for whey utilization and reduce environmental pollution. Nowadays technologies, such as ultra filtration and spray drying, are non-expensive and allow the separation of different fractions of whey (lactose, whey proteins as whey protein concentrate, lactalbumin lactoglobulin, etc.) that are commercialized in the food and pharmaceutical industries. Furthermore, whey proteins provide an excellent way to fortify foods increasing the nutritional quality of cheese, dairy desserts, bakery products, etc. (Pescuma et al., 2008).

2.2. Whey Proteins

In bovine whey, there are five major protein fractions with defined molecular weights, i.e., immunoglobulins (Igs), mainly immunoglobulin-G (IgG), bovine serum albumin (BSA), α-lactalbumin (α -La), β-lactoglobulin (β-Lg) and proteose peptones (Merina et al., 2001).

Whey protein has an exceptional biological value that exceeds that of egg protein by about 15%, the former benchmark, and a range of other common edible proteins. Biological value is a measure of the percentage of a given nutrient (e.g., protein) that is utilised by the body. In essence, biological value refers to how well and how quickly the body can utilise the protein consumed. In this regard, whey protein excels and is the protein of choice for body builders, elite athletes, and those whose health is compromised (Smithers, 2008).

As the proteins are made up of amino acid chains, the importance of whey proteins are based on their amino acid constituents which play crucial role through health benefits. Whey protein is a rich source of the essential amino acids when

compared with other typical food proteins and is also rich in the branched chain amino acids (leucine, isoleucine, and valine) (>20%, w/w). These latter amino acids are thought to play a role as metabolic regulators in protein and glucose homoeostasis, and in lipid metabolism, and as such may play a role in weight control. Whey protein is a rich and balanced source of the sulphur amino acids (methionine, cysteine) . These amino acids serve a critical role as anti-oxidants, as precursors to the potent intracellular anti-oxidant glutathione, and in one-carbon metabolism (Smithers, 2008). Basic properties of whey proteins are given in Table 2.2.

Table 2.2. Protein profile of whey and basic properties of whey proteins (Source: Madureira et al., 2007; Doultani et al., 2004).

Protein	**Concentration (g/l)**	**Molecular weight (Da)**	**Number of amino acids residues**
β-Lactoglobulin	3.2	18 277	162
α-Lactalbumin	1.2	14 175	123
Bovine serum albumin (BSA)	0.4	66 267	582
Immunoglobulins (A, M and C)	0.7	25 000(light chain)+50 000-70 000(heavy chain)	-
Lactoferrin	0.1	80 000	700
Lactoperoxidase	0.03	70 000	612
Glycomacropeptide	1.2	6700	64

In long term storage in order to provide maximum benefit from whey proteins, the major criterion that must be taken into consideration is the minimum exposure of heat. The temperature and the duration of heating process have significant importance for protecting the nutritive value of whey proteins in processes especially spray drying of proteins.

Whey protein denaturation begins with the multiple-reaction process, initial swelling of the protein structure when it is first exposed to heat. As the intensity of the heat treatment increases, the whey proteins unfold, aggregate, and sediment through a multiple-reaction process. In various whey systems, calcium, total solids, and pH affect whey protein denaturation (Hollar and Parris, 1995).

Table 2.3. Biological functions of whey proteins
(Source: Madureira et al., 2007).

Protein	Biological function
Whole whey proteins	Prevention of cancer -Breast and intestinal cancer -Chemically induced cancer Increment of gluthatione levels -Increase of tumor cell vulnerability -Treatment of HIV patients Antimicrobial activities Increment of satiety response -Increment in plasma amino acids cholecystokinin and glucagon-like peptide
β-Lactogulobulin	Transporter -Retinol -Palmitate -Fatty acids -Vitamin D and cholesterol Enhancement of pregastric esterase activity Transfer of passive immunity Regulation of mammary gland phosphorus metabolism
α-Lactalbumin	Prevention of cancer Lactose synthesis Treatment of chronic stress-induced disease
Bovine Serum Albumin	Fatty acid binding Anti-mutagenic function Prevention of cancer Immunomodulation -Disease protection through passive immunity
Immunomodulation	Antibacterial activity -HIV Antifungal activity Opioid activity

It is known that, not even taking industrial processing into account, the concentrations of individual proteins in natural milk are influenced by several genetic and environmental factors, seasons, nutritional regime of the cows, age of the cows, stage of lactation, etc. (Bordin et al., 2001).

The principal methods available for isolation of proteins from biological fluids at commercial scales are chromatography and filtration. A number of techniques have been used to separate and analyze milk proteins: salt and organic solvent fractionation,

starch gel electrophoresis, polyacrylamide gel electrophoresis, isoelectric focussing, as well as combinations of them. Ion-exchange chromatography on DEAE cellulose and open-column gel permeation have been widely used, but the most significant advances have been achieved by applying high performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) techniques. These techniques reduce analysis times from days or even weeks to just hours. HPLC and FPLC methods are also useful in isolating compounds present in small concentrations, and the separations carried out on a laboratory scale can be transferred to an industrial scale. Practically all known mechanisms have been employed in the separation of proteins by chromatography, such as separations based on molecule size (gel permeation chromatography), on charge (ion exchange chromatography), on hydrophobicity (reversed phase chromatography and hydrophobic interaction chromatography), and even on combinations of these various mechanisms (Gonzalez et al., 1990).

Most of the separation techniques given above are valid for laboratory scale, where as the industrial scale requires the applicability of separation methods for gross amounts.

2.3. Applications of Whey

Dairy industries all over the world generate great amounts of whey per litre of milk processed, depending upon the processes employed, products manufactured and housekeeping exercised. About 50 % of total world cheese whey production is treated and transformed into various food products, of which about 45 % is used directly in liquid form, 30 % in the form of powdered cheese whey, 15 % as lactose and byproducts from its removal, and the rest as cheese–whey–protein concentrates (Panesar et al., 2006). In order to make protein concentrates it may be exposed to ultrafiltration. The filtrate is whey permeate, which still contains almost the total lactose and salts derived from milk, resulting in a high COD of 20-60 kg 0_2/t. The disposal of the whey permeate is still a problem. Because of the high chemical oxygen demand (COD). It is not economic and mostly not possible to treat it in sewage treatment plants. A promising way to use whey permeate in high quantities is the fermentation of the lactose to lactic acid by lactic acid bacteria (Börgardts et al., 1998).

The whey proteins have high nutritional value and can be used to manufacture different types of food products. Large amounts of whey proteins are used for infant formula, yogurt, ice cream, and beverages. Attempting to maximize the extraction of these proteins from whey and separating them has been a challenging task. The impurities in whey make the extraction process relatively difficult. In addition, the similarities between α–La and β-Lg make it even more difficult to separate these proteins. Reserchers have developed a method to produce enriched fractions of α–La and β-Lg from cheese whey by concentrating the whey protein using ultrafiltration followed by pH adjustment of the concentrate. However, it isreported that separation of proteins using selective thermal precipitation is more promising than using the ultrafiltration route, provided that proper conditions including initial protein concentration, pH, and length of precipitation time are maintained. It was shown that the tendency of α–La to aggregate is higher under specific conditions including at pH values near the isoelectric point (pH 4.2–4.6) and in a temperature range of 50 ^{o}C–65 ^{o}C. It was observed that the tendency to aggregate increased with protein concentration. The effect of citrate on the precipitation of whey proteins was studied and the addition of citrate leading to α–La rich fractions at temperatures around 35 ^{o}C was observed (Börgardts et al., 1998; Elshereef et al., 2008).

Research has shown that the two major whey proteins, α-La and β-Lg, become unstable at temperatures above 65 ^{o}C. When heated above this temperature, protein denaturation occurs resulting in protein aggregation and precipitation. The response to thermal treatment varies with different types of proteins, which results in different proteins precipitating out of the solution in different proportions making separation possible. Therefore, heat-induced aggregation and precipitation is an important treatment process in the manufacture of many dairy products, and is used to modify functional properties with the goal of ensuring food safety of the product. Functional, physical and chemical properties of milk such as texture, heat stability, foaming properties and rheological behavior are all affected by the heat treatment. The rate at which whey proteins aggregate is controlled by process conditions such as protein concentration, pH and temperature and the presence of other components. Different solutions of β-Lg and α-La were considered in that case. These proteins are the predominant proteins that make up about 70 % of all the proteins in whey and are key to the functional properties and characteristics of whey (Elshereef et al., 2008). Of the whey proteins, α-La has the lowest denaturation temperature, 62 ^{o}C, followed by BSA

at 64 ^{o}C, Ig at 72 ^{o}C, and β-Lg at 78°C. β-Lg denatures more quickly than α-LA at 85°C and at other temperatures in heated milk and whey systems. The overall effect of heating is greater on β-Lg than on α-La. Because α-La can renature upon cooling, it is considered to be the most thermostable whey protein. However, it was found that, although purified α–La renatured upon cooling, the α- La present in heated whey protein concentrate (WPC) did not (Hollar and Parris, 1995).

Several methods have been proposed for whey valorisation. In this respect, lactose converting microorganisms have been evaluated for the production of potable and fuel-grade alcohol, kefir-like whey drinks, lactic acid, baker's yeast, single cell protein (SCP) as livestock feed, probiotic starter cultures for fermented milk products and cheese ripening. In the frame of extensive efforts to reduce starter culture and baker`s yeast production costs and valorisation of the majority of global whey production, research on microbial thermal drying techniques was considered. An integrated technology for starter culture production from whey for use in cheese ripening is another application area of whey. The development of this environmentally friendly technology aimed to the valorisation of the total whey production through the production of a large number of products such as bulk chemicals (potable and fuel-grade alcohol) and various other foodstuffs of high nutritional and added value (baker's yeast, protein enriched animal feedstock, whey drinks simulating kefir, and starter cultures for cheese ripening or as probiotic food additives) (Koutinas et al., 2009).

Whey protein concentrate is a mixture of all the whey proteins obtained by membrane concentration of whey. Whey protein concentrate retains significant amounts of the minerals, lipids, and lactose from whey. Whey protein isolate (WPI) is a higher quality, higher value mixture of the whey proteins, which is devoid of most, but not all, of the minerals, lipids, and lactose found in whey. Fractionated whey proteins have the greatest value, resulting from unique properties. For example, α-La has a large potential market in the manufacture of human infant formula (Zihao and Kefeng, 1995).

The advent of improved and more cost-effective processing technologies and production procedures (i.e., ultrafiltration, reverse osmosis, and electrodialysis) has made possible a dramatic increase in production of whey protein concentrate (WPC). However, WPC produced using different procedures may not demonstrate the same functionality or stability in standard formulations (Börgardts et al., 1998; Chen and Ju, 1998; Rojan et al., 2007).

For goals of whey purification different technologies can be used. One of them is membrane separation, because of the possibility to separate from aqueous phase dispersed particles of different sizes (bacteria, lipid drops, solid particles using microfiltration, or cold sterilization; proteins, using ultrafiltration; salts and lactose are removed by hyperfiltration). Reverse osmosis allows the one-set removing of all dry substances from whey. However membrane processes sometimes make difficult to operate revealing phenomena of fouling and membrane breaking and others (Izmailova and Yampolskaya, 1998).

CHAPTER 3

LACTIC ACID

3.1. Characteristics of Lactic Acid

Lactic acid ($CH_3CHOHCOOH$) is an organic acid (α-hydroxy-propionic-acid) with two isomeric forms. L-(+) and D-(-) lactic acid are two optical isomers (Figure 3.1). Lactic acid was first isolated from sour milk by the Scheele in 1780. Later on Lavoisier named this component "acide lactique" which is the possible origin of the current terminology in 1789. It was known to be a milk component until the discovery of Pasteur, in 1857, that it was a metabolite generated by certain microorganisms during fermentation. The first commercial microbial production was started in 1881 by Avery in the United States (Vijayakumar et al., 2008; Wee et al., 2006). The two optically active forms of lactic acid are shown in Figure 3.1. The physical prperties of lactic acid are given in Table 3.1.

L-(+)Lactic Acid D-(-)Lactic acid

Figure 3.1. Isomeric forms of lactic acid.

Lactic acid can be produced by humans, animals, plants and microorganisms. Both isomeric forms of lactic acid can be polymerized and polymers with different properties can be produced depending on the composition. High levels of D-(-) lactic acid are harmful to humans, bringing out the importance of L-(+) lactic acid in food and

pharmaceutical industries. The worldwide market for this valuable chemical is growing every year, which was 68,000 tons/year in 2006 (Vijayakumar et al., 2008).

Table 3.1. Physical properties of lactic acid.

Molecular weight	90.08 g/mole
Melting Point D(-) or L(+) DL(varies with composition)	52.8-54^{o}C 16.8-33^{o}C
Boiling point DL	82^{o}C at 0.5 mmHg
Dissociation constant(K_a at 25^{o}C)	1.37x10^{4}
Heat of combustion(ΔH_c)	1361 kj mol^{-1}
Specific heat(C_p at 20^{o}C)	190 J mol^{-1} ^{o}C

3.2. Lactic Acid Production Technology

Lactic acid can be produced in two ways; either microbial production or chemical synthesis. Of the 90 % of lactic acid produced worldwide every year are made by lactic acid bacterial fermentation and the rest is produced synthetically by the hydrolysis of lactonitrile. Fermentative production has the advantage that by choosing a strain of lactic acid bacteria (LAB) producing only one of the isomers, an optically pure product can be obtained, whereas synthetic production always results in a racemic mixture of lactic acid (Hofvendahl and Hahn-Hägerdal, 2000; Joglekar et al., 2006). Fermentation is the most adequate means to obtain the pure isomers L(+) or D(-) lactic acid, and actually it is possible to choose a lactic acid bacterium capable of producing one of the stereoisomers because of its taxonomic characteristics (Raya-Tonetti et al., 1999).

3.2.1. Chemical Production

The synthetic manufacture of lactic acid on a commercial scale began around 1963 in Japan and in the United States. The commercial process for chemical synthesis is based on lactonitrile. Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction occurs in liquid phase at high atmospheric pressures. The crude lactonitrile is recovered and purified by distillation. It is then

hydrolyzed to lactic acid, either by concentrated HCl or by H_2SO_4 to produce the corresponding ammonium salt and lactic acid. Lactic acid is then esterified with methanol to produce methyl lactate, which is removed and purified by distillation and hydrolyzed by water under acid catalyst to produce lactic acid and the methanol, which is recycled. This process is represented by the following chemical reactions (Narayanan et al., 2004).

(a) Addition of Hydrogen Cyanide

$$\underset{\text{Acetaldehyde}}{CH_3CHO} + \underset{\text{hydrogen cyanide}}{HCN} \xrightarrow[\text{catalyst}]{} \underset{\text{lactonitrile}}{CH_3CHOHCN} \quad (3.1)$$

(b) Hydrolysis by H_2SO_4

$$\underset{\text{Lactonitrile}}{CH_3CHOHCN} + H_2O + 1/2\underset{\text{sulphuric acid}}{H_2SO_4} \longrightarrow \underset{\text{lactic acid}}{CH_3CHOHCOOH} + 1/2\underset{\text{ammonium salt}}{(NH_4)_2SO_4} \quad (3.2)$$

(d) Esterification

$$\underset{\text{lactic acid}}{CH_3CHOHCOOH} + \underset{\text{methanol}}{CH_3OH} \longrightarrow \underset{\text{methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O \quad (3.3.)$$

(e) Hydrolysis by H_2O

$$\underset{\text{methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O \longrightarrow \underset{\text{lactic acid}}{CH_3CHOHCOOH} + \underset{\text{methanol}}{CH_3OH} \quad (3.4)$$

During chemical synthesis racemic mixture of lactic acid is obtained which is the existence of both L(+) and D(-) lactic acid.

3.2.2. Microbial Production

Microorganisms that can produce lactic acid can be divided into two groups: bacteria and fungi. The microorganisms selected for recent investigations of the biotechnological production of lactic acid are listed in Table 3.2.

Table 3.2. Microorganisms used in the biotechnological production of lactic acid (Source: Vijayakumar et al., 2008; Wee et al., 2006).

Microorganism	**Lactic acid** γ **(g.L^{-1})**	**Yield** Y **(g.g^{-1})**	**Productivity** P **(g.L^{-1}.h^{-1})**
Rhizopus oryzae ATCC 52311	83.0	0.88	2.6
Rhizopus oryzae NRRL 395	104.6	0.87	1.8
Enterococcus faecalis RKY1	144.0	0.96	5.1
Lactobacillus rhamnosus ATCC 10863	67.0	0.84	2.5
Lactobacillus helveticus ATCC 15009	65.5	0.66	2.7
Lactobacillus bulgaricus NRRL B-548	38.7	0.90	3.5
Lactobacillus casei NRRL B-441	82.0	0.91	5.6
Lactobacillus plantarum ATCC 21028	41.0	0.97	1.0
Lactobacillus pentosus ATCC 8041	21.8	0.77	0.8
Lactobacillus amylophilus GV6	76.2	0.70	0.8
Lactobacillus delbrueckii NCIMB 8130	90.0	0.97	3.8
Lactococcus lactis ssp. *lactis* IFO 12007	90.0	0.76	1.6
Lactobacillus delbrueckii IFO 3202	60.3	0.95	3.4
Streptococcus bovis 148	_	0.88	14.7
Rhizopus oryzae	93.8	0.77	1.38
Lactobacillus paracasei	88-106	0.91-0.95	3.31-3.67
Lactobacillus lactis	109	0.93	1.09
Enterococcus faecalis RKY1	102	0.97	4.87
L. salivarius sp. *salivarius ATCC 11742*	28.0	0.92	11
L. amylovorus ATCC 33622	93.0	0.52	2.0
L. plantarum ATCC14917	_	_	2.0
L. acidophilus R	8.60	0.17	
S. thermophilus	18.0	0.50	5.9

Although most investigations of lactic acid production were carried out with lactic acid bacteria (LAB), filamentous fungi, such as *Rhizopus*, utilize glucose aerobically to produce lactic acid. *Rhizopus* species such as *R. oryzae* and *R. arrhizus* have amylolytic enzyme activity, which enables them to convert starch directly to L(+)-lactic acid. Fungal fermentation has some advantages in that *R. oryzae* requires only a simple medium and produces L(+)-lactic acid, but it also requires vigorous aeration because *R. oryzae* is an obligate aerobe. In fungal fermentation, the low production rate, below 3 $g.(L.h)^{-1}$, is probably due to the low reaction rate caused by mass transfer limitation. The lower product yield from fungal fermentation is attributed partially to the formation of by-products, such as fumaric acid and ethanol (Wee et al., 2006).

3.2.3 Characteristics of Lactic Acid Bacteria

Lactic acid bacteria (LAB) consist of the Gram-positive genera: *Carnobacterium, Enterococcus (Ent), Lactobacillus(Lb); Lactococcus (Lc), Leuconostoc (Leu), Oenococcus, Pediococcus (Ped), Streptococcus (Srr), Tetragenococcus, Vagococcus,* and *Weissella.* LAB are cocci, with the exception of lactobacilli and carnobacteria which are rods, unable to synthesize ATP by respiration, and that have lactic acid as the major end product from energy-conserving fermentation of sugars. Most LAB are facultatively anaerobic, catalase negative, nonmotile and nonspore forming bacteria. They have high acid tolerance and survive at pH 5.0 and lower. Their acid tolerance gives them a competitive advantage over other bacteria. The optimal temperature for growth varies among the members of genera from 20 ^{o}C to 45 ^{o}C. Most of them are considered GRAS (Generally Regarded as Safe), but some strains of LAB e.g. *Streptococci* are pathogenic. All LAB genera belong to the *Clostridium* which in addition to give LA is also metabolized into formate and acetyl-CoA by pyruvate formate lyase (PFL). In the presence of oxygen PFL is inactivated, and an alternative pathway of pyruvate metabolism becomes active via pyruvate dehydrogenase (PDH), resulting in the production of carbon dioxide, acetyl-CoA and NADH. Lactic acid bacteria are also capable of forming other products, e.g. flavors such as diacetyl and acetoin or bacteriocins (Hofvendahl and Hahn-Hägerdal, 2000).

On the basis of the nature of fermentation, LAB are classified into homofermentative and heterofermentative. Homofermentative LAB produce virtually a

single product, i.e., lactic acid, whereas the heterofermentative LAB produce other products such as ethanol, diacetyl, formate, acetoin or acetic acid, and carbon dioxide along with lactic acid. Lactic acid-producing organisms, most of which are anaerobic, utilize pyruvic acid, which is the endproduct of Embden–Meyerhof pathway. The conversion of pyruvic acid to lactate can be effected by either of the two enzymes, L-lactate dehydrogenase or D-lactate dehydrogenase. The stereospecificity of the lactic acid depends on the type of organism, whose enzyme is involved in the process of lactic acid production (Rojan et al., 2007). Metabolic pathways of homofermentative and heterofermentative LAB are illustrated in Figure 3.2.

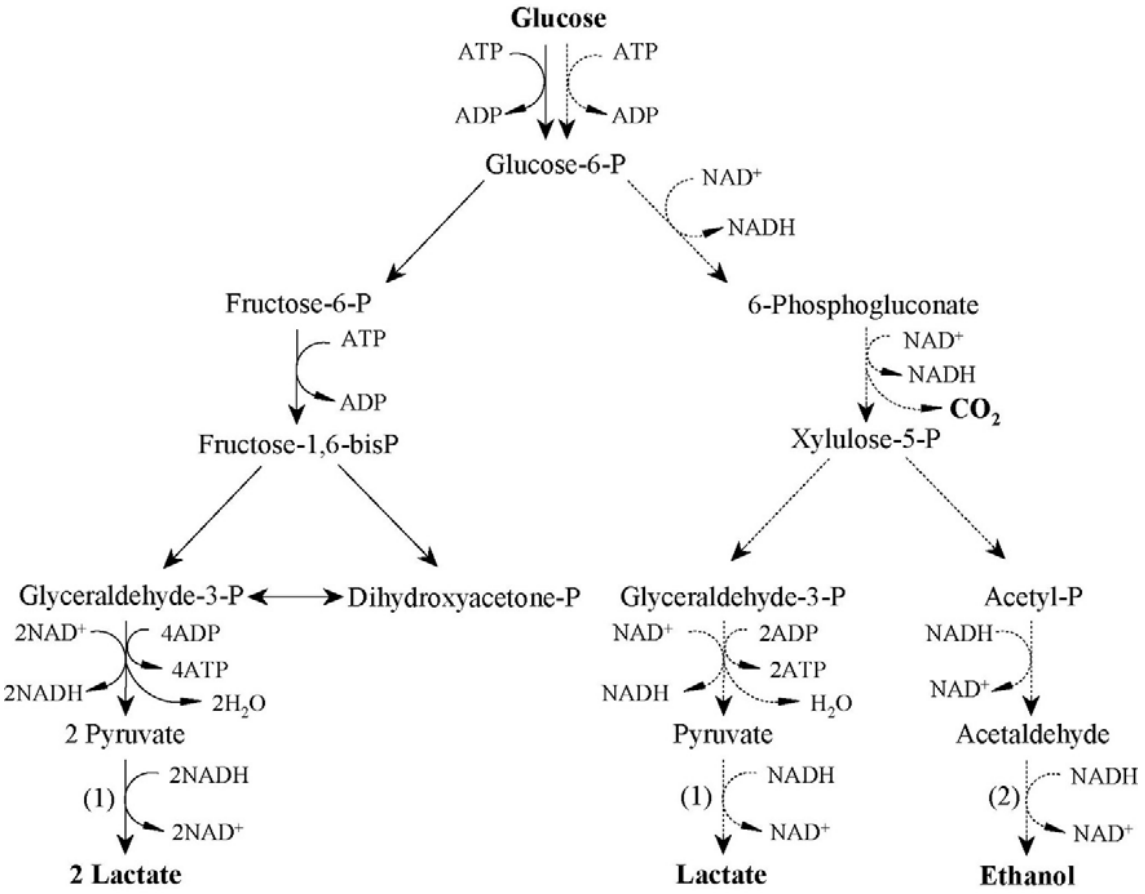

Figure 3.2. Metabolic pathways of homofermentative (solid line) and heterofermentative (dotted line) lactic acid bacteria: P, phosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); (1), lactate dehydrogenase; (2), alcohol dehydrogenase (Source: Wee et al., 2006).

Most LAB produce only one isomeric form of LA, but sometimes there is a slight production of the other isomer. The lactate dehydrogenase (LDH) is stereospecific, giving either D- or L-lactic acid. Which isomeric form(s) of the enzyme present in the LAB mainly determines the isomery of the lactic acid produced. Some of the LAB and their lactic acid configurations are given in Table 3.3.

Table 3.3. Isomeric forms of lactic acid.
(Source: Vijayakumar et al., 2008)

Microorganism	**Homoferm.**	**Heteroferm.**	**Configuration**
Lactobacillus delbrueckii	+	–	D(–)
Lactobacillus lactis	+	–	D(–)
Lactobacillus bulgaricus	+	–	L(+)
Lactobacillus casei	+	–	DL
Lactobacillus plantarum	+	–	DL
Lactobacillus curvatus	–	+	DL
Lactobacillus brevis	–	+	DL
Lactobacillus fermentum	–	+	DL
Sporolactobacillus inulinus	+	–	D(–)
Streptococcus faecalis	+	–	L(+)
Streptococcus cremoris	+	–	L(+)
Streptococcus lactis	+	–	L(+)
Leuconostoc mesenteroides	–	+	D(–)
Leuconostoc dextranicum	–	+	D(–)
Pediococcus damnosus	+	–	DL
Bifidobacterium bifidum	–	+	L(+)

Besides the process parameters also affects the isomeric forms of lactic acid produced. For some applications, such as poly-lactic acid synthesis, an optically pure product or a racemic mixture of constant composition is desirable. For the L-lactic acid producing *Lb. amylophilus, Lb. delbrueckii* and *Lb. rhamnosus*, no D-isomer was produced when the pH was varied or when the amount of nutrients was changed. On the other hand, only D-lactic acid was formed *by Lb. delbrueckii spp. bulgaricu*s in batch and continuous culture, from glucose and lactose, and when the amount of nutrients was

changed. The composition of the racemate formed by *Lb. plantarum* changed with aeration and amount of NaCl. Comparing batch with continuous culture, the amount of the predominant isomer was higher in the former. The amount of the predominant isomer also increased with increasing pH and amount of substrate, but decreased with increasing temperature and when the pH was uncontrolled (Hofvendahl et al., 2000).

3.3. Fermentation Process

Lactic acid is most commonly produced in the batch mode, but numerous examples of continuous culture exist, as well as some fed-batch and semicontinuous/repeated batch fermentations. When comparing batch and continuous fermentation modes, the former gave higher latic acid concentrations and yields in most of the studies. This is mainly that all substrate is used in the batch mode, whereas a residual concentration remains in the continuous one. On the other hand, the continuous mode generally resulted in higher productivities. The major reason is probably that the continuous cultures were run at a high dilution rate, where the advantage over the batch mode is most pronounced. Varying the dilution rate in continuous culture affects both the substrate and nutrient concentrations. However, the effects on the yields and productivities were inconclusive. Fed-batch, semicontinuous and repeated batch mode gave higher yields than the batch mode (Hofvendahl et al., 2000).

More than half of the total consumption of lactic acid is produced in industrial scale traditionally in simple batch fermentations with low productivities. In order to get higher productivities and therefore lower production costs, techniques have been developed for raising the concentration of biomass in the reactor by separating the fluid. This leads to higher volumetric productivities. For this purpose, the most promising bioreactor system seems to be the stirred tank reactor with cell recycles. The biomass is separated by filtration from the fermentation broth and recycled to the reactor. The product stream is free of solids and can directly be processed for product recovery. In the last years many researchers have worked with the investigation of this type of reactor and high lactic acid productivities have been achieved. But there are still problems with fouling of the filtration membrane and the stability of the culture over long fermentation times with a high recycling ratio needs to be further investigated (Börgardts et al., 1998).

At present, batch fermentation is generally employed in industrial sca1e lactic acid production. About 70 % of total lactate produced although continuous processes with cell recycling or cell immobilization systems have been proposed. The restriction imposed by lactic acid on its fermentation due to the lowering pH property as a result of lactic acid formation has been avoided by an extractive fermentation technique employing ion-exchange resin (Vaccari et al., 1993). Within this process the produced lactic acid can be removed from the medium, thus the pH was kept at constant level for the microorganism to grow and to convert sugar into lactic acid by preventing its product inhibition ability.

Lactic acid produced through the fermentation process, has to cope with the problems of purification to meet the required quality standards. An attempt to improve the fermentative production is possible by proper design of an industrial process involving low capital cost for the plant. Also, the low energy costs both in its fermentation and purification are required. In the commercial interest, the investment cost should be minimised, which is possible only when the cell density in fermenter is high. It means that the inhibitory effect of the product on process kinetics must be minimised. Based on these requirements, the extractive bioconversion technique is one of the approaches to achieve the commercially viable lactic acid production (Srivastava and Roychoudhury, 1999).

To increase volumetric production in lactic acid fermentations, high cell density has been achieved through growth of biofilm on activated carbon or cell immobilization in gelatin beads. Removal of inhibitory product has been achieved using both liquid extractants and solid adsorbents either in a product stripping side stream or added directly to the continuous stirred tank reactor. *In situ* product removal during the fermentation has the potential to minimize process waste streams by obviating the need for reactor pH control and lactic acid precipitation (Kaufman et al., 1994).

Lactic acid fermentation is well known for its sensitivity. Several integrated fermentation-separation systems have been used to reduce end-product inhibition and thus to improve overall process efficiency. These systems attempted to increase the productivity of lactic acid and biomass. However, these two targets are interrelated: an increase in biomass concentration will improve the productivity of lactic acid since lactic acid is a partial growth-associate product, while high lactic acid concentration will cause the inhibition of microbial cell growth which in turn results in a low production rate of lactic acid (Ye et al., 1996).

Depending on the type of microorganism whether homofermentative or heterofermentative, the product composition changes. The homofermentative lactic acid bacteria produce lactic acid around 90 %. The rest of the composition is made up of secondary products. For homofermenative microorganism this value is around 50 %.

3.4. Factors Affecting Lactic Acid Fermentation

In the case of lactic acid fermentation, the process mainly depends on the living organism, the microorganisms. These microorganisms need some vital requirements to provide their viability performing lactic acid fermentation. They need nutrients, mineral supplements, carbon, and nitrogen sources. Additionally, the temperature and pH range for their growth should be provided. Aseptic conditions must be supplied. Otherwise, lactic acid can not be produced which is known to be a growth associated product. Furthermore, obtaining a higher productivity and yield is directly related with the optimum growth conditions and the levels of the fermentation medium constituents.

3.4.1. Effect of Temperature

The effect of temperature on the production of LA has only been studied in a few reports. The temperature giving the highest productivity was in some cases lower than the temperature resulting in highest LA concentration and yield, whereas in others the same temperature gave the best results in all categories. For *Lb. amylophilus*, which is known to grow at 15°C but not at 45°C, the optimal temperatures were 25 and 35°C for maximum productivity and yield, respectively. For *Lb. casei* and *Lb. paracasei* the optimal temperature was reported to be between 37 and 44°C, which is contradictory to the information that the strains grow at 15 but not at 45°C. In agreement with previous observations, *Lc.lactis* and *Lb.rhamnosus* exhibited the highest yields and productivities at 33 to 35°C and 41 to 45°C, respectively (Hofvendahl et al., 2000).

One of the studies was about the investigation of the cultivation temperature on the solid-state fermentation (SSF) of lactic acid production by controlling the growth temperatures at 22, 30, 35, and 40 °C. The results from measuring the residual starch and reducing sugar in 4h and 8h indicated that there was an increase in starch hydrolysis and reducing sugar accumulation as the temperature increased from 22 to 30 °C, and a

further increase from 30 to 40 °C resulted in a slight improvement for the saccharification in both *Rhizopus oryzae 2062* and *Rhizopus oryzae 36017* cultures. The lactic acid production and biomass growth were affected by the temperature. The fermentation performance by the *Rhizopus arrhizus 36017* was appeared to be relatively less sensitive than by the *Rhizopus oryzae 2062* with respect to the temperature. Consequently, 30 °C appeared to be an optimum cultivation temperature for both saccharification and fermentation by the Rhizopus species in the SSF (Vijayakumar et al., 2008).

3.4.2. Effect of pH

The fermentation pH is either set at the beginning and then left to decrease due to acid production, or it is controlled by base titration, or by extraction, adsorption, or electrodialysis of LA. The effect of pH has been studied by fermenting at various pH values. In all cases, titration to a constant pH resulted in higher or equal LA concentration, yield and productivity in comparison with no pH control. Removing LA by electrodialysis and extraction, including aqueous two-phase systems, were successful in some of the studies, whereas in others titration gave the same or better results. The optimal pH for LA production varies between 5.0 and 7.0. A pH below 5.7 was only optimal for *Lb.* strains, which are known to tolerate lower pH than *lactococci* (Hofvendahl et al., 2000).

An investigation was completed about the influence of culture pH on lactic acid fermentation from molasses at pH 5.0–9.0. Although the optimum pH for cell growth of *Enterococcus faecalis RKY1* was seen to be 8.0, the lactic acid fermentation at pH 7.0 was completed faster than that at pH 8.0 (Vijayakumar et al., 2008). Several studies are resulted with similar findings which is, although being in the suitable growth pH range, the fermentation pH may differ from the optimum cell growth pH.

3.4.3. Effect of Carbon Sources

The lactic acid production has received much attention due to its numerous uses in the food and biochemical industries. The substrates such as, soybean milk, corn, sulfite waste liquor and potatoes have been used for lactic acid production (Srivastava et

al., 1992). In lactic acid fermentation the selection of the substrate depends on the following criteria; fast fermentation rate, high lactic acid yields, low cost, low levels of contamination, little or no by product formation, ability to be fermented with little or no pre-treatment and year round availability. Generally lactic acid is produced from carbohydrates. The most widely used sources are glucose, lactose, maltose, and sucrose that can be supplied either in pure form or as a constituent of a crude feedstock. The most widely used crude feedstocks are molasses and whey. Molasses is the by-product of sugar industry and contains high levels of sucrose.

Cheese whey liquid waste is generated during the manufacture of cheese. Whey contains high levels of organic matters and its direct discharge into the drain, without further treatment, constitutes an environmental hazard. The idea of converting this waste into valuable products attracted the attention of researchers whom have analyzed several applications of whole cheese whey and/or its major components, lactose and proteins are (Joglekar et al., 2006; Wee et al., 2006; Hofvendahl et al., 2000).

It is also possible to use renewable resources as substrates, such as starch and cellulose in fermentative production. Renewable resources do not give any net contribution of carbon dioxide to the atmosphere as the limited oil and fossil-fuel-based sources. Cellulose, hemicellulose and starch are the most abundant compounds in the world and when hydrolyzed to mainly glucose they are fermentable by a number of microorganisms. Hemicellulose, in contrast to starch and cellulose, contains pentoses, which give rise to by-products such as acetate and ethanol, decreasing the lactic acid yield. Fermentative lactic acid production from renewable resources comprises the following steps: First pretreatment of substrate including hydrolysis to sugars, next fermentation of sugars to lactic acid, then separation of bacteria and solid particles from the broth, and finally purification of lactic acid (Hofvendahl et al., 2000).

3.4.4. Effect of Nitrogen Sources

Traditionally, the most common nutrients for the preparation of fermentative media are yeast extract and peptone, which turn out to be very expensive being able to account for almost 30 % of the total cost of the process. Because of this, the search for alternative, financially competitive nutrient sources is particularly interesting (Vijayakumar et al., 2008).

The medium composition has been investigated from many aspects, including the addition of various mass concentrations of nutrients in the form of yeast extract, peptone or corn steep liquor. The addition of nutrients and higher nutrient mass concentrations generally had a positive effect on the lactic acid production (Vijayakumar et al., 2008).

The addition of nutrients and higher nutrient concentrations generally had a positive effect on the lactic acid production. MRS medium, which contains yeast extract, peptone and meat extract, was superior to yeast extract, which in turn was better than malt extract. This reflects the complex nutrient demands of LAB. Yeast extract alone at high concentration gave higher lactic acid production than yeast extract and peptone in low amounts, but the opposite resulted when the concentration of yeast extract was kept constant and peptone was added. Whey treatment also affects the outcome of fermentation. Whole Wheat Flour did not supply enough nutrients for *Lc. lactis*. This was overcome by adding hydrolyzing enzymes, α- and gluco-amylase and a protease, releasing nutrients in forms of amino acids from the flour (Hofvendahl et al., 2000).

As the LAB require high level of nutrient supplementation like amino acids, vitamins etc., yeast extract was supplemented as best nutrient. The addition of malt-combing nuts, waste from the industry of barley malting, were employed to reduce the high cost of such supplements. Whey protein hydrolyzate was supplemented for lactic acid production from whey. Soybean meal and cottonseed were used as the inexpensive nitrogen source. Addition of mustard powder in pickle brine increases the rate of acid production. Those nutrients could substitute partially for yeast extract. However, large amount of their supplementation contributed to an increase in the concentration of impurities, corresponding to the increase in separation cost and the decrease in lactic acid recovery. Wheat bran hydrolyzate or wheat bran extract was also used as the nitrogen source. Nutrient supplementation raises the cost of production, as unutilized nutrients will raise the purification cost. The amylase-treated rice bran and wheat bran was used for the DL lactic acid production by *Lactobacillus sp.,* as they contain several nutritional factors besides carbohydrate. The lactic acid production with wheat starch was studied. The addition of protease in the medium enhances the lactic acid production; the productivity increased up to 1.5 g l^{-1} h^{-1} from 0.23 g l^{-1} h^{-1}. When the protease, along with wheat starch, was supplemented with peptide, vitamins, and amino acids, the yield was raised to 2.2, 2.4, and 2.8 g l^{-1} h^{-1}, respectively. It was reported that

using protease-treated wheat bran, around tenfold decrease in supplementation of the costly medium component, like yeast extract, was achieved together with a considerable increase in the lactic acid production level. Various nitrogen sources like silkworm larvae, yeast autolyzate, dry yeast, and shrimp waste as a replacement of yeast extract in cane juice medium were investigated. At the same concentration of nitrogen sources (1% w/v), addition of silkworm larvae, yeast autolyzate, and shrimp waste all led to increases in lactic acid production more than that attained with yeast extract. But colony forming unit and cell dry weight were highest with yeast extract (Rojan et al., 2007).

3.4.5. Effect of Mineral Salts

Mineral salts play a vital role in the lactic acid fermentation. The components, K_2HPO_4, sodium acetate, sodium sulfate, $FeSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot 4H_2O$ were found to be significant. The components KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, tri-sodium citrate and sodium succinate were found to be less significant on lactic acid products (Vijayakumar et al., 2008).

Lactic acid fermentation with whey powder was performed by the supplementation of K_2HPO_4, KH_2PO_4, $MgSO_4$, and $MnSO_4 \cdot 4H_2O$ by *L. casei*. It was obtained that Mn^{+2} was essential for lactic acid fermentation of whey. It was reported that it is a constituent of lactate dehydrogenase playing a role in the metabolic pathway (Büyükkileci, 2000).

3.5. Purification of Lactic acid

In recent years fermentation processes have become more industrially successful because of the increasing demand for naturally produced lactic acid. The major drawback of processes is the high cost of recovery, purification and concentration of the acid, which represents a considerable portion of the lactic acid production costs.

Highly purified lactic acid as monomer is required for the chemical synthesis of polylactide. As with most polymerizations, monomer purity is highly critical in the synthesis of polylactides and a purity of 99.9% or higher is usually required with the starting lactide material. However, the purification of lactic acid obtained from bacterial fermentation is difficult due to its low vapor pressure (boiling temperature of 122 ^{o}C at

0.0164 atm), its tendency to undergo self-esterification and the presence of troublesome impurities. The conventional methods for the recovery of pure lactic acid were crystallization, extraction with a solvent and filtration-carbon treatment-evaporation (Choi and Hong, 1999).

Conventional lactic acid purification from fermentation broth consisted in recovering lactate as a calcium salt, but this technique is expensive and produces wastes with an important contaminant load. Actually alternative processes have been developed such as electrodialysis, solvent extraction and ion exchange. The extractive fermentation of lactic acid was recently proposed as an alternative technology. A fluidized bed column for integrative protein purification from unclarified culture broth was developed to diminish the overall time of conventional downstream processing and increase the product yield (Raya-Tonetti et al., 1999).

Cross-flow filtration has been demonstrated to be an efficient process for high cell density culture with high productivity of lactic acid by continuous removal of fermentation broth from fermenter and recycling biomass. A productive process may not be achieved in practice since nutrients, especially the carbon source, are not efficiently utilized due to removal of fermentation broth for control of lactic acid concentration. Also a low concentration of lactic acid in the effluent from the fermenter is a burden on the recovery of lactic acid in downstream processes. To improve the performance of conventional biomass recycling fermentation, the spent broth should be re-used through recovery of lactic acid *in situ* with an appropriate downstream process. The spent broth from which lactic acid was extracted is then re-used for subsequent cell recycle fermentations after being supplemented with some necessary nutrients and a carbon source. A novel integrated fermentation system was developed in this study to carry out biomass recycle and efficient reutilization of broth media by coupling a biomass recycling fermentation to an ion-exchange resin column (Ye et al., 1996).

Since it is the product recovery cost rather than the feedstock or fermentation cost that dictates the production economics of the process, considerable recent work has been carried out to study methods of purification. One method that has received widespread attention is the extraction with organic solvents. However, since this method presents a number of problems related to the toxicity of the solvent, its selectivity, distribution ratio of the solute and solvent recovery, other separation processes have been investigated. For example, a number of authors have analyzed the application of liquid membrane and reverse osmosis and Prigent has proposed and studied lactic acid

recovery in a two-stage process consisting of ultrafiltration and electrodialysis. However, in spite of all these developments at the scientific level, to date, few have found their way into commercial practice. The reason for the apparent lack of success in industrial developments is that they are at present fairly costly. An alternative to all these methods that would eliminate much of downstream processing costs associated with the concentration of the acid, would be a technology focused on the purification of the fermentation broth to obtain a lactic acid solution suitable for use as preservative in the food industry. Taking into account that the fermentation broths do not contain lactic acid but lactate (because during the fermentation process to produce lactic acid a neutralizing agent is used to keep the medium at an optimum pH value), ion exchange using cationic resins could be a possible method for obtaining a lactic acid solution from the broths. Although NaOH is a common neutralizing agent in lactic fermentations, the Na^+ ion becomes a common cation to be removed from the fermentation broths (Rincon et al., 1997).

The traditional recovery process of lactic acid from fermentation broth is quite complicated, in the conventional proceses; lactic acid has been recovered from the fermentation beer by precipitation of calcium lactate with calcium hydroxide. In this recovery, calcium lactate is precipitated, recovered by filtration, and converted to lactic acid by addition of sulfuric acid. The dilute lactic acid product is then sequentially purified using activated carbon, evaporation and crystallization. These stages account for up to 50% of the production costs. On the other hand, the accumulation of lactic acid product in the fermentation broth often inhibits further product formation. Reactor productivities are low and the products are obtained in dilute form. The effects of end product inhibition can be reduced by removing toxic metabolic products from the broth *in situ* by several methods. Fermentation products, such as lactic acid, can be removed from the broth by conventional liquid-liquid extraction. This process requires high volumes of solvent. Like many such processes, the product recovery and purification contribute significantly to the cost of production. As an alternative, Liquid Surfactant Membrane (LSM) extraction has been proposed. Interest in liquid surfactant membranes over other separation techniques is the large surface area available for mass transfer which results in a fast rate of separation. In spite of these apparent advantages, very few industrial applications have been reported so far. Several drawbacks were shown to hinder implementation, mainly complexity of operation and swelling in LSMs. The use of supported liquid membranes for the recovery of lactic acid offers unique advantages.

Some of the advantages are lower energy consumption, higher separation factors in a single stage and the ability to concentrate lactic acid during the separation. However, supported liquid membrane often suffers from membrane instability (Turhan and Etzel, 2004).

The growth of microorganisms may be limited either by the availability of nutrients or by the accumulation of inhibitory metabolic products. Thus, lactic acid inhibition of fermentation can be avoided by extractive fermentation techniques. Studies were performed by attaching an ion exchange resin-packed column to a fermenter for the separation of lactic acid. The recirculation of the fermentation broth through the resin to the fermenter minimized the inhibition of the batch fermentation by lactic acid. An innovative process for the recovery of lactic acid from fermentation broths is described. Fermentation was carried out in a Continuous Stirred Tank Fermenter (CSTF) with an ion exchange resin system for the removal of lactic acid from the fermentation broth. Lactate was adsorbed as it was formed on to a strongly acidic anion exchange resin in the carbonate form placed inside a column and eluted with ammonium carbonate during the course of fermentation. The resulting ammonium lactate was passed through a strong cation exchange resin in the H^+ form to obtain lactic acid. By this means, it was possible to maintain an actively growing culture in a medium of low lactate concentration. The utilization of the fermenter-ion exchange resin system offers the advantages of eliminating the inhibitory effects of lactate at the same time, reducing the costs of recovery and purification of the lactic acid from the fermentation broth (Monteagudo and Aldavero, 1999).

Chen and Ju examined the activated carbon in lactic acid fermentation from glucose using *Lactobacillus delbrueckii*. The cells were also rapidly adsorbed on activated carbon, which will limit its application in lactic acid fermentation. The problem originates from the structure of such materials, which has a wide pore size distribution. The interior is accessible by all sizes of molecules since the pore size ranges from micrometer to angstrom level. The competition by other heavier hydrocarbons in the broth for adsorption sites will reduce the selectivity and the capacity of the activated carbon (Chen and Ju, 1998). An alternative method of recovery of lactic acid from fermentation broth, based on adsorption on Silicalite molecular sieves, has been investigated. Silicalite pellets have pore sizes suitable for adsorption of lactic acid, yet which can exclude larger size components of the fermentation broth,

such as proteins. It also has low affinity for water and it is characterized by high thermal and hydrothermal stability (Aljundi et al., 2005).

3.6. Applications of Lactic Acid

Lactic acid is a useful product in the food industry as a biologically produced acidulant and preservative. It is used in cosmetic industry, in medical applications, in pharmaceutical area, in leather processing and in textile industries. Furthermore, lactic acid is widely used as a starting material for chemical synthesis, because of its optical activity and its hydroxyl and carboxyl moieties. Lactic acid has the potential of becoming a very large volume chemical intermediate, produced from renewable resources for use as a feedstock for biodegradable plastics and other environmentally friendly green compounds. But until now, the extensive use of lactic acid in chemical industry is hampered by the high production costs of optically pure (biologically produced) lactic acid (Börgardts et al., 1998; Wee et al., 2006; Joglekar et al., 2006).

Nowadays, the industrial application of this acid as a precursor for polylactic acid polymers requires one of the isomers to produce high quality products for biomedical applications and drug delivery (Raya-Tonetti et al., 1999).

Polylactide, which is known to be biocompatible and biodegradable, can be used as biodegradable polymer for bulk product and is now widely used for many biomedical applications. In surgical suture production, drug delivery systems, and the internal bone fixation applications polylactide is used (Choi and Hong, 1999).

Poly-L (+)-lactic acid is a polymer used in medical applications such as sutures, scaffold materials for artificial organs and implantable drug delivery systems (Vaccari et al., 1993). Fundamental applications of lactic acid are tabulated in Table 3.4.

Table 3.4. Applications and commercial uses of lactic acid and its salts. (Source: Wee et al., 2006; Vijayakumar et al., 2008)

Chemical industry	-Descaling agents -pH regulators -neutralizers -chiral intermediates -green solvents -cleaning agents -slow acid releae agents -metal complexing agents
Chemical feedstock	-propylene oxide -acetaldehyde -acrylic acid -propanoic acid -2,3-pentanedione -ethyl lactate -dilactide -poly(lactic acid)
Cosmetic industry	-moisturizers -skin lightning agents -pH regulators -anti-acne agents -humectants -anti-tartar agents
Food industry	-acidulants -preservatives -flavours -pH regulators -improving microbial quality -mineral fortification
Pharmaceutical industry	-parenteral solution -dialysis solution -mineral preparations -Tablettings -prostheses -surgical sutures -controlled drug delivery systems

CHAPTER 4

ZEOLITES

4.1. Chemistry and Crystal Structure of Zeolites

Cronstedt, a Swedish mineralogist discovered zeolites in 1756. Zeolite means, "boiling stones" in Greek words. Since then, about 50 natural species of zeolites have been recognized. The most common ones are analcime, chabazite, clinoptilolite, erionite, ferrierite, heulandite, laumontite, mordenite, and phillipsite. Besides, more than 100 species have been synthesized in the laboratory. Some of the well known synthetic zeolites are zeolites A, X, Y and ZSM-5. Zeolites are crystalline, hydrated aluminosilicates of alkali and earth metals that possess infinite, three-dimensional crystal structures. They are characterized by an ability to lose and gain water reversibly and to exchange some of their constituent elements without major change of structure (Mumpton, 1985).

Zeolites belong to the family of the tectosilicates, where the SiO_4 tetrahedra form three-dimensional supercages. In their structure, some Si atoms are substituted by Al atoms, resulting in a negatively charged structure that originates from the difference between the $(AlO_4)^{-5}$ and $(SiO_4)^{-4}$ tetrahedra. These negative sites are balanced by counterions, usually alkaline and alkaline earth cations, which can be substituted by other cations, thus, providing zeolites with the property of ion exchange (Valdes et al., 2006).

In the zeolite framework, each aluminum atom introduces one negative charge on the framework which must be balanced by an exchangeable cation (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, etc.). The exchangeable cations located within the framework play a crucial role in adsorption and thermal properties of the zeolites (Çakıcıoğlu-Özkan and Ülkü, 2005).

Zeolites are tectosilicates, that is, they consist of three-dimensional frameworks of silicon-oxygen $(SiO_4)^4$ tetrahedral, wherein all four corner oxygen atoms of each tetrahedron are shared with adjacent tetrahedral. This arrangement of silicate tetrahedral reduces the overall oxygen:silicon ratio to 2:1, and if each tetrahedron in the framework contains silicon as its central atom, the structures are electrically neutral, as is quartz

(SiO_2). In zeolite structures, however, some of the quadrivalent silicon is replaced by trivalent aluminum, giving rise to a deficiency of positive charge. This charge is balanced by the presence of mono and divalent elements such as sodium (Na^+), calcium (Ca^{2+}), and potassium (K^+) elsewhere in the structure. Thus, the empirical formula of a zeolite is of the type:

$$M_{2/n}O \cdot Al_2O_a \cdot xSiO_2 \cdot y\,H_2O \qquad (4.1)$$

where M is any alkali or alkaline earth element, n is the valence charge on that element, x is a number from 2 to 10, and y is a number from 2 to 7. The empirical and unit-cell formula of clinoptilolite, the most common of the natural zeolites, is:

$$(Na,K)_2O \cdot Al_2O_3\ 10SiO_2 \cdot 6\,H_2O \qquad (4.2)$$

or

$$(Na_4K_4)\ (Al_8Si_{40})\ O_{96}.24H_2O \qquad (4.3)$$

Elements or cations within the first set of parentheses in the formula are known as exchangeable cations; those within the second set of parentheses are called structural cations, because with oxygen they make up the tetrahedral framework of the structure. Loosely bound molecular water is also present in the structures of all natural zeolites, surrounding the exchangeable cations in large pore spaces. Whereas the framework structures of quartz and feldspar are dense and tightly packed, those of zeolite minerals are remarkably open and void volumes of dehydrated species as great as 50 percent are known . Each zeolite species has its own unique crystal structure and, hence, its own set of physical and chemical properties. Most structures, however, can be visualized as SiO_4 and AlO_4 tetrahedral linked together in a simple geometrical form. This particular polyhedron is known as a truncated cube-octahedron. It is more easily seen by considering only lines joining the midpoints of each tetrahedron, as shown in Figure 4.1.

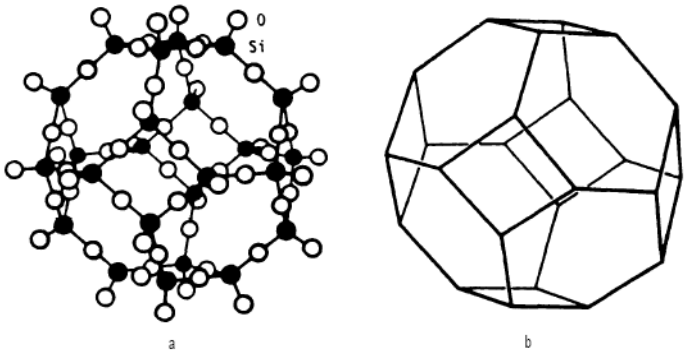

Figure 4.1. Typical polyhedron of zeolites.
(Source: Mumpton, 1985)

Once water is removed from a zeolite, considerable void space is available within both the simple polyhedra building blocks and the larger frameworks formed by several polyhedra. Three and two dimensional framework structure is represented in Figure 4.2. Although water and other inorganic and organic molecules would appear to be able to move freely throughout a dehydrated zeolite framework, the passage ways leading into the simple polyhedra are too small for molecules to pass; however, ports or channels up to 8 Å in diameter lead into the large, three-dimensional cavities (Mumpton, 1985).

The crystalline skeleton may be formed by a three-dimensional combination of tetrahedra TO_4 (T = Si, Al, B, Ga, Ge, Fe, P and Co) bonded by oxygen atoms. The T–O–Si links result in rings of great variety that are responsible for zeolites cage and channel framework and also for their capacity for molecular discrimination, in both size and shape, as well as their molecular sieve character. Due to their rigid three-dimensional network, they exhibit this property without significant structural changes and are free of shrink-swell behavior. However, the process depends on so many characteristics of the zeolite, the solution and the ions present, that the behavior of zeolites does not exactly follow the normal rules of ion exchange (Valdes et al., 2006).

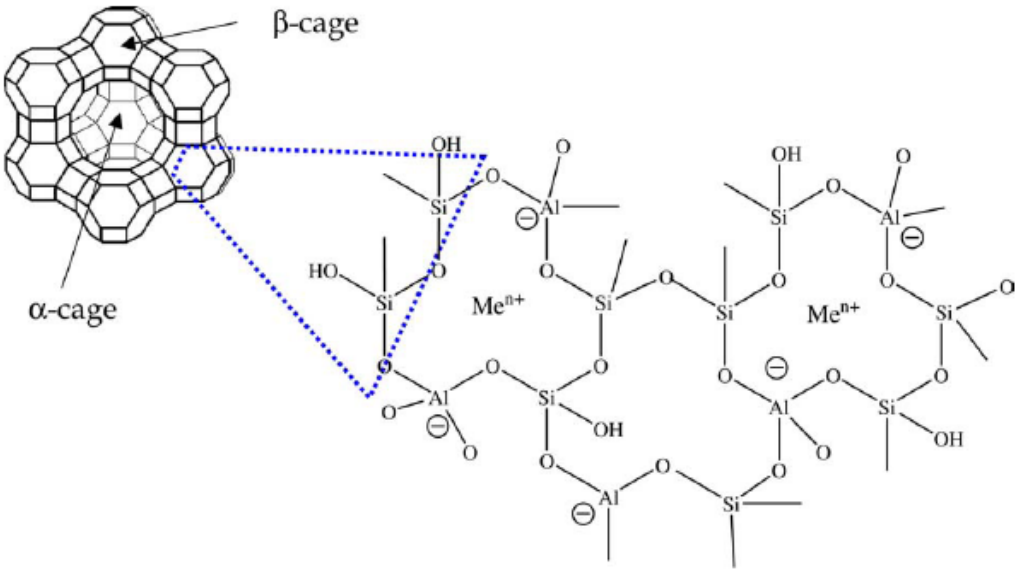

Figure 4.2. Three and two dimensional representation of framework structure of zeolites (Source: Valdes et al., 2006).

4.2. Properties of Zeolites

A zeolite has an infinite, open, three dimensional structure. It is further able to lose and gain water reversibly and to exchange extra framework cations, both without change of crystal structure. The large structural cavities and the entry channels leading into them contain water molecules, which form hydration spheres around exchangeable cations. On removal of water by heating at 350–400°C, small molecules can pass through channels, but larger molecules are excluded-the so called "molecular sieve" property of crystalline zeolites. The uniform size and shape of the rings of oxygen in zeolites contrasts with the relatively wide range of pore sizes in silica gel, activated alumina, and activated carbon (Mumpton, 1999).

Due to the diffusion limitations of guest molecules in micropores, the catalytic adsorption or other guest transport dependent performance of molecular sieve materials may be strongly coupled with particle morphology. Therefore molecular sieve crystal and shape manipulation, as well as controlling the formation of molecular sieve particle assemblies are important aspects of molecular sieve practice (Drews and Tsapatsis, 2005).

In spite of their molecular-sieving properties, their adsorption capacity and especially their ion-exchange possibilities, zeolites have been used in analytical separation methods. There are many studies dedicated to ion-exchange chromatography

with zeolites, mostly focused on the interaction between zeolite and exchanged ions, determination of selectivity series, obtaining adsorption isotherms. In the simplest approach to separation, zeolites can be used as sorbent materials for charged molecules. The attainable enrichment and clean-up will depend primarily on the affinity of the zeolite for the selected target molecules (Valdes et al., 2006).

Furthermore, zeolites adsorb polar molecules with high selectivity. The adsorption selectivity for H_2O, however, is greater than for any other molecule. The weakly bonded extraframework cations can be removed or exchanged readily by washing with a strong solution of another cation. The cation exchange capacity (CEC) of a zeolite is basically a function of the amount of Al that substitutes for Si in the framework tetrahedra; the greater the Al content, the more extraframework cations needed to balance the charge. Natural zeolites have CECs from 2 to 4 milliequivalents/g (meq/g), about twice the CEC of bentonite clay. Unlike most noncrystalline ion exchangers, e.g., organic resins and inorganic aluminosilicate gels, the framework of a crystalline zeolite dictates its selectivity toward competing ions. The hydration spheres of high field-strength cations prevent their close approach to the seat of charge in the framework; hence, cations of low field strength are generally more tightly held and selectively exchanged by the zeolite than other ions. Some of the selected physical properties are tabulated in Table 4.1. Clinoptilolite has a relatively small CEC (2.25 meq/g), but its cation selectivity is:

$$Cs > Rb > K > NH4 > Ba > Sr > Na > Ca > Fe > Al > Mg > Li \qquad (4.4)$$

This preference for larger cations, including NH_4 , was exploited for removing NH_4-N from municipal sewage effluent and has been extended to agricultural and aquacultural applications. Most zeolites in volcanogenic sedimentary rocks were formed by the dissolution of volcanic glass (ash) and later precipitation of micrometer-size crystals, which mimic the shape and morphology of their basalt counterparts Sedimentary zeolitic tuffs are generally soft, friable, and lightweight and commonly contain 50–95% of a single zeolite; however, several zeolites may coexist, along with unreacted volcanic glass, quartz, K-feldspar, montmorillonite, calcite, gypsum, and cristobalite/tridymite. Extrinsic properties of the rock (e.g., siliceous composition, color, porosity, attrition resistance, and bulk density) are also important in many applications. Thus, the ideal zeolitic tuff for both cation-exchange and adsorption applications should

be mechanically strong to resist abrasion and disintegration, highly porous to allow solutions and gases to diffuse readily in and out of the rock, and soft enough to be easily crushed (Mumpton, 1999).

Table 4.1. Selected physical properties of important zeolites.
(Source: Mumpton, 1999)

Zeolite	Representative unit-cell formula	Channel dimensions, Å	Thermal stability (relative)	CEC, meq/g
Analcime	$Na_{10}(Al_{16}Si_{32}O_{96}).16H_2O$	2.6	High	4.54
Chabazite	$(Na_2Ca)_6(Al_{12}Si_{24}O_{72}).40H_2O$	3.7 x 4.2	High	3.84
Clinoptilolite	$(Na_3K_3)(Al_6Si_{30}O_{72}).24H_2O$	3.9 x 5.4	High	2.16
Erionite	$(NaCa_{0.5}K)_9(Al_9Si_{27}O_{72}).27H_2O$	3.6 x 5.2	High	3.12
Faujasite	$(Na_{58})(Al_{58}Si_{134}O_{384}).240H_2O$	7.4	High	3.39
Ferrierite	$(Na_2Mg_2)(Al_6Si_{30}O_{72}).18H_2O$	4.3 x 5.5	High	2.33
Heulandite	$(Ca_4)(Al_8Si_{28}O_{72}).24H_2O$	4.0 x 5.5 4.4 x 7.2 4.1 x 4.7	Low	2.91
Laumonitte	$(Ca_4)(Al_8Si_{16}O_{48}).16H_2O$	4.6 x 6.3	Low	4.25
Mordenite	$(Na_8)(Al_8Si_{40}O_{96}).24H_2O$	2.9 x 5.7 6.7 x 7.0	High	2.29
Phillipsite	$(NaK)_5(Al_5Si_{11}O_{32}).20H_2O$	4.2 x 4.4 2.8 x 4.8 3.3	Medium	3.31
Linde A	$(Na_{12})(Al_{12}Si_{12}O_{48}).27H_2O$	4.2	High	5.48
Linde X	$(Na_{86})(Al_{86}Si_{106}O_{384}).264H_2O$	7.4	High	4.73

Regions that have undergone past volcanic activity are likely to contain significant deposits of zeolite minerals. Because of their inherently fine crystal size, zeolites are not easily identified by ordinary microsscopic techniques and have often been missed by geologists and mineralogists studying these formations. In general, zeolite identification requires sophisticated X-ray diffraction and electron microscopic equipment (Mumpton, 1999).

The high probability of finding minable deposits of zeolites in countries where there has been considerable volcanic activity in the past, supported the studies for searcing the deposits. In the early and late 1970s, the scientists found out large distribution of such materials in volcanogenic sedimentary rocks in Turkey (Mumpton, 1999).

The outer surface of solids is normally characterized by a peculiar reactivity, due to (a) loose binding of particles to the structure, (b) presence of primary or induced polarity and (c) frequent occurrence of surface defects. Zeolites, in particular, exhibit large, negatively charged, external surfaces, balanced by hydrated inorganic cations, which results in possible interactions either with other cations or with polar molecules (Colella, 2007).

Their high surface area, and therefore their high adsorption capacity, as well as their ion-exchange properties make them very useful in many fields. Another important inherent property of aluminosilicate zeolites is their catalytic action due to their strongly acidic nature. The terminal hydroxyl groups in the Si–OH...T framework are considered Brönsted-acid sites and the interaction of hydroxyl oxygen with a T atom produces Lewis-acid sites. Their technological importance in the petroleum industry arises precisely from their use as acid catalysts. Zeolites can be considered as catalytic micro-reactors (Valdes et al, 2006). Within the last decade, numerous studies have been devoted to understand the physico-chemical properties of zeolites. Some of the principal advantages of zeolites are their low cost of extraction, their availability in great volumes, and their excellent stability in chemical and thermal processes. They can also be submitted to diverse treatments in order to give them desired physical and chemical properties. Those same properties that make zeolites an important material in petrochemistry, environmental science, agriculture and many other fields could be exploited in analytical chemistry (Valdes et al, 2006).

4.3. Natural Zeolite-Clinoptilolite

Clinoptilolite, one of the most commonly observed natural zeolite mineral, is a member of the heulandite group. Its framework structure consists of three channels. The channel A and B, 10-member and 8-member rings, respectively; are parallel to each other while the channel C, 8-member ring, intersects the channel A and B. Strong

specific interaction of the cationic sites with water vapor, due to high dipole moment of the water leads to selective adsorption of water by the zeolites (Çakıcıoğlu-Özkan and Ülkü, 2005).

Natural zeolites are crystalline aluminosilicates of alkaline and alkaline-earth metals. They possess many desirable ion exchange, molecular sieving and catalytic properties, which make them valuable minerals. The availability of large volumes of internal space is one of the most desirable characteristics of zeolites for sorbent applications. Window dimensions generally depend on the structure of the zeolite, but the "fine tuning" can be achieved by variation of the cation type which is located at preferred sites within the framework of the zeolite. Larger suitable volumes are associated with the presence of small cations including protons. In addition, the composition of the zeolite framework (Si/Al ratio) has a significant effect on the sorptive properties of the adsorbent. There are two main types of modification procedure, which can be used to form proton exchanged clinoptilolite (H-CLN); ammonium exchange followed by calcination or direct ion exchange with dilute acid solution (Kurama et al., 2002). The treatment of the natural zeolitic tuff with HCl causes the dealumination (hydrolysis of Al–O–Si bonds) besides the exchange of the cations by hydronium ions (H_3O^+) (Çakıcıoğlu-Özkan and Ülkü, 2005).

One of the chemical property of the zeolites is the Si/Al ratio. In general zeolites have a low Si/Al ratio and therefore a high degree of ion-exchange capacity. If the Al content and thus the ion-exchange capacity of a zeolite is reduced, it is said to become more hydrophobic or organophilic in its sorptive characteristics (Klint and Eriksson, 1997).

Despite the fact that zeolitic Si–OH–Al groups are well known as donors of protons towards molecules acting as proton acceptors (Kukulska-Zazac et al., 2006). As inorganic support materials, zeolites offer interesting characteristics, such as mechanical and chemical resistance as well as high surface area. They have the advantage that the basic/acidic nature of the material can be modified by varying the Si/Al ratio or by introducing different metals (Me) into the crystalline framework and by changing the Si/Me ratio. Furthermore, zeolite acidity can be modified by exchanging extra-framework metal cations with H^+. Finally, zeolites are known to be stable both in wet and dry conditions (Tavolaro et al., 2007).

Zeolites, in particular natural zeolites, present, interesting surface properties, which make them able to interact with big organic molecules and cations, which are

unable to enter their microporous structure. In recent years surface activity has deeply been evaluated, testing a series of attractive applications in environment and health-care fields. Particular attention will be paid to clinoptilolite-rich tuffs, in consideration of their wide availability in many tens of deposits all over the world (Colella, 2007).

Electrokinetic properties of fine particles in an aqueous solution such as the isoelectric point (iep) and potential determining ions (pdi) play a significant role in understanding the adsorption mechanism of inorganic and organic species at the solid/solution interface (Ersoy and Çelik, 2002).

Direct hydrogen ion exchange occurs readily in a zeolite to a limited extent (10±15 wt.%) with water washing. Achievement of higher exchange requires use of a solution with a greater proton concentration, i.e. dilute acid. Since most zeolites are acid sensitive, their applications are limited. But silica rich zeolites such as mordenite, clinoptilolite and erionite can be directly treated with suitable reagents (e.g. acids, chelating agents, salts) to increase the sorption capacity of sorbent. In zeolites containing a high percentage of silica, clinoptilolite was of special interest to early research. They pointed out that the sorption capacity of clinoptilolite could be increased up to mild acid concentration. Since then several researchers have shown different modifications to clinoptilolites which determine their catalytic applicability (Kurama et al., 2002).

4.4. Applications of Zeolites

The discovery of natural zeolites as large, widespread, mineable, near-monomineralic deposits in tuffaceous sedimentary rocks in many countries suported the importance of these useful industrial minerals whose exciting surface and structural properties have been exploited in industrial, agricultural, environmental, and biological technology. Like talc, diatomite, wollastonite, chrysotile, vermiculite, and bentonite, zeolite minerals possess attractive adsorption, cation-exchange, dehydration–rehydration, and catalysis properties, which contribute directly to their use in pozzolanic cement; as lightweight aggregates; in the drying of acid-gases; in the separation of oxygen from air; in the removal of NH_3 from drinking water and municipal wastewater; in the extraction of Cs and Sr from nuclear wastes and the mitigation of radioactive fallout; as dietary supplements to improve animal production; as soil amendments to

improve cation-exchange capacities (CEC) and water sorption capacities; as soilless zeoponic substrate for greenhouses and space missions; in the deodorization of animal litter, barns, ash trays, refrigerators, and athletic footwear; in the removal of ammoniacal nitrogen from saline hemodialysis solutions; and as bactericides, insecticides, and antacids for people and animals. Besides, uses in pollution control, the handling and storage of nuclear wastes, agriculture, and biotechnology are also forthcoming (Mumpton, 1999).

There are several studies related with the natural zeolites from Gördes. The structure of clinoptilolite under the effect of microwave were investigated by Akdeniz and Ülkü (2007). The effect of HCl treatment on the water vapor adsorption characteristics of clinoptilolite rich natural zeolites were studied by Çakıcıoğlu-Özkan and Ülkü (2004). Top and Ülkü has investigated the antibacterial behaviour of exchanged clinoptilolite and the cation exchange behavior of natural zeolites (2004). Narin, studied the carbon monoxide adsorption in clinoptilolite, and the release properties of nitric oxide adsorbed natural zeolites (2001; 2009).

Synthetic molecular sieves are being used extensively as adsorbents for the purification of gaseous waste in commercial applications. They have been used extensively to remove water and carbon dioxide from natural/waste gas streams. Another important application of clinoptilolites involves the removal of corrosive H_2S from methane gas produced during the anaerobic digestion of sludge in municipal sewage treatment plants (Çakıcıoğlu-Özkan and Ülkü, 2005).

Based on the strong water–clinoptilolite interactions, several processes with different applications of water adsorption could be mentioned such as air drying, energy storage, hydrocarbon drying applications. In separation (gas or liquid) technology, the existence of water hinders the adsorption of the others since it is selectively adsorbed by various adsorbents, especially by the zeolites. On the other hand the presence of water determines the product obtained in catalytic reactions. Adsorption of small water molecule (molecular diameter: 2.66 A $^{\circ}$) is also used to obtain information regarding the impurities in natural zeolite tuff and characterize the microporous adsorbents such as microporous carbon and zeolite. The water vapor adsorption on cation exchanged natural zeolite, HZSM-5 and carbon have been studied in the analysis of the interaction mechanism between water and energetically heterogeneous surface (Çakıcıoğlu-Özkan and Ülkü, 2005).

The use of natural zeolites for environmental remediation (organic compounds and heavy-metal ions) is well established. Also, natural zeolites have been found to be helpful in the control of malodors (e.g., hydrogen sulfide and ammonia) emanating from confined livestock-rearing areas, kennels, pet shops, zoos and pet-litter trays (Valdes et al., 2006).

Natural zeolites have found applications as fillers in the paper industry, as lightweight aggregate in construction, in pozzolanic cements and concrete, as ion-exchangers in the purification of water and municipal sewage effluent, as traps for radioactive species in low-level wastewaters from nuclear facilities, in the production of high purity oxygen from air, as reforming petroleum catalysts, as acid-resistant absorbents in the drying and purification of natural gas, and in the removal of nitrogen compounds from the blood of kidney patients. Besides they are used as animal feed, fertilizer carrier, odor controller, oil absorbent, catalysts, desiccant, gas absorbents. The applications and potential applications of both synthetic and natural zeolites depend, of course, on their fundamental physical and chemical properties. These properties are in turn related directly to the chemical composition and crystal structure of individual species (Mumpton, 1985).

Natural zeolites have been used as lightweight aggregate, as soil amendments in agronomy and horticulture, as energy exchangers in solar refrigerators, as dietary supplements in animal diets, as consumer deodorizers, in taking up ammonia from animal manures, and as ammonia filters in kidney-dialysis units. From their use in construction, to their role as hydroponic (zeoponic) substrate for growing plants on space missions, to their recent success in the healing of cuts and wounds, natural zeolites are now considered to be full-fledged mineral commodities, the use of which promise to expand even more in the future (Mumpton, 1999).

Large-scale cation-exchange processes using natural zeolites demonstrated the effectiveness of clinoptilolite for extracting NH_4^+ from municipal and agricultural waste streams. The clinoptilolite exchange process in sewage treatment plant removes >97% of the NH_4^+ from effluent (Mumpton, 1999).

In general, natural zeolites do not compete with their synthetic counterparts in adsorption or catalytic applications because of their inherent lower adsorption capacities and, to some extent, to the presence of traces of Fe and other catalyst ‘‘poisons.’’ Most natural materials have smaller pore openings than the synthetics. Despite the low cost of the natural materials (a few cents per kilogram), the economics of hardware

construction, activation, and regeneration favor the more expensive synthetics, for most adsorption applications (Mumpton, 1999).

In agriculture by using clinoptilolite-rich tuff as a soil conditioner, significant increases in the yields of wheat (13–15%), eggplant (19–55%), apples (13–38%), and carrots (63%) were reported. A substrate consisting of a specially cation-exchanged clinoptilolite and a synthetic apatite containing essential trace nutrients for use as a plant-growth medium in Shuttle flights. This formulation may well be the preferred substrate for vegetable production aboard all future space missions and in commercial green houses.

Natural zeolites are increasingly being used in biomedical applications (Pavelic et al., 2001). Natural phillipsite and certain synthetic zeolites were found to be effective filter media to remove NH_4^+ from the dialysate of kidney patients during hemodialysis, thereby allowing the cleansed saline solution to be used repeatedly in recirculating home and portable dialysis systems. Zeolites, especially the natural varieties, are substantially less expensive than the zirconium phosphate ion exchanger currently used. Inexpensive, indigenous natural zeolites are being studied as buffers to reduce stomach acidity and to treat stomach ulcers. External application of zeolite powder has been found to be effective in the treatment of athlete's foot and to decrease the healing time of wounds and surgical incisions (Mumpton, 1985).

Since many biological processes are related to properties exhibited by zeolites, these materials have also been studied and used in the medical field recently. Their biological activity has been tested in anticancer therapy and for the treatment of diarrhea. It is evident from the literature that zeolites (both natural and synthetic) are versatile materials in their application, yet the situation is not exactly the same as far as analytical applications are concerned (Valdes et al., 2006).

The best known and documented positive biological activity of natural clinoptilolite is its action as antidiarrheal drug. Furthermore, some of them seem to have antibacterial property. The clinoptilolite has antioxidative and immunostimulatory effects, and it has been used as an adjuvant to anticancer therapy. Some of the studies performed with the animals have reproduced inspiring results. Clinoptilolite treatment with a variety of tumor types led to improvement in the overall health status, prolongation of life span, and decrease in tumor size. Local application of clinoptilolite to skin cancers effectively reduced tumor formation and growth. If the content of heavy metals (Pb, Cd, Zn, etc.) is high, that may cause negative effects in higher organisms.

Based on these results the adsorbent qualities and ion exchange properties of clinoptilolite could be effective on viruses too (Grce and Pavelic, 2005).

Although the pore sizes of zeolites are too small to allow proteins to pass or enter, the external surfaces can adsorb proteins and zeolites can be used in the purification of proteins. It is considered two mechanisms: the adsorption of proteins on zeolite and the protein-protein interactions. The protein-protein interaction can be manipulated by the charge on protein and the ion composition of the solution. Purification can be done by the removal of undesired proteins from crude protein preparation or by adsorption and subsequent elution of proteins (Klint and Eriksson, 1997).

The physical methods are focused on the removal of mycotoxins by different adsorbents added to mycotoxin-contaminated diets with the hope of being effective in the gastro-intestinal tract more in a prophylactic rather than in a therapeutic manner. Most studies related to the alleviation of mycotoxicosis by the use of adsorbents are focused on aluminosilicates, mainly zeolites and hydrated sodium calcium aluminosilicates (HSCAS), and aluminosilicate-containing clays, all consisting of aluminates, silicates and some interchangeable ions, mainly alkali metal and alkaline earth metal ions. The applicability of aluminosilicates for the adsorption of mycotoxins has been studied for more than 20 years (Huwig et al., 2001).

The surface adsorption of proteins on heterogeneous supports is important in a wide variety of medical and biochemical phenomena and in many practical cases such as a biomaterial implant in a living body, the cell growth in a culture, or the functionality of a biosensor. It is known that proteins are able to distinguish between non-biological materials by binding selectively to the surfaces of metals, metal oxides, metal carbonates and semiconductors that vary in their chemical composition. The investigation of interactions between specific proteins and inorganic supports in order to understand and optimize protein production in biotechnology is of interest. Recently, there has been attention to the immobilization of proteins on nanoparticles. Nanoparticles can retain the bioactivity of proteins to a large extent. Zeolites are inorganic materials which have a highly ordered structure and can be synthesized with a nanocrystallline size and they are well-tolerated by microorganisms, and therefore normally compatible with biochemical analyses. These materials are receiving increased interest as biomaterials. In fact, zeolite containing antibiotic toothpaste and antimicrobial orthopedic agents were reported in recent patents (Tavolaro et al, 2007).

Sakaguchi and his research team has investigated to introduce a new carrier, the zeolites for the recovery of biopolymers, namely proteins. They have summarised that the morphology of each of the zeolites and the relationship to the three-dimensional structures of biopolymers should be considered. Some proteins that adsorbed to zeolites with high Si/Al ratios could bind to the zeolites at a pH above the pI value. Each of the biopolymers tended to bind well at or around its pI to zeolites with a high Si/Al ratio, suggesting that ion-exchange capability rather than hydrophobicity has the strongest influence on adsorption. Adsorption at or above the pI is probably related to hydrophobic interactions between zeolites and biopolymers. One possibility is that, since proteins cannot bind to silicalite (which has no Al), the Al molecules in zeolite may play a role in adsorption. Alternatively, since silicalite has no mesopores, their number and size might be important. Under conditions in which Coulomb's force-mediated adsorption and repulsion do not occur, hydrophobic interactions may occur at many points on the surface of the mesopores by surface-to-surface interactions, resulting in the adsorption of biopolymers. Furthermore, since proteins would shrink at the pI due to loss of their surface charge, they would be more readily adsorbed into the mesopores. In conclusion, there are three physicochemical principles that may underlie adsorption: (1) below the pI, mainly Coulomb's attractions may occur, as in ion-exchange chromatography, (2) at the pI, hydrophobic interactions (a kind of van der Waal's attraction) plus the three-dimensional mesopore structure are involved, (Valdes et al., 2006) above the pI, the sum of the Coulomb's repulsions and attractions (such as hydrophobic interactions) and substitution reactions of water on the Al molecule with a protein amino base might be important. At high Si/Al ratios in the presence of a small amount of Al and with mesopores between the zeolite particles, maximal absorption was seen at the pI, suggesting a dependence both on the number of hydrophobic interaction points on the mesopores and on the mesopore morphology (Sakaguchi et al., 2005; Munsch et al., 2001; Matsui et al., 2001).

Sakaguchi and his research team has submitted the representation in Figure 4.5 for better understanding of the mechanism between zeolites and proteins at different pH values with respect to the isoelectric point (Sakaguchi et al., 2005).

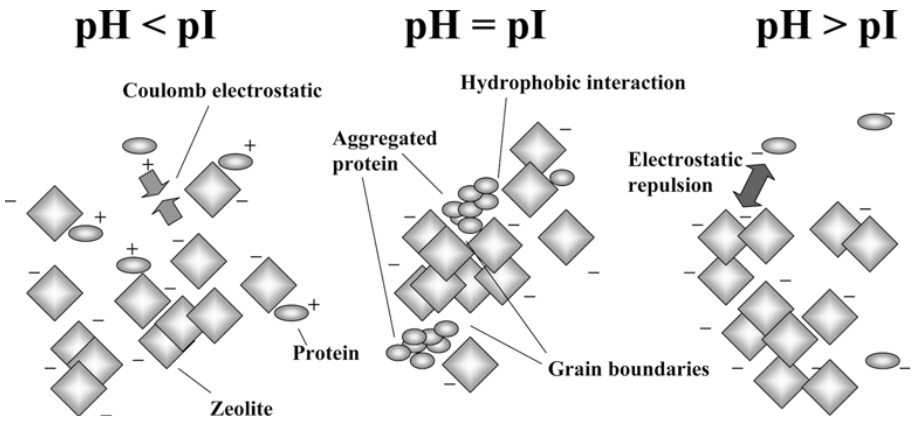

Figure 4.5. Zeolite protein interactions at different pH values.
(Source: Sakaguchi et al.,2005)

In one of the recent studies, the adsorption characteristics of bovine serum albumin (BSA) on various zeolite crystals synthesized in hydrothermal conditions were reported. The equilibrium and kinetics characteristics of the protein on these materials was studied by varying the incubation temperature and time, the protein concentration and the pH values. The results were reported as although the interaction of the BSA with the zeolites occur exclusively on the external crystal surface, the chemical composition and structure of framework influence the percentage of adsorption. The analysis of the influence of temperature on the BSA adsorption revealed that increasing the temperature of the incubation, the immobilization of the protein increases, according to the opposite behaviour observed for the mesoporous materials. Concerning the effect of the pH values, at the operational pH 4.8 both aluminum-free zeolite crystals and aluminium containing zeolite crystals have different values of immobilization percentage, but all the prepared structures have highest adsorption (Tavolaro et al., 2007; Chiku et al., 2003).

During fermentation studies natural zeolites were offered to be used as a carrier to immobilize yeast cells. The use of immobilization cells has been suggested as an effective means for improving ethanol fermentation. The immobilization of cells lead to higher cell densities with consequent increases in reaction rates and enzyme productivities. However, immobilized yeast cells on polymeric carbohydrates have not been used for continuous fermentation for long periods because of the weakness of the carrier. Furthermore, ceramic beads and glass beads are easily broken by wearing in a fluidized-bed reactor development of a novel mechanical stable carrier, which is obtained by heating natural zeolite at high temperature and crushed to optimum size for

yeast immobilization. When the abilities of natural zeolite carrier were compared with glass beads, the capacity for immobilization and alcohol fermentation activity were, respectively, 2-fold higher and 1.2-fold higher than that of glass beads. Continuous alcohol fermentation was stable for over 21 days without breakage of the carrier (Shindo et al., 2001).

Application of zeolite NaY in yeast fermentation as a pH regulator was successfull. The presence of zeolite allowed the maintenance of the pH around 3.7 in the fermentation medium, leading to consumption of all the initial glucose and so to a higher ethanol concentration. Thus, zeolite could act as a pH regulator allowing the fermentation of high glucose concentrations (Castellar et al., 1998).

An alternative method of recovery of lactic acid from fermentation broth, based on adsorption on Silicalite molecular sieves, has been investigated. Silicalite pellets have pore sizes suitable for adsorption of lactic acid, yet which can exclude larger size components of the fermentation broth, such as proteins. It also has low affinity for water and it is characterized by high thermal and hydrothermal stability. Although the Silicalite showed lower capacity for lactic acid than other polymeric adsorbents, this process still has the advantages of simplicity in operation and use of additional chemicals (other than water) can be eliminated (Aljundi et al., 2005).

CHAPTER 5

ADSORPTION OF PROTEINS

Adsorption is one of the separation techniques applied to recover the proteins from aqueous solutions. Adsorption is simply the attachment of the liquid or gas molecules in an interfacial surface called adsorbent. Depending on the involved forces, adsorption is classified into two; physical adsorption and chemical adsorption. Physical adsorption involves intermolecular interaction forces which are known as van der Waals forces. In chemical adsorption electrons are transferred and shared between the adsorbate and adsorbent resulting a chemical bond formation. In phsical adsorption, both monolayer adsorption and multilayer adsorption can take place. In chemical adsorption, after the first layer is covered with adsorbate molecules, adsorption stops. Within a wide range of pressure and temperature, chemical adsorption is possible. Chemical adsorption is frequently irreversible resulting a difficulty in desorption and during desorption high temperatures or modification of pH and eluent concentrations are required (Ülkü and Mobedi, 1991; Wiel and Wesselingh, 1988).

5.1. Adsorption Kinetics and Adsorption Equilibria

Adsorption equilibrium is experimentally determined. The data is used to construct a correlation namely adsorption isotherms. Adsorption isotherms is the representation of adsorbed amount as a function of pressure or concentration at constant temperature (Ülkü and Mobedi, 1991). The expression adsorption equilibria for this study involves the uptake of proteins as adsorbate onto natural zeolites. The basic factors affecting adsorption equilibrium are; the nature of the adsorbent, the nature of adsorbate, temperature and the concentration values. Measurement of adsorption isotherm is an important step in the characterization of the interaction between the adsorbent and adsorbate.

In order to optimize the adsorption system, the most appropriate correlation for the equilibrium curves should be established. Several isotherm equations are used to describe the equilibrium nature of adsorption where Langmuir and Freundlich isotherm

models are most widely used adsorption equilibrium expressions (Kavitha and Namasivayam, 2007). The linear plots of Langmuir and Freundlich equations shows the applicability of the model for the adsorbents studied. Langmuir adsorption isotherm assumes that adsorption occurs on a homogenous adsorbent surface. The surface is made up of identical sites that are available equally and energetically equivalent with each sites carrying equal numbers of molecules. There is no interaction between adsorbate molecules. On the other hand, Freundlich isotherm assumes that the adsorption occurs on heterogeneous surface at sites with different energy of adsorption and with non-identical adsorption sites that are not always available (Ayyappan et al., 2005; Qada et al., 2006). The equilibrium adsorbed amount is calculated by equation (5.1) where C_o: initial solute concentration, V; working volume and m; adsorbate amount.

$$q_e = \frac{(C_0 - C_e)V}{m} \tag{5.1}$$

The Langmuir isotherm is expessed as in equation (5.2). The linearized form of equation is used to fit the experimental data with the isotherm model.

$$q_e = \frac{K.q^m.Ce}{1 + K.Ce} \tag{5.2}$$

K; adsorption equilibrium constant and q^m; maximum adsorption capacity with fairly rapid attainment of a well-defined plateau and high affinity to a solid. One of the characteristics of the Langmuir isotherm is given by a separation factor R_L. The calculated value of R_L indicates the type of isotherm. R_L=0, the isotherm is irreversible; 0< R_L<1, the isotherm is favorable; R_L=1, isotherm is linear and R_L>1, isotherm is unfavorable.

$$R_L = \frac{1}{1 + KC_o} \tag{5.3}$$

Sometimes the adsorption data formally obey the Freundlich equation where the constant K_f is a measure of adsorption capacity and a is a measure of affinity (Magdassi, 1996).

$$q_e = K_F.Ce^{1/n} \tag{5.4}$$

5.2. Adsorption Mechanism

Adsorption equilibrium and adsorption kinetics are two physicochemical aspects evaluating the adsorption process as a unit operation. Adsorption equilibrium is reached when the concentration of the adsorbate in the bulk solution is in dynamic balance with that of the interface. The adsorption equilibrium analysis is the most important fundamental information used to determine the capacity of adsorbent. Both the adsorption capacity and the kinetic behavior of the adsorbent are of great importance for the lab-scale and large scale applications. The kinetics analysis is a useful tool to get the time required to reach the equilibrium regarding the completion of adsorption. The kinetic process of adsorption is explained by several mathematical models where in case more than one mechanism may be responsible as a rate determining step (Ho et al., 2000; Ho and McKay, 1998; Qiu et al., 2009). The optimization and scale up of adsorbent beds requires that the equilibrium and mass transfer of adsorbent-adsorbate system are fully understood in the formulation of kinetics of sorption in solid-liquid systems. The interaction between the adsorbent-adsorbate is responsible for the nature of equilibrium between them and affected by the resistances of mass transfer in the establishment of that equilibrium.

The interaction between proteins and adsorbents do not occur instantaneously. Heat and mass transfer is controlled by the adsorption rate. The controlling mechanisms of adsorption rate are explained by mathematical models. These models describing the adsorption data have been explained by adsorption reaction models and adsorption diffusion models. Diffusion models are basicly given as external fluid film and intraparticle diffusion, however subdivisions are existing depending on the assumptions and boundary conditions of the systems investigated.

Adsorption of a protein from a bulk solution onto an adsorbent involves a number of steps. These steps are assessed in detail with adsorption models. The

adsorption diffusion models are classified into four distinct steps. As a starting point, the adsorbate molecule is transported from the bulk solution to the external layer of the adsorbent. Depending on the system, both diffusive and the convective forces may be in progress. In practice due to severe mixing, this step is known to be very fast when it is compared with the movement of adsorbate molecule through the external layer of particle and inside the particle that are governing the latter steps. For the subsequent steps convective forces through the pores are negligible thus only diffusion mechanism takes place. Second step involves the diffusion of the adsorbate molecule across the external liquid film surrounding the adsorbent. This external film diffusion can be investigated by the assumptions of linear approach or non-linear approach and also dimensional analysis model. The third step is intraparticle diffusion which is also called internal diffusion. This step is examined in two aspects. The pore diffusion and surface diffusion. In case of pore diffusion, the solute diffuses through the fluid filling the pores hence intercrystalline macropore diffusion and intracrystalline micropore diffusion are involved. Then the protein molecule is adsorbed. In addition to that, for surface diffusion the solute diffuses in an amorphous and homogeneous sphere. The latter is also called as homogeneous solid diffusion model (HSDM). The fourth and last step is the chemical or physical interactions of protein molecules with the active sites on the adsorbent which is also called mass action or surface reaction. The adsorption/desorption chemical reaction takes place at this step. The rate of surface reaction can be determined with the adsorption kinetic reaction models (Baup et al., 2000; Ülkü and Mobedi, 1991; Hall et al., 1966; Qiu et al., 2009; Ho et al., 2000; Gerente et al., 2007; Gebauer et al., 1997; Plazinski et al., 2009).

Since consideration of all those factors complicate the analyses, attempts have been made for simplification by assuming of controlling resistances and neglecting other resistances in comparison.

5.2.1. Adsorption Reaction Models

For the adsorption reaction models; Lagergren presented first order rate equation to describe the kinetic process of liquid-solid phase adsorption in 1898. It is believed to be the earliest model describing the adsorption rate with respect to the adsorption capacity. the Lagergren`s first order rate equation has been called pseudo-first-order to

distinguish kinetic equations based on adsorption capacity from solution concentration (Qiu et al., 2009). The equation can be presented as follows:

$$\frac{dq_t}{dt} = k_{p1}(q_e - q_t) \tag{5.5}$$

where q_e and q_t (mg/g) are the adsorption capacities at equilibrium and at time t (min), respectively. k_{p1} (min^{-1}) is the rate constant for the pseudo-first-order kinetic model (Ho and McCay, 1998). After integration and applying boundary conditions t=0 to t=t and q_t=0 to q_t= q_t, the integrated form of equation becomes:

$$\log (q_e\text{-}q_t)=\log(q_e)\text{-}\frac{k_{p1}}{2.303}\ t \tag{5.6}$$

In general, if the film diffusion is the rate controlling step, the slope of the plots of equation (5.6) will vary inversely with the particle size, the film thickness and with the distribution coefficient (Ho and McKay, 1998).

When the rate of adsorption is a pseudo-second order mechanism, the kinetic rate equation is presented as:

$$\frac{dq_t}{dt} = k_{p2}(q_e - q_t)^2 \tag{5.7}$$

where q_e and q_t are the adsorption capacity at equilibrium and at time t, respectively (mg/g) and k_{p2} is the rate constant of pseudo-second order mechanism (g/mg.min). By the application of boundary conditions t=0 to t=t and q_t=0 to q_t= q_t, the integrated form of equation (5.6) becomes

$$\frac{t}{q_t} = \frac{1}{k_{p2}q_e^2} + \frac{1}{q_e}t \tag{5.8}$$

and where h, the initial adsorption rate as q_t/t approaches zero (mg/g.min)

$$h=k_{p2}q_e^2 \tag{5.9}$$

equation (5.8) becomes

$$\frac{t}{q_t} = \frac{1}{h} + \frac{1}{q_e} t \tag{5.10}$$

The plot of t/q_t against t of equation (5.10) should be linear if pseudo-second order is applicable. The slope and intercept is used to determine q_e and k_{p2} and h. The evaluation of rate constant k_{p2} is reported as; if the adsorption is chemically rate controlled, the slope will be independent of particle diameter and flow rate and will depend only on the concentration of the sorbate in solution and the temperature (Ho and McKay, 1998).

Another reaction model approach is the Elovich reaction equation which is expressed as:

$$\frac{dq_t}{dt} = \alpha \exp(-\beta q_t) \tag{5.11}$$

α is the initial adsorption rate (mg/g.min) and β is the desorption constant(g/mg) during experiment. The Elovich equation is simplified with the assumption $\alpha\beta t >> 1$ and applying the boundary conditions $q_t=0$ at $t=0$ and $q_t=t$ at $t=t$, hence equation (5.11) becomes

$$q_t = \beta \ln(\alpha\beta) + \beta \ln(t) \tag{5.12}$$

the constants are calculated from the slope and the intercept of the plot with the axis q_t against ln(t) (Ho and McKay, 1998).

5.2.2. Adsorption Diffusion Models

In large scale processes sometimes the rate determining step is transport in the bulk of solution. But by rapid mixing the effect of transport in the solution is eliminated that is no longer a rate limiting step in several experimental adsorption systems (Ho et

al., 2000). So, in the case of adsorption diffusion models; the film diffusion, intraparticle diffusion and surface reaction are considered for liquid-solid adsorption system. For physical adsorption mass action is a rapid process and can be neglected for kinetic study. Therefore, the kinetic process of adsorption is always controlled by liquid film diffusion and/or intraparticle diffusion. Hence adsorption diffusion models are important to describe the process of film diffusion and/or intraparticle diffusion (Qiu et al., 2009).

External Film Diffusion: If the adsorbent particle is nonporous or diffusion within the particle is very rapid, than adsorption is observed on the outer surface of the particle. Diffusion takes place through the laminar film surrounding the external surface of the particle. The adsorbate concentration at the particle surface is in equilibrium with the fluid phase concentration (Karger and Ruthven 1992; Ülkü, 1991). The standard linear rate expresion is used to express the transport rate through the external laminar film:

$$\frac{d\bar{q}}{dt} = k_f a(C-C^*) \tag{5.13}$$

The film mass transfer coefficient is k_f and a is the specific surface area for the spherical adsorbent particle which is $a=3/R_p$. The value of q averaged over a particle is presented as $\bar{q}$. If the equilibrium relationship is observed than $q^*=KC$. The equilibrium adsorbed phase concentration is represented as q^*. The boundary conditions can be written as:

$$t<0, \quad C=q=0$$

$$t>0, \; C=C_\infty=q_\infty/K$$

Thus;

$$\frac{\bar{q}}{q_\infty} = 1 - \exp\left[-\frac{3k_f t}{KR_p}\right] \tag{5.14}$$

This equation may be rearranged and the film diffusion mass transfer rate equation is presented as;

$$\ln(1-\frac{\bar{q}}{q_\infty})= -R`t \tag{5.15}$$

$$R`=\frac{3k_f}{R_pK} \tag{5.16}$$

R_p is the particle radius K is the constant of linear equilibrium (q=KC) and k_f is the film mass transfer coefficient. When the film diffusion is the rate limiting step, the plot of $\ln(1-q_t/q_e)$ versus t should be a straight line and the slope is –R` (Qiu etal., 2009). If film diffusion is rate controlling, the constant of the rate equation vary inversely with the particle size and the film thickness. Adsorption studies carried out at different agitation speeds resulted the film diffusion is strongly affected by agitation (Ho et al., 2000).

Dimensionless numbers can also be used to determine the experimental film mass transfer coefficients. The correlation is written as;

$$Sh = \frac{k_f(2R_p)}{D_m} = f(\mathrm{Re}, Sc) \tag{5.17}$$

$$Sh= 2.0+1.1Sc^{1/3}Re^{0.6} \tag{5.18}$$

For an isolated particle in a stagnant fluid the Re is obtained as; 0 and Sherwood approaches 2.0 (Karger and Ruthven 1992; Ülkü, 1991). There are several empirical sorrelations for Sherwood number and some of them are given in Table 5.1.

Table 5.1. Empirical external mass transfer correlations.
(Source: Ko et al., 2001)

Empirical Correlation	Range of Validity
$Sh= 5.4Re^{1/3}Sc^{1/4}$	0.04<Re<30
$Sh= 4.3Re^{0.35}Sc^{1/4}$	0.1<Re<3
$Sh= 4.58Re^{1/3}Sc^{1/3}$	0.4<Re<10
$Sh=\frac{1.09}{\varepsilon} Re^{1/3}Sc^{1/3}$	0.016<Re<55
$Sh= 2.4\varepsilon^{0.66} Re^{0.34}Sc^{1/3}$	0.04<Re<52
$Sh=\frac{1.13}{\varepsilon} Re^{0.21}Sc^{1/3}$	Re<10
$Sh=1.85\left(\frac{1-\varepsilon}{\varepsilon^2}\right)^{1/3} Re^{1/3}Sc^{1/3}$	Re/(1- ε)<10
$Sh= Re^{0.28}Sc^{1/3}$	Re<10

Macropore Diffusion: There are several mechanisms taking place in macropore diffusion. The fundamentals are; molecular diffusion, Knudsen flow, surface diffusion and Poiseuille flow. In liquid systems, molecular diffusion is almost always dominant. Molecular diffusion takes place especially in large pores and at higher pressures. There happens the collision between the diffusing molecules. The effective pore diffusivity under molecular diffusion control is calculated as:

$$\varepsilon_p D_p = \frac{\varepsilon_p D_m}{\tau} \tag{5.19}$$

where ε_p; porosity of particle, τ; tortuosity factor, D_p; pore dffusivity, and D_m; molecular diffusivity.

Knudsen diffusion is dominant in small pores and at low pressures which is the result of collisions between the diffusing molecules and the pore wall (Karger and Ruthven 1992; Ülkü, 1991). The Knudsen diffusivity in a straight cylindrical pore is given as:

$$D_K= 9700\,\bar{r}\,(T/M)^{1/2} \tag{5.20}$$

D_K; Knudsen diffusivity (cm^2/s), $\bar{r}$; average pore diameter, T; Temperature, M;molecular weight. The combined effect of molecular diffusion and Knudsen diffusion can be estimated by:

$$\frac{1}{\varepsilon_p D_p} = \frac{\tau}{\varepsilon_p}\left(\frac{1}{D_K} + \frac{1}{D_m}\right) \tag{5.21}$$

If micropore diffusion is rapid in the system, the concentration gradient within microparticles is uniform but there will be a concentration gradient through macroparticles. Hence macropore resistace is dominant. The expression is derived with the assumption of the local equilibrium reached between the adsorbed phase and fluid phase (Karger and Ruthven 1992; Ülkü, 1991). The pore diffusivity is assumed to be independent of concentration:

$$\left(1-\varepsilon_p\right)\frac{\partial q}{\partial t} + \varepsilon_p \frac{\partial C}{\partial t} = \varepsilon_p D_p\left[\frac{\partial^2 C}{\partial R^2} + \frac{2}{R}\frac{\partial C}{\partial R}\right] \tag{5.22}$$

The macropore diffusion control with linear equilibrium (q^*=KC) is obtained and can be written as;

$$\frac{\partial C}{\partial t} = \frac{\varepsilon_p D_p}{\varepsilon_p + (1-\varepsilon_p)K}\left[\frac{\partial^2 C}{\partial R^2} + \frac{2}{R}\frac{\partial C}{\partial R}\right] \tag{5.23}$$

The boundary conditions are given as:

$$C(R,0)=C_o, \qquad q(R,0)=q_o=KC_o$$

$$C(R_p,t)=C_\infty, \qquad q(R_p,t)=q_\infty=KC_\infty$$

$$\left.\frac{\partial c}{\partial R}\right|_{R=0} = \left.\frac{\partial q}{\partial R}\right|_{R=0} = 0$$

so the solution for uptake curve can be written as;

$$\frac{q_t}{q_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-n^2\pi^2 t D_p \varepsilon_p}{R_p{}^2(\varepsilon_p + K - K\varepsilon_p)}\right) \tag{5.24}$$

Micropore Diffusion: When the system is isothermal and only micropore diffusion resistance exists, the diffusion equation for a spherical article is:

$$\frac{\partial q}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}\left[r^2 D_c \frac{\partial q}{\partial r}\right] \tag{5.25}$$

$t<0$, $C=C_o$, $q=q_o$, (r and t, independent)

$t\geq 0$ $C=C_\infty$ $q(r_c,t)=q_\infty$,

$t\rightarrow \infty$ $C=C_\infty$ $q(r,t)\rightarrow q_\infty$,

$$\left.\frac{\partial q}{\partial r}\right|_{r=0} = 0$$

D_c is the intracrystalline diffusivity. If the spherical particle is initially free of solute, and the concentration of solute at the surface remains costant, external film resistance can be neglected according to the costant surface concentration. This approach was developed by Crank.

$$\frac{q_t}{q_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-n^2\pi^2 D_c t}{r_c{}^2}\right) \tag{5.26}$$

Hence the uptake curve for the long time region can be expressed as:

$$\frac{q_t}{q_\infty} = 1 - \frac{6}{\pi^2}\exp\left(\frac{-\pi^2 D_c t}{r_c{}^2}\right) \tag{5.27}$$

by neglecting the higher order terms in order to simplify the equation for the initial region of uptake curve in a short time region, the following expression is obtained. The system is assumed isothermal, and diffusivity is assumed constant which occurs if concentration change is small (Karger and Ruthven 1992; Ülkü, 1991).

$$\frac{q_t}{q_\infty} = \frac{6}{\sqrt{\pi}}\left(\frac{D_c t}{r_c^{\,2}}\right)^{1/2} \tag{5.28}$$

It is obvious that the plot of uptake versus the square root of time should be linear. The expression q_t/q_∞ is representing the uptake curve.

Biot number is used to make a comparison between the internal mass transfer resistance and external mass transfer resistance that is a helpful tool to determine the controlling mechanism (Ülkü and Mobedi, 1991).

$$Bi = \frac{k_m r}{3\varepsilon_p D_p} = \frac{Sh}{6}\frac{D}{\varepsilon_p D_p} \tag{5.29}$$

k_m; external mass transfer coefficient, r; radius of particle (m), ε; porosity of particle, D_p; pore diffusivity (m^2/sec) since Sherwood number is always greater than or equal to 2 and pore diffusivity (D_p) < molecular diffusivity (D)/ τ (Tortuosity factor), Biot number is obtained as Bi>τ/3ε. There are several approaches for the limitation of Biot number for the discrimination between external mass transfer and internal mass transfer.mechanisms. For Biot number<<1, the adsorption rate is external mass transfer resistance controlled. While for Bi>>100, pore diffusion is the predominant mass transfer controlling mechanism (Maiti et al., 2009).

The uptake may be controlled by macropore diffusion, micropore diffusion or the combination of them in a particle. When the adsorption rate is controlled by macropore diffusion, there exists a concentration gradient within the particle. The adsorption rate is dependent to the particle size. But in case of micropore diffusion controlling system, the assumption of constant concentration gradient through the particle is applicable. Thus the rate of adsorption is not dependent to the particle size of adsorbent (Ülkü and Mobedi, 1991).

According to Weber-Morris another approach which is obtained from the simplification of Crank`s solution given in equation (5.28) that explains the intraparticle diffusion model is expressed by the equation

$$q_t = k_d t^{1/2} \quad (5.30)$$

k_d is the intraparticle diffusion rate constant. When the intraparticle diffusion is the only rate limiting step, the plot of q_t versus $t^{1/2}$ should go through origin. Otherwise, the adsorption kinetics may be controlled by film diffusion and intraparticle diffusion simultaneously (Qiu et al., 2009). Many researchers reported the plots of adsorption capacity versus the square root of time are linear while the initial curved portions of the are attributed to boundary layer diffusion effects. The linear portion is due to intraparticle diffusion effects (Ho et al., 2000).

When the mechanism is not confirmed, system variables over the entire adsorption range from the beginning until the equilibrium is reached, should be tested. The basic variables are: agitation speed, particle diameter, temperature, pH, concentration and adsorbent dose. Many kinetic models and correlation coefficients should be used to test the experimental adsorption data. (Ho and Mc Kay, 1998). For example the mathematical expression may be enhanced by correlating the intraparticle diffusion parameter k_d as a function of adsorbent particle size, solution temperature, adsorbate concentration etc. (Gerente et al., 2007).

Ho and his coworkers reported the suitable approach for the selection of adsorption model with respect to the several studies given in literature as analysing the square root of the residence time. The plot of q_t vs time is a linear line with a slope of k_d, diffusional rate parameter. When the straight line passes through the origin the model is controlled by diffusion (Ho et al.,2000). The adsorbents having pore sizes as micropores, mesopores and macropores, three linear sections can be obtained. Additionally, the effect of system variables on k_d can be analysed for the confirmation of the diffusion method. The product of k_d times adsorbent mass vary linearly with adsorbent mass and k_d values vary with the square root of concentration that are indicating the intraparticle diffusion. On the other hand when k_d vary with the reciprocal of the particle diameter, the rate controlling step is surface mass transfer (Ho et al., 2000). During adsorption studies at least two to three variables are useful before elucidating the rate determining adsorption process.

In order to describe the external film diffusion on a theoretical basis, the proposed route is plotting the experimental kinetic adsorption data for diffusion sorption model namely intraparticle diffusion. When the plot of q_t versus $t^{1/2}$ do not pass through the origin, there is external film mass transfer or boundary layer which is the evidence of external film controling mechanism.

The analysis of external film diffusion can be done by performing different adsorption batches at different agitation speeds. The mass transfer resistance is reduced as a result of the shear effect on the film boundary layer by agitation. It can be seen by the plots of adsorbate concentration versus time (Ho et al., 2000).

It should be noticed that during adsorption a controlling mechanism may occur solely or a combination of two mechanisms sequentially. The film diffusion is the rate controlling step at the initial period due to the availability of external surface layers, however, as the time period increases, the external surface sites become saturated and the rate controlling step moves to reaction inetic control or to internal diffusion (Gerente et al., 2007).

Mohanty and his research team reported that there happens gradual increase in adsorption as the particle size decreases. The smaller particles have higher solid-liquid interfacial areas resulting higher adsorption rates. The temperature effect was also investigated. The adsorption was increased as the temperature increased which was attributed to endothermic systems suggesting chemisorption is taking place. It was explained by the hydrogen bond formation between the adsorbate and adsorbent. So the adsorption capacity was said to be dependent on the chemical interaction between the functional groups on the adsorbent surface and the adsorbate that should increase as a result of temperature increase (Mohanty et al., 2006).

The effect of temperature on the adsorption process is explained by two major changes. The increase in temperature results an increase on the rate of diffusion of the adsorbate molecules across the external boundary layer and inside pores of the adsorbent particle owing to the decrease in the viscosity of the solution. Furthermore, changing the tempertaure is changing the equilibrium capacity of the adsorbent for adsorbate (Wang and Zhu, 2006).

5.3. Factors Affecting Protein Adsorption

The possible interactions and their strength between natural zeolite and proteins are complicated. They could consist of acid-base reactions. The simplest method of protein immobilization is adsorption. Adsorption of proteins on solid surfaces is a very important phenomenon since it is a common application in many fields such as biology, medicine, biotechnology, and food processing and sometimes they play an important role in a system's performance. In particular many chromatographic separations, such as hydrophobic, displacement, and ion-exchange chromatographies, are based on the tendencies of proteins for the support. Protein adsorption at implanted biomaterials is believed to play an important role in determining their biocompatibility with various biological systems. Another importance of protein adsorption onto solid surfaces is the extensive use of immunodiagnostic reagents, in which antibodies are adsorbed on latex or gold particles or on polystyrene plates. The varied functions, structures and its interactions are needed to be well understood (Magdassi, 1996; Nakanishi et al., 2001).

Adsorption at solid/liquid interfaces has some features. An interface is formed between two different phases during adsorption. The standard free energy of this interface is usually higher than the standard free energy of bulk phase. As a result, the interface has the tendency of thermodynamic stability by adsorbing the solute molecules. The adsorbed substances are different from the solvent molecules. Due to the adsorption, the structure and the functions of adsorbed molecules change more or less on the surface (Nakanishi et al., 2001).

The chemical nature of the adsorbent and its properties (charge, hydrophobicity, etc.) determine the mode and strength of binding, as well as, in many cases, the conformational changes in adsorbed protein molecules. For the specific interactions the solid surfaces can be easily modified. Usually, different from fluid surfaces, solid surfaces are not chemically or energetically uniform, and their heterogeneity may result in nonuniform adsorption of protein layers. Finally, during adsorption from solutions competition between a protein and a solvent takes place. A large number of different carriers for protein adsorption have been described: glass, silicas, clays, insoluble inorganic salts, ceramics, metals and metal oxides, molecular sieves , carbon, many types of neutral and charged polymers, and carriers of mixed types, obtained by

deposition of lipid layers (e.g., lecithin and cholesterol) on a solid support (Magdassi, 1996).

There are several techniques used for observing protein adsorption behavior. The conventional technique used to determine the adsorbed amount is measurement of the decrease in solute concentration after treating with adsorbent. Besides, the adsorbed amount and their changes is obtained by ellipsometry due to the change in polarized light state on reflection. Fluorescence spectroscopy and Fourier transform infrared spectroscopy determine the conformation of proteins adsorbed on surfaces with respect to the change in spectrum. With atomic force microscopy three dimensional image of protein adsorbed surface can be obtained. The amount of irreversibly adsorbed proteins is measured by radioisotope labeled molecules. The amount and thickness of adsorbed proteins are obtainable with radiotracer technique (Nakanishi et al., 2001). The factors influencing the functional properties of proteins and the ability to regulate these properties are of great importance. These properties determine the adsorption behavior of proteins.

The main molecular properties of proteins are size, charge, structural properties, stability, amphipathicity, and lipophylity which are responsible for their surface activity. Proteins form multiple interaction points with the surface (e.g., 77 contact points for an albumin molecule and 703 contact points for the fibrinogen molecule adsorbed on silica). The irreversibility of protein adsorption is usually seen in case of multipoint binding if irreversible denaturation is not governing in system. The rates of desorption are much lower than those of adsorption, and in many cases it is often impossible to obtain the equilibrium state for desorbing the adsorbed protein. In other words, the formation of one or several bonds with the surface increases the probability of adsorption of closer sites of the identical molecule. As a result, desorption of a protein molecule requires the simultaneous breakage of a large number of bonds. This corresponds to a considerable difference between the activation energies for the adsorption and desorption processes (Magdassi, 1996).

Protein adsorption at the solid/liquid interface is influenced by the surface charge of the molecule and the surface charge of the solid. The overlap of the electrical double layers at the sorbent and the protein surfaces results in electrostatic attraction if they have opposite charges and in repulsion if their charges have the same sign. Besides, of great importance is the repulsion between the protein molecules in adsorbed layers, which is minimized at their isoelectric points.The charge, its density and

distribution in the protein molecule have a great affect on the surface activity. Proteins frequently show greater surface activity near the isoelectric point (pI), because Coulombic repulsion between the same charged adsorbed molecules decrease. In case of adsorption at ionic surfaces the main factor is, the net opposite charge of the protein molecule with the adsorbed surface, which may contribute to the enthalpic part of the adsorption free energy. Also, heterogenous distribution of ionic sites on the surface of a protein can lead to attractive electrostatic interactions between the sites and the surface even when the net charge of the protein is same with the surface. The pH of the solution determines the charge of the proteins that are comprised of both charged amino and carboxyl groups and the interacting surfaces consequently is responsible for the attractive and repulsive effects (Tavolaro et al., 2007; Magdassi, 1996; Krohn and Tsapatsis, 2005; Klint and Eriksson, 1997; Munsch et al., 2001).

Although electrostatic forces are very important, they do not dominate the adsorption process solely. In many cases q^m is at a maximum value near the isoelectric points of the protein/sorbent complex. One possible explanation of this behavior is that increased lateral electrostatic repulsion of the equally charged protein molecules prevents the formation of close-packed monolayers. Another explanation takes into account that the structural stability of the protein molecules is maximal and the conformational rearrangements are minimal when adsorption proceeds at a pH equal to the pI of the protein/sorbent complex. The dehydration of surface and protein causes release of water molecules which enhances the adsorption of protein on hydrophobic surface. On the contrary, affinity of adsorption onto hydrophilic surface decreases (Magdassi, 1996, Giacomelli et al., 1997).

The properties of proteins, type of surface and operating conditions are of great concern on the amount of proteins adsorbed. In particular, the charge, the size, the structure related with the stability of protein, amino acid composition and the steric conformation plays significant role on the amount of adsorbed proteins. At room temperatures denaturation of proteins is negligible. The proteins with a rigid structure, as a result of intramolecular (e.g., disulfide) bonds, possess (at least partially) the native conformation, and structural rearrangements do not contribute to the adsorption process. "Soft" protein molecules undergo structural rearrangements at interface and should be more surface-active than "rigid" proteins, because more contacts with a surface could be formed and because the configurational entropy gain favors the adsorption. About the structure of proteins, high internal stability possessing proteins are called hard proteins.

The protein structure related with the internal stability affect their adsorption such as β-Lactoglobulin which is a hard protein. If there is not electrostatic attraction, β-Lactoglobulin adsorb on hydrophilic surfaces in small quantity. If the proteins and the surface have the same charge repulsive effect due to electrostatic interaction is seen. The repulsion can be reduced by increasing the ionic strength. The soft proteins that have low internal stability such as bovine serum albumin, human serum albumin, immunoglobulin, α-Lactalbumin and hemoglobin are known to be adsorbed independent of electrostatic interactions on all surfaces (Nakanishi et al., 2001; Magdassi, 1996).

The chemical differences are also very important because the balance of polar, nonpolar and charged amino acid side chains determines the surface activity of proteins. i.e., the possibility and mode of their location at interfaces of different types. Another very important property of proteins is the hydrophobicity. It influences adsorption and orientation of proteins at interfaces and in many cases correlates with surface activity (Magdassi, 1996).

The rate of protein adsorption onto solids is usually slow. For various protein-adsorbent systems, the period of time required to obtain maximum adsorption is observed from several tens of minutes to several hours. It is not so common that the adsorption ends up after several minutes or continues for 24 h and longer. The importance of diffusion is also obvious at the first step of adsorption from protein mixtures. For the initial period, the interface accommodates the protein molecules with the largest diffusion coefficients, and next these molecules may be exchanged by other molecules with higher affinity to the surface. The question of whether the adsorption of proteins onto solids is reversible or irreversible is very important for correct estimation of physicochemical characteristics of the process (Magdassi, 1996). Only in this case the isotherm represents thermodynamic equilibrium, and the standard Gibbs energy of adsorption can be determined as:

$$\Delta G^{o}_{ads} = \Delta H^{o}_{ads} - T\Delta S^{o}_{ads} \qquad (5.31)$$

The adsorption of proteins (especially of those with the rigid globular structure) is usually inherently reversible, and the irreversibility is only apparent and has kinetic origins. This provides the possibility to estimate from the experimental isotherms some physicochemical characteristics of the protein adsorption process, such as adsorption

equilibrium constants K and corresponding standard free energies ΔG^{o}_{ads} (or their lower limits, if the irreversible entropy and enthalpy changes cannot be completely ignored). Initial high slopes of adsorption isotherms indicate, usually, a high affinity of proteins for the solid/liquid interfaces. The ΔG^{o}_{ads} values calculated from the Langmuir isotherms are usually in the range between -6 and -12 kcal/mol for various protein-adsorbent systems (Magdassi, 1996).

The protein adsorption on solids is often endothermic and that the driving force of the process is the positive ΔS^{o}_{ads}. The main contributions to large positive values of entropy can arise from sorbent surface dehydration and from the conformational rearrangement of protein molecules upon adsorption (Magdassi, 1996). The studies revealed that protein amounts adsorbed at high temperatures are usually higher than the amounts adsorbed at room temperatures. The cause for that behavior is attributed to the cysteine residues of proteins which form a multilayer on the adsorbent surface. As an example β-Lactoglobulin has five cysteine residues. Four of them form disulfide linkages and the last is free which is highly reactive in forming disulfide linkages. At higher temperatures the protein molecule undergoes a confomational change and the internal cysteine residue becomes available to form a linkage or exchange with the adsorbent surface. These disulfide linkages are responsible for the higher adsorbed amount of proteins at higher temperatures (Nakanishi et al., 2001). In one of the recent researches concerning the immobilization of BSA on the surface of inorganic supports, the results revealed that BSA adsorbed amounts were highest at 30^{o}C where the results at 18^{o}C were higher than 4^{o}C (Tavolaro et.,2007).

The molecular dimensions of proteins are estimated as a*x*b*x*c and are considered as having two conformations. One with a long axis is known as end-on-type and the other with a short axis called side-on type. The initial concentration of protein may cause a reconformation of protein layer on surface. At low concentrations reconformation of protein is negligible. Where as at high protein concentrations as a first step without changing a conformation fast adsorption is observed. Subsequently, the adsorbed layer thickness gradually increase as a result of conformational change (Nakanishi et al., 2001). The surface properties of the protein molecules, especially hydrophobicity, as well as the degree of the sorbent surface hydrophobicity, strongly affect the protein adsorption behavior. When a protein is adsorbed on a solid surface with high hydrophobicity, considerable conformational changes, due to hydrophobic

interactions, can take place. It was observed that the conformation of fibronectin does not change upon adsorption onto hydrophilic silica but changes significantly upon adsorption onto chemically modified hydrophobic silica (Magdassi, 1996). Protein adsorption usually observed in an irreversible mode even at room temperature except for the hard proteins. Hard proteins adsorb mostly reversible but also partly irreversible adsorption is observed depending on the type of adsorbent. The reason for irreversible adsorption is not well known in detail however, one of the possible explanation is given as the protein structural deformation due to the increasing adsorption time. The hydrophobic peptide in the protein molecule tends to interact with a hydrophobic region of the surface, so multipoint attachment is recognized (Nakanishi et al., 2001).

Hydrophobic and electrostatic interactions favors the adsorption of proteins on solid surfaces. The research performed with β-Lactoglobulin presented that increasing the hydrophobicity by modification of surface resulted higher adsorbed amounts. In addition to that in a BSA adsorption study as the surface is modified with hydrophilic agents, the adsorbed amount decreased. Electrostatic interactions also play an important role as a result of protein and surface charges in solution pH`s. Nevertheless, proteins having high internal stability show less tendency for adsorption even the surface and protein are in opposite charges (Nakanishi et al., 2001). Krohn and Tsapatsis have performed a study about the adsorption of amino acids. The effect of hydrophobicity was investigated by adsorption of phenylalanine. When the hydrophobic nature of the adsorbent material was increased, the amount of adsorbed phenyalanine decreased (Krohn and Tsapatsis, 2005).

Magdassi reported about the size and the molecular weight of the protein molecules that their effect is not much studied on the adsorption of proteins to solid surfaces. They are likely to be not much important for adsorption on solid surfaces. Besides, it is proposed that there will be the affinity of larger protein molecules to be selectively adsorbed if all other conditions are kept constant (1996). However, Ismail and his collegues showed that the adsorption isotherm strongly dependent on molecular size of the adsorbate than the matrix pores. It is concluded as, adsorption capacity is affected by the difference in molecular weight and the size of two proteins (2005).

Adsorption from mixtures usually proceeds as a competitive process. It is also important to emphasize that by changing solution conditions, e.g., ionic strength or pH, those may affect the properties of only some of the proteins in the mixture and hence

decrease or increase the adsorption tendencies depending on the overall physicochemical effects (Magdassi, 1996).

CHAPTER 6

MATERIALS AND METHODS

6.1. Materials

Natural zeolites were used as adsorbents throughout the study. The raw form was mined from Western Anatolia; namely Gördes/MANİSA region. The samples having 75-150 μm particle size were prepared. Throughout, the study not only the natural form of zeolite but also the dealuminated form, Na-exchanged form and Mn-exchanged form of natural zeolites were utilized.

For protein adsorption whey/whey powder and for fermentation studies whey powder was used as carbon source. They were obtained from Pınar Dairy Products, Inc (İzmir, Turkey). The homofermentative microorganism was *Lactobacillus casei* NRRL B-441 which was used in lactic acid fermentation. This organism was supplied from United States Department of Agriculture, National Center for Agricultural Utilization Research.

The chemicals and their properties, used during this study are submitted in Appendix A. All the solutions were prepared by using ultrapure water. The chemicals used for HPLC analysis were HPLC grade and the others were analytical grade.

6.2. Methods

The methods are submitted starting from the preparation of natural zeolites and modified zeolites. In adddition to that characterization techniques are explained. Batch adsorption equilibrium studies of Bovine Serum Albumin (BSA) and whey proteins were studied. The effects of pH, initial protein concentration, solid/liquid ratio, elemental composition of the medium, particle size, agitation speed, temperature, conditioning, purity and dealumination were investigated. Moreover, desorption studies of BSA and whey proteins were performed. The effects of elution pH, protein source and washing step were determined. Furthermore, batch fermentation studies for lactic

acid production with natural zeolites were performed. The effect of natural zeolite on fermentation pH and their effect as elemental source in fermentation was studied.

6.2.1. Natural Zeolite Preparation

The natural zeolite tuff was washed by using deionized water to remove the main impurities. Next, the tuff was coarse crushed without using any equipments but providing the force by hitting at each other. Thus the material having dimensions around 5 cm was ready to be fed into the tribomechanical mill. The particle size of outlet product was below 500 μm roughly. In the following step, dry screening was applied with the mesh size of 500 μm, 150 μm, and 75 μm screeens. Natural zeolites between the ranges of 75-150 μm was wet sieved by deionized water to remove the fine particles, until clear washing solution was obtained. The 75-150 μm sieved part was collected and wet washed twice in a thermal stirrer up to boiling temperature with a solid/liquid ratio of 1/10. This step was performed to remove the water soluble impurities. The supernatant was poured away and the precipitate was dried in the oven at 105 ^{o}C overnight. For storage they were kept inside well closed containers to protect from moisture exposure at room temperature. The adsorbent material having particle size between 75-150 μm was ready to be used througly in the study. Since the whole properties are of great importance both in adsorption and lactic acid fermentation studies, the prepared natural zeolite was characterized by several instrumental techniques determining crystal structure (XRD), particle size distribution (Sedigraph), elemental composition (quantitavely-ICP, semi quantitative-EDX), crystal morphology (SEM), bonding structure (FTIR), surface area and pore size distributions (Micromeritics) and the presence of loosely and tightly bound water (TGA). The characterization techniques are given in subsequent headlines and the resuls of these characterization studies are presented in Chapter 7 Results and Discussion session.

6.2.2. Modified Zeolite Preparation

Besides the natural form, dealuminated natural zeolites were also studied. Dealumination of natural zeolites was performed by treating 5 g of natural zeolite with 1 M HCl, 5 M HCl, 8 M HCl, and 10 M HCl solution in 100 ml working volume. The

solutions were kept in a thermally controlled shaking waterbath GFL 1092 at 99^{o}C for 3 hours. Next, the acid solutions were poured out and the solid residue was washed several times with ultrapure water to remove the Cl^- ions. The washing step was repeated until the pH of the washing water was ensuring the pH of the ultrapure water (UPW). Besides, $AgNO_3$ was used as an indicator to detect the Cl^- ions present in the washing water. $AgNO_3$ forms a precipitate namely AgCl as white opaque color in the presence of Cl^- ions. The adsorbents were dried in oven overnight at 65 ^{o}C.

Na-exchanged natural zeolites were prepared in microwave determined by the procedure defined by Akdeniz and Ülkü (2007). The applicability of Na-exchanged form in lactic acid fermentation studies were investigated. 1 N NaCl solution was prepared and the solution was treated with natural zeolite having both 1/10 and 1/50 Solid-Liquid ratios inside microwave MARS 5-CEM. The mixtures were put into closed teflon containers including teflon stirrer bars. The exchange process was performed at 80 ^{o}C for 1 hour. Later on the liquid part was poured away, refreshment of solution with 1 N NaCl stock solution was carried out. They were let to be exchanged for a second 1 hour time period at 80 ^{o}C under 1200 watt. At the end, the liquid part was removed and the solid part was dried in oven at 65 ^{o}C overnight. They were labeled and stored in airtight sample containers.

The experimental protocol followed for Mn-exchange process was performed in a thermally controlled shaking waterbath GFL 1092. The initial solution concentration used for Mn-exchange process was 1000 ppm $Mn(NO_3)$. 5 g natural zeolite with 250 ml Mn solution was placed inside 500 ml closed bottles and the mixture was directed to exchange process under the agitation speed of 185 rpm at 25 ^{o}C for 24 hours. Later on, the liquid part was removed and the solid part was dried in oven at 65 ^{o}C overnight.

All the zeolite samples were placed inside well closed containers. During the study attention was paid for the minimum moisture exposure. First all the experimental preparations completed, next natural zeolites were transferred inside flasks to preserve constant moisture content.

6.2.3. Adsorbent Characterization

The particle size distribution of the natural zeolite samples (75-150 μm) were analyzed by Micromeritics Sedigraph 5100. The natural zeolite particles were mixed

with 0.1 % calgon solution followed by ultrasonic treatment for 15 minutes in order to disperse the particles.

The surface area, pore size and pore volume of natural zeolites were measured by the ASAP 2010 M Micromeritics Instrument. Degassing conditions were 350 ^{o}C for 24 hours. Nitrogen gas was used at 77 K for the adsorption and desorption studies onto natural zeolites.

The crystals and the general morphology of natural zeolites were visualized by Scanning Electron Microscope by using Philips XL-30S FEG. Besides this instrument was also utilized for energy dispersive X-ray analysis.

The X-ray diffractograms of natural zeolite powders were obtained by Philips X`pert Pro. The crystalline phases were identified for natural zeolites. It was a beneficial tool to see the effect of modification applications by the appearance and disappearance of minerologic constituents.

Thermo Gravimetric Analysis were conducted by Shimadzu TGA-51 H by a 10 ^{o}C heating rate up to 1000 ^{o}C. The flowing gas was N_2 with a flow rate of 40 ml/min. Differential Thermal Analysis of natural zeolites were performed by DTA-50 Shimadzu.

The elemental composition of the natural zeolites and the element composition in aqueous solutions were measured by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP) with axial plasma Liberty Series II-Varian. The solid zeolite samples were treated with lithium tetraborate. The samples were dissolved in HNO_3 solution adjusting the 2.5 % acid concentration for the final solution. The liquid samples did not require a treatment with lithium tetraborate but acidified in the HNO_3 with the 2.5 % final acid concentration. Multielement standard solutions and silisium standard solutions were used to prepare calibration curves for the element compositions.

The infrared spectroscopy of natural zeolites were measured by Fourier transform infrared spectroscopy (FTIR). The natural zeolites in dry form were pelletized with potassium bromide by the mass ratio of sample to KBr ;1/50. The solid mixture was ground and exposed to pressure by hydraulic press. The infrared spectrum was obtained with FTIR 8400 S Shimadzu from 400 to 4000 cm^{-1}.

6.2.4. Protein Adsorption

Adsorption studies were performed in batch mode inside temperature controlled orbital shaker Max Q-4000 and Gerhardt thermoshaker. The adsorption studies were performed at constant temperature.Bovine serum albumin (BSA) as a standard protein, whey powder and whey were used for adsorption studies. BSA and whey powder were dissolved in 10 mM potassium phosphate buffer solution.

The adsorption study of natural zeolite and Bovine Serum Albumin(BSA) as a function of concentration was performed in a 50 ml working volume. 2g. natural zeolite was added into the protein solution. Samples were taken against 24 hours. Initially sampling was carried out by short time intervals, however after 1 hour sampling was continued every hour. Sampling volume was 400 μl to provide constant working volume. Samples were obtained and centrifuged at 3000 rpm for 60 seconds to provide separation between solid and liquid part without a change in protein content as a result of centrifugal forces. The supernatant was stored in refrigerator at 4 ^{o}C for kinetics and equilibrium measurements. The protein concentration determination in experiments were performed with standard Bovine Serum Albumin (BSA) solution and whey protein by UV-Visible Spectrophotometer. The assay used for total protein determination was Lowry method. Detailed procedure for Lowry analysis is given in Appendix C. In addition HPLC equipped with Zorbax GF-250 gel permeation column was used to determine the major whey proteins one by one. The operating conditions are given in Appendix D.

The effect of pH was studied by adjusting the 10 mM potassium phosphate buffer solution at 3 different pH values. 1 N HCl solution was used to obtain lower pH values. The adjusted pH values were pH 6.6, pH 4.9 and pH 3.2. The pH of ultrapure water was 5.6. The kinetics study for sorption was observed for 24 hours at 25^{o}C. The agitation speed was 150 rpm.

The elemental composition for BSA-natural zeolites and whey powder-natural zeolites medium during protein adsorption was studied. The adsorption and release of cations by the natural zeolites inside liquid medium were analysed by ICP. BSA solutions were prepared with water and 10 mM potassium phosphate solution at pH 3.2, 4.9 and 6.6. The solutions for whey powder system were prepared with 10 mM potassium phosphate solution at pH 3.2. The samples were acidified to 2.5 % final

concentration with HNO_3. For BSA-natural zeolite system initial and final elemental compositions were measured. But for whey powder-natural zeolite system elemental kinetics during the 24 hour adsorption study at 25 ^oC and 150 rpm was analysed.

Particle size effect was investigated for whey powder-natural zeolite system at three different natural zeolite dimensions. 0.4 g whey powder was dissolved in 10 mM potassium phosphate buffer solution. 2 g of natural zeolite particles smaller than 38μm, between 38-75 μm and 75-150 μm were treated with 50 ml whey powder solution. Sampling was conducted by each minute during the initial 5 minutes period for small particle size batches. The equilibrium was observed for 24 hours at 25^oC by 150 rpm agitation speed.

The influence of dealuminated zeolites were investigated by addion of 2 g adsorbents into 50 ml solutions in which 0.4 g. whey powder was dissolved. Under the speed of 150 rpm agitation, natural zeolites treated with 1M HCl, 5M HCl, 8M HCl and 10M HCl were investigated for protein adsorption. The temperature was 25^oC.

Agitation speed effect for the equilibrium adsorption of whey proteins were observed for 50 rpm, 100 rpm, 150 rpm and 200 rpm. The solid/liquid ratio was 1/25 and the temperature was kept at 25 ^oC for 24 hours. 0.4 g whey powder was dissolved in 50 ml potassium phosphate buffer solution which has 1.016 g/l initial protein concentration.

The effect of temperature was investigated for BSA-natural zeolite, whey powder-natural zeolite and whey-natural zeolite systems. The temperatures were set at 15 ^oC and 25 ^oC. The solid/liquid ratio was 1/25 and the agitation sped was 150 rpm. The BSA and whey powder solutions were prepared with 10 mM potassium phosphate solution with a pH value of 3.2. Whey was readily used without pH adjustment. The working volume was 50 ml.

Experimental studies were conducted for investigating the adsorption study at different initial concentrations. For BSA solutions having 0.4 g/l, 0.6 g/l, 0.8 g/l, 1.0 g/l, 2.0 g/l, 3.0 g/l, 4.0 g/l and 5.0 g/l initial concentrations were prepared with 10 mM potassium phosphate buffer solution at pH 3.2. For whey proteins, 0.2 g, 0.4 g, 0.6 g, 0.8 g, 1.0g, 2.0 g, 3.0g, 4.0 g and 5.0 g. whey powder was dissolved in 10 mM potassium phosphate buffer solution at pH 3.2. the corresponding whey protein concentration values were; 0.508 g/l, 1.016 g/l, 1.524 g/l, 2.032 g/l, 2.54 g/l, 5.08 g/l, 7.62 g/l, 10.16 g/l and 12.7 g/l respectively. the solid/liquid ratio was 1/25, the

temperature was 25 ^{o}C and the agitation speed was 150 rpm. Sampling was carried out for 24 hours.

The effect of solid/liquid ratio during adsorption of proteins in BSA solution and whey powder solution were studied. The experiments were performed at 25 ^{o}C for 24 hours. The agitation speeed was 150 rpm. Initial BSA concentrations were 2 g/l and the initial whey protein concentrations were 12.7 g/l. The solid/liquid ratio values for BSA were; 1/12.5, 1/17, 1/25, 1/50, 1/67, 1/100, 1/200 and 1/500. the ratios for whey proteins were; 1/25, 1/33, 1/50, 1/100 and 1/500.

In order to compare the differences between the conditioned natural zeolites and readily used natural zeolites, the effect of conditioning was investigated. Natural zeolites were treated with 50 ml buffer solution without proteins for 1 hour at 150 rpm and 25 ^{o}C before adsorption has started. The liquid part was separated with centrifugation. 50 ml BSA solutions with 1.0 g/l, 2.0 g/l, 3.0 g/l, 4.0 g/l and 5.0 g/l initial concentrations were prepared and addded into conditioned natural zeolite containing flasks. Adsorption was performed at 25 ^{o}C for 24 hours under the agitation speed of 150 rpm.

The effect of purity for natural zeolites on the protein adsorption was studied. The experiment was made with natural zeolite samples having two purity values obtained as 73 % purity and above 95 % purity. The purity values were calculated considering the main clinoptilolite peaks and their intensities. The seven peaks observed in XRD diffractograms and the ratio of them to the sum of characteristic peaks were used to calculate the purity values. The reference material was Idaho which has 95 % purity. Besides that, the particle size for the former was 75-150 μm and 7 μm for the latter sample. The experiment was performed for 24 hours at 25 ^{o}C, 150 rpm and the initial protein concetration was 1.016 g/l and the solid liquid ratio was 1/25.

6.2.5. Protein Desorption

Desorption of proteins were studied with protein adsorbed natural zeolites. Different eluents were used. Polyethyleneglycol, water and 10 mM potassium phosphate buffer solution were the preliminary desorption liquids. The effect of elution buffer pH on the desorption of proteins was investigated. 10 mM potassium phosphate buffer solution having pH values at 3.2, 4.9, 6.6 and 8.9 were prepared and used during

washing and desorption steps. Desorption studies were performed for BSA-natural zeolites, whey powder-natural zeolites and whey-natural zeolites adsorption systems.

At the end of adsorption study the liquid part was poured out and the residual mixture was transferred into 15 ml falcon tubes. They were subjected to centrifugation at 3000 rpm for 60 seconds in Hettich Zentrifugen. The liquid part was removed. The solid part was washed with 5 ml adsorption buffer by 3 times. The protein adsorbed natural zeolites were vortexed by Yellowline TTS 2 at 1600 rpm for 1 minute. The washed solid part was tranferred into 100 ml flasks with 50 ml elution buffer. The flasks were installed inside shaker at 150 rpm, at 25 ^{o}C. Samples were collected by various time intervals. Liquid samples of washing step and desorption part were analyzed for protein contents.

The effect of washing step applied before desorption was investigated. Two experimental sets for protein adsorption study were performed. After protein adsorption, one of the set was washed as in the preceeding session and for the other set it was performed without washing the adsorbent. For the latter case, the adsorbed samples were centrifuged for 60 seconds at 3000 rpm to remove the liquid part. The solid part was treated with 50 ml desorption liquid at 150 rpm and 25 ^{o}C. The samples were collected until the equilibrium was reached. All the conditions, concentrations and amounts were identical except the washing step during the experiment. The protein content for the desorption samples and the washing buffer samples were analysed with Lowry protein determination method at 750 nm.

6.2.6. Natural Zeolites in Fermentation

Litmus milk was used as culture propagation medium. Lyophilized form of *Lactobacillus casei* NRRL B-441 was activated in this medium. 101 g litmus milk powder was dissolved in 1 liter distilled water. 20 ml of suspension was poured into 25 ml heat stable glass bottles and were sterilized at 121 ^{o}C for 5 minutes in an autoclave with respect to the appropriate preparation procedure.

The maintenance of culture was done by propagating 10 % (v/v) *Lactobacillus casei* NRRL B-441 in 20 ml sterile litmus milk suspensions. The microorganism was incubated at 37 ^{0}C for 24 hours by at least 15 day intervals in the incubator and stored in the refrigerator at 4 ^{o}C. For the fermentation experiments 24-hour-old fresh cultures

were used as the inocula. The components and concentrations of fermentation media are given in Table 6.1.

Table 6.1. Ingredients used for the preparation of the fermentation media. (Source: Büyükkileci, 2000)

Component	**Concentration (g/l)**
Whey powder	80
Yeast extract	5
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4$	0.2
$MnSO_4.H_2O$	0.05

The medium components were sterilized separately. 250 ml erlen meyer flasks were used for shake flask experiments. Each with a working volume of 125 ml were used for the batch fermentation studies. The pH control was provided by using 3 g $CaCO_3$ in batch fermentation experiments. Fermentations were carried out at 37 ^oC, with a stirring rate of 150 rpm. The experimental set was named as control fermentation operated under preceeding compositions and conditions. 500 µl samples were collected and analysed against various time intervals. The samples were centrifuged at 14 000 rpm for 10 minutes and diluted with the mobile phase used in HPLC (High Pressure Liquid Chromatography) measurements.

The applicability of natural zeolites, as pH regulator, as mineral source for the fermentation media and as adsorbents were studied. From this viewpoint natural zeolites in aqueous lactose and lactic acid solutions, in fermentation media and in cell-free fermentation broth were investigated.

For determining the effect of natural zeolites on fermentation pH 3 g, 5g, 8g, and 15 g natural zeolites were added inside fermentation media with and without the pH regulating constituent, $CaCO_3$. The mixtures were agitated continuously in an orbital shaker at 37^oC and 150 rpm to ensure homogenous dispersion. The amount of lactose and lactic acid concentrations in solution were taken by pipetting small amounts of solution at short time intervals starting from the initial time until the equilibrium was

reached. Samples were centrifuged and diluted with 5mM H_2SO_4 for further analyses. The lactose and lactic acid concentrations in solution were determined by using HPLC. The pH of the process was monitored at various times during fermentation.

The behavior of Na-exchanged zeolites inside fermentation media without $CaCO_3$ addition were studied. The lactose and lactic acid concentration changes were observed for the whole fermentation period. The operating conditions were 37^oC and 150 rpm.

The effect of natural zeolites as elemental source during fermentation studies was investigated. The mineral salts KH_2PO_4, K_2HPO_4, $MgSO_4$, and $MnSO_4.H_2O$ were put in control fermentation set but those mineral salts were not added for 5 g natural zeolite containing fermentation sets. In addition to that to see the influence of manganase on fermentation individually, the study was performed with no manganase addition in the absence and presence of 5 g natural zeolites. The pH profile was observed for the whole fermentation period. The behavior of Mn-exchanged natural zeolites in fermentation was observed. 5 g Mn-exchanged zeolites were added inside fermentation media after sterilization. The pH profile was moditored for 32 hours.

Lactose adsorption was applied by dissolution of whey powder. Samples were harvested from the flasks of whey powder solution. The solution was prepared by dissolving 10 g of whey powder within 10 mM potassium phosphate buffer solution. The test period was started as soon as natural zeolite was transferred inside the medium. The samples were diluted with mobile phase for detection in HPLC. The agitation condition was 150 rpm and the temperature was set at 37 ^oC.

Lactic acid solutions were prepared from a L(+)-lactic acid solution. Adsorption kinetics experiments for both natural zeolites and acid treated natural zeolites were performed as batch mode in well stirred vessels. 25 ml of 5 % aqueous lactic acid solutions and 3 g zeolites were treated at 37 ^oC for 24 hours.

The adsorption behavior of natural zeolite and synthetic zeolite ZSM-5 was studied in 25 ml 5 % lactic acid solution, in cell free fermentation broth. 3 g adsorbents were added inside liquid medium at 37 ^oC and 150 rpm. The effect of synthetic zeolite ZSM-5 during fermentation process was observed and the pH profile was monitored.

The lactose and lactic acid analyses were performed with HPLC and total protein determination was done by spectrophotometer where as the whey proteins were investigated one by one with HPLC. The detailed description of analytical conditions, procedures and the instrument specifications were given in Appendix D.

CHAPTER 7

RESULTS AND DISCUSSION

7.1. Characterization of Clinoptilolite

Local natural zeolite, clinoptilolite was obtained as large particles. They were pretreated to be used during the experimental study. Characterization techniques were applied to the natural zeolite samples to determine the structural features and to provide consistency in experimental part of the study.

7.1.1. Particle Size Distribution

Particle size analyzer Sedigraph 5100 was used to measure particle size distribution of natural zeolite. As shown in Figure 7.1, 96% of the particles were lower than 150 μm in size and 20 % of the particles were below 30 μm.

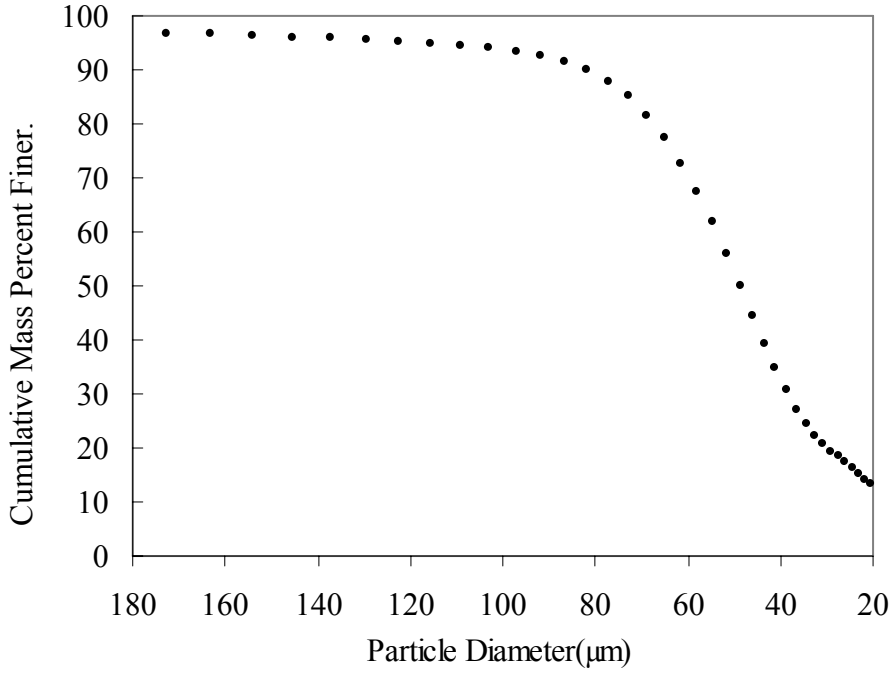

Figure 7.1. Particle size distribution of clinoptilolite.

7.1.2. Surface Area Measurements

BET and Langmuir methods were used to determine surface areas, pore size and volume of clinoptilolite. The surface area is an important parameter for high adsorption capacity. The pore diameter is also an important property which affects the diffusion in adsorption mechanism. The values obtained for clinoptilolite are tabulated in Table 7.1.

Table 7.1. The adsorption and desorption measurements of clinoptilolite.

Area	**Clinoptilolite**
BET Surface Area (m^2/g)	32.1786
Langmuir Surface Area (m^2/g)	46.0988
Micropore Area (m^2/g)	11.5855
Volume	
Micropore Volume (cm^3/g)	0.006134
Pore Size	
Average Pore Diameter (4V/A by BET) Å	62.0796
H-K Method	
Median Pore Diameter Å	11.3835

7.1.3. Scanning Electron Microscopy Analysis (SEM)

Clinoptilolite posseses the regular platelike structure. It is a common morphology of heulandites. The morphology of clinoptilolite shows the characteristic clinoptilolite crystal structure. The SEM micrograph is shown in Figure 7.2.

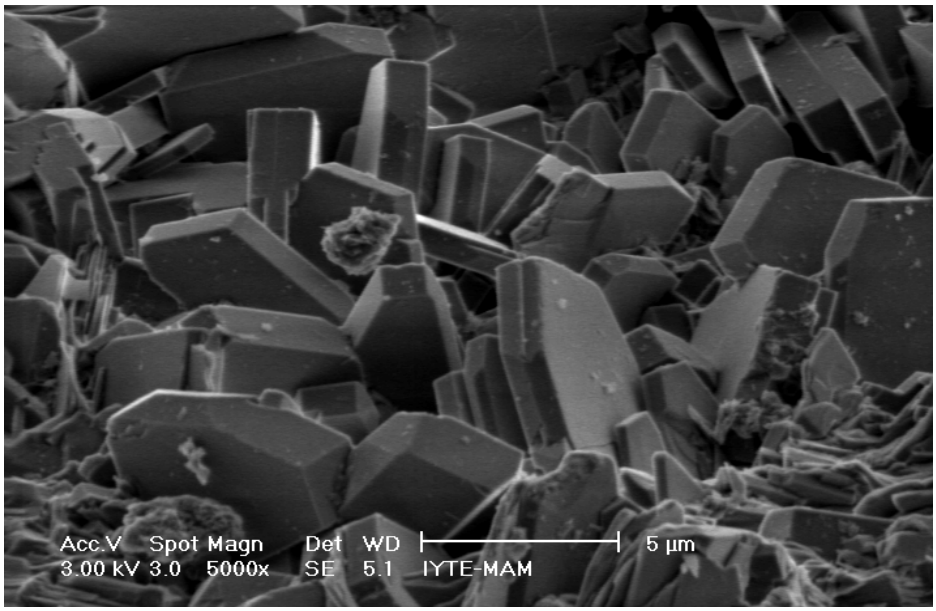

Figure 7.2. Natural zeolite crystals (75 -150 µm).

7.1.4. X-Ray Diffraction (XRD) Analysis

The X-Ray diffractogram of natural zeolite is given in Figure 7.3. The XRD pattern of clinoptilolite has four main peaks. The characteristics peaks takes place at 9.8^o, 11.15^o, 22.3^o and 30.1^o and denoted by arrows.

The positions and the intensities of the samples are of great importance. They are used to identify the phases existing in natural zeolites. The results indicate the compositional characteristics of the sample. In order to interpret the dominant phase X-Ray diffractograms are required. With respect to the peak intensity values and their positions, percentage of the sample purity is determined. The purity of the natural zeolite samples are calculated as 73 %. No specific purification step was applied. But it is known that grinding to smaller particle sizes, thermal treatment and centrifugation are the useful steps to increase the purity of samples. Higher percentage values of purity can be required for their usage in health applications.

The samples are rich in clinoptilolite content. On the other hand, the other phases existing in smaller amounts are; quartz ($2\Theta=26^o$) and feldspar ($2\Theta=27^o$).

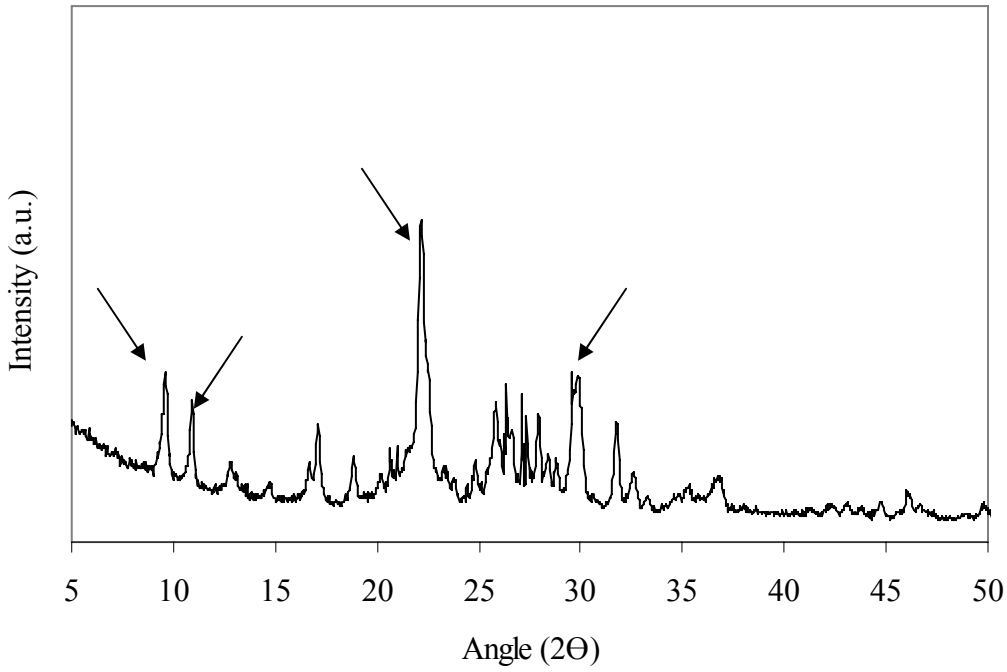

Figure 7.3. XRD Pattern of clinoptilolite (75-150μm).

7.1.5. Thermal Gravimetric Analysis (TGA)

The TGA curve for clinoptilolite is given in Figure 7.4. The percent weight losses of clinoptilolite when exposed to high temperatures were given in Table 7.2. these were related with the decrease in water content. The water content of clinoptilolite were categorized into three classes. External water, loosely bound water and tightly bound water. The external water was released up to 85^{o}C, and loosely bound water was rapidly lost up to 285 ^{o}C. The clinoptilolite started to loose the tightly bound water after 285 ^{o}C. The average percent weight losses up to 800^{o}C was determined as 11.71 %.

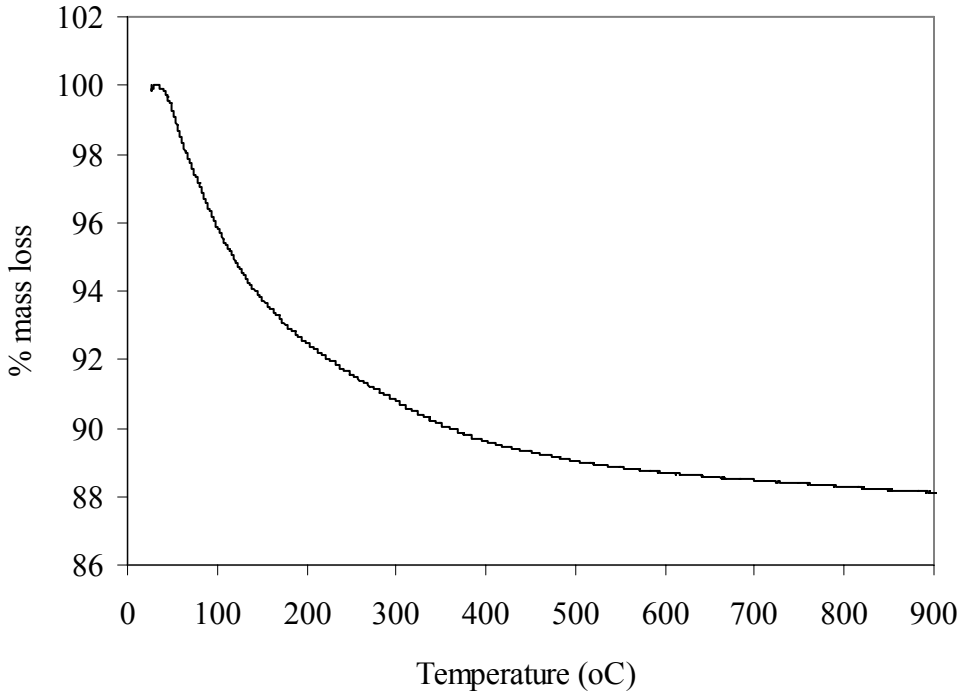

Figure 7.4. TGA curve of clinoptilolite.

Table 7.2. The percent weight losses of external, loosely bound and tightly bound water for clinoptilolite.

External Water	**Loosely Bound Water**	**Tightly Bound Water**		**Total**
Below 85^{o}C	(85^{o}C-285^{o}C)	(285^{o}C-500^{o}C)	Above 500^{o}C	
2287	5.7399	2.0089	0.7354	11.71

7.1.6. Differential Thermal Analysis (DTA)

The DTA curve belonging to clinoptilolite is given in Figure 7.5. The endotherm could be related to the dehydration of clinoptilolite and the exotherm was related with the structural changes of clinoptilolite. Dehydration around 58 ^{o}C is considered.

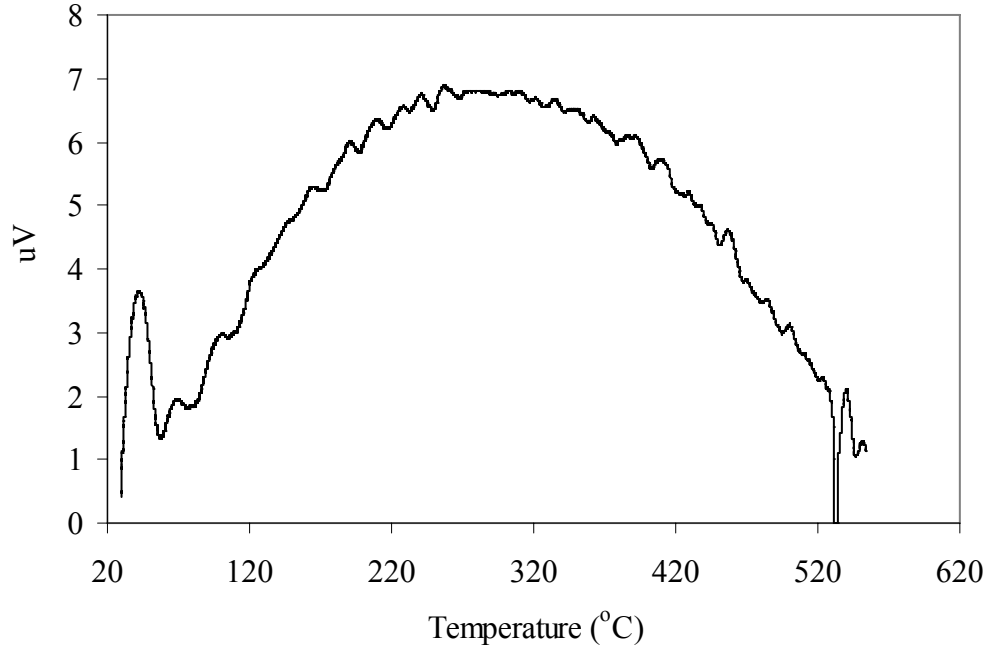

Figure 7.5. DTA curve of clinoptilolite.

7.1.7. Elemental Analysis

Table 7.3. The elements composition in oxide form.

Elements in Oxide Form	**Chemical Composition by weight(%)**
Al_2O_3	13.88
CaO	2.24
Fe_2O_3	1.29
K_2O	7.04
MgO	0.33
MnO	0.03
Na_2O	2.20
SiO_2	72.98
Si/Al	**5.26**

The chemical composion of clinoptilolite in oxide form is given in Table 7.3. The Si/Al ratio can be related with the thermal stability and hydrophobicity of natural zeolites. As the Si/Al ratio increases, clinoptilolite gain more hydrophobic character

which is very important for adsorption studies in aqueous solutions. The Si/Al ratio of natural zeolite sample is 5.26. The range for clinoptilolite is 4.5-5.5.

7.2. Preparation and Modification of Natural Zeolite

The adsorbent material that is clinoptilolite rich natural zeolite was used throughout the study. The importance of the pretreatment procedure appears when the SEM images were compared. In Figure 7.2 all steps were completed where as Figure 7.6 belongs to the sample which was not exposed to wet sieving and wet washing steps. The zeolite crystals were obviously seen in Figure 7.2. The outer surface of the natural zeolite samples were covered with finer particles of natural zeolite, and such impurities formed a barrier over the natural zeolite crystals.

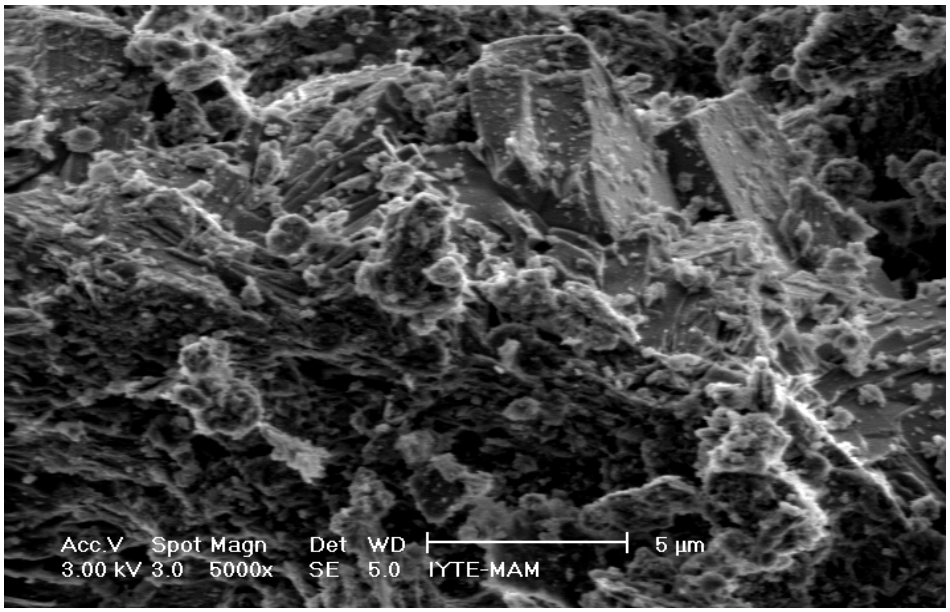

Figure 7.6. Dry sieved natural zeolite (75-150 µm).

The semi-quantitative element composition of natural zeolites were measured by EDX where the results are tabulated in Table 7.4 The weight percent distribution of element composition for these two samples were identical. The EDX results could give general idea about the samples since the system takes samples of few representative points and the results were given as percentage value. The amount of Si, Al, Ca, K, Mg, Na and O atoms on the natural zeolite surface were measured.

Direct acid treatment is one of the main types of modification methods applicable to obtain proton exchanged clinoptilolite (H-CLN); the other method is ammonium exchange followed by calcination. Direct ion exchange with dilute acid solution is known to increase the sorption capacity of clinoptilolite up to mild acid concentration. However, depending on the molar concentration of acidic solution, the modification leading structural and textural changes occurring during process should be taken into consideration (Kurama et al., 2002). The acid treatment removes the Al^{+3} ions from the structure resulting with an increase of Si/Al ratio. This ratio is a characteristic tool to determine the hydrophobicity. As the ratio increases the natural zeolites gain a more hydrophobic character.

The SEM images of acid treated natural zeolite samples are given in Figure 7.7. and Figure 7.8. There was no visual difference between images of natural zeolites and acid treated zeolites. However, if the EDX results of natural zeolites were compared with 1M HCl treated samples and 5 M HCl treated samples, the results showed that the weight percentage of Al content decreased as the concentration of HCl increased as a result of dealumination process.

Table 7.4. EDX results of representative samples.

Wt %	**Dry sieved only**	**Natural zeolite**	**1M HCl**	**5M HCl**
O	48.65	47.56	47.1	49.66
Na	1.3	1.35	0.26	0.72
Mg	0.18	0.41	0.22	0.3
Al	7.59	8	6.19	3.03
Si	36.32	36.35	42.75	44.11
K	4.33	4.49	2.57	1.42
Ca	1.64	1.84	0.9	0.76
Total %	100	100	100	100

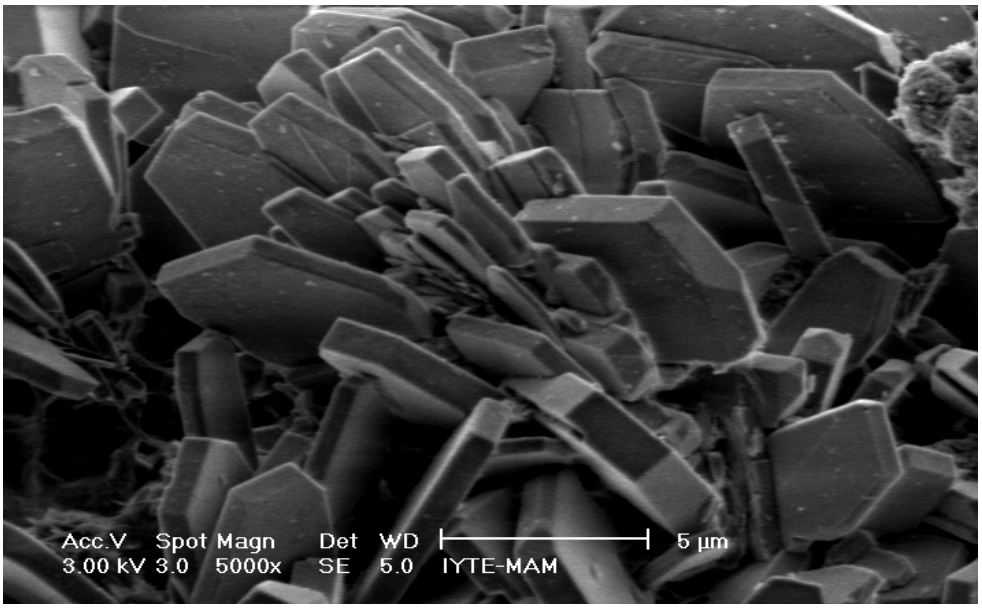

Figure 7.7. Natural zeolite modified by 1 M HCl (75-150 µm, solid-liquid ratio 1/20).

In addition to these preliminary findings of the characterization techniques, ICP and X-ray results supported them quantitatively to define the natural zeolite. ICP analyses give exact elemental composition of the natural zeolite structure in concentration values and X-ray diffractograms are useful tools to get the idea about the distribution of crystallinity leading to the purity of samples.

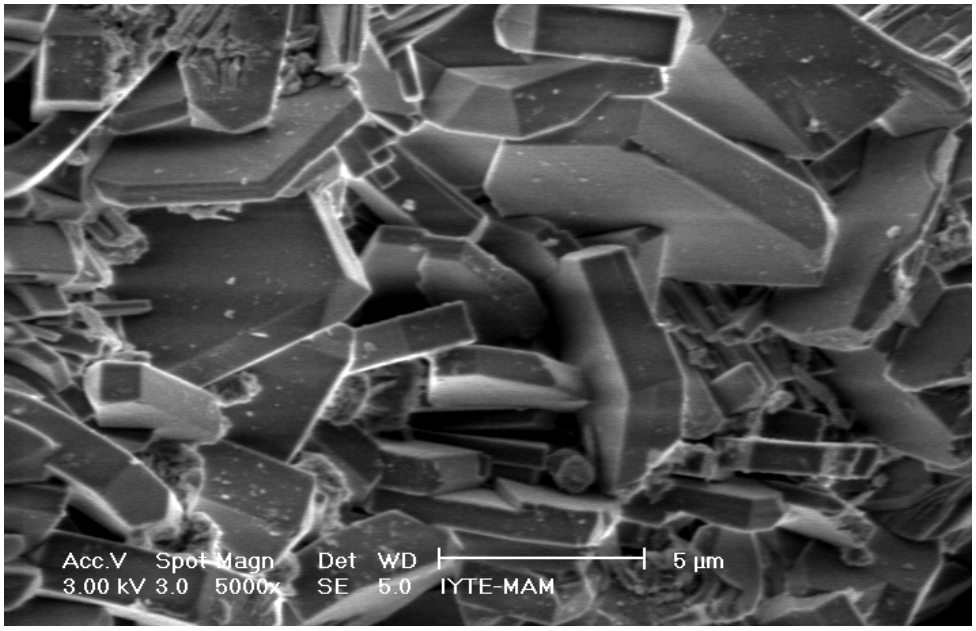

Figure 7.8. Natural zeolite Modified by 5 M HCl (75-150 µm, solid-liquid ratio 1/20).

The X-ray diffractograms of natural zeolite and dry sieved natural zeolite are depicted in Figure 7.3 and 7.9, respectively. The position of characteristic peaks belonging to the clinoptilolite phases do not change. Among those figures the crystal structure was same with almost same intensities. All the crystalline phases belonging to the natural zeolite samples are clearly visible in Figure 7.3. and Figure 7.9. The main peaks identifying the clinoptilolite phases are at positions; 9.8^o, 11.15^o, 22.3^o and 30.1^o. The X-ray diffraction analysis of natural zeolites showed that the positions and intensities of reflections correspond well with the literature data (Kurama et al., 2002).

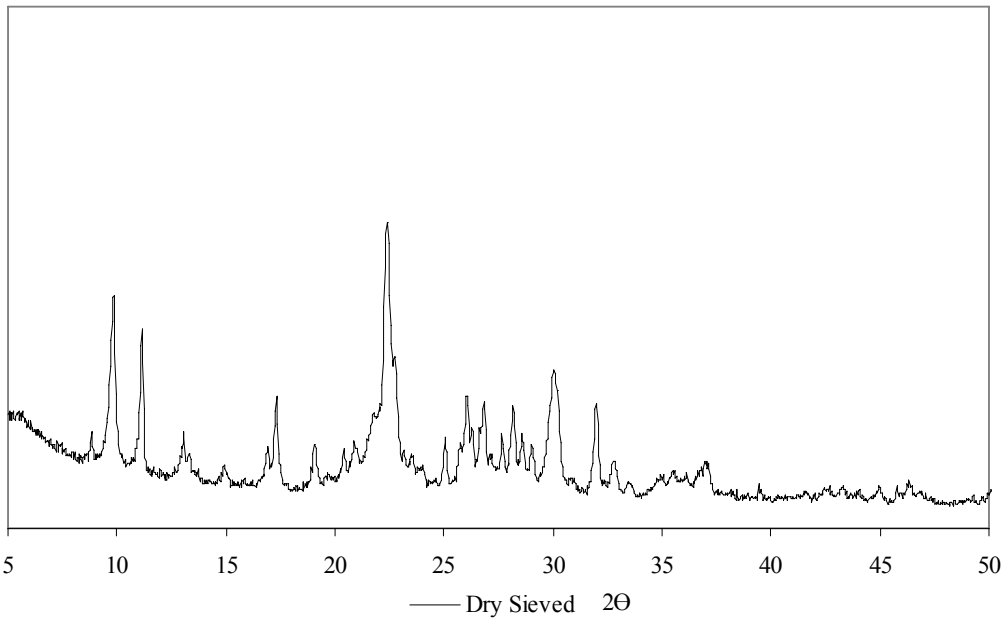

Figure 7.9. The X-ray diffraction pattern of natural zeolite (75-150 μm, dry sieved).

However, the X-ray diffractograms of acid treated natural zeolites are significantly different from the diffractograms of natural zeolites as seen in Figure 7.10. The concentration of acid solution, treated with natural zeolites for dealumination process has a great effect on the crystal structure of natural zeolites. The position of the main peaks did not change for natural zeolite and modified zeolite with 1 M HCl solution. But the intensities of the characteristic peaks highly decreased. On the other hand, for the acid solutions higher than 5 M HCl concentrations, the peak positions are dramatically changed. In that case the intensities were almost disappeared. The natural zeolite and the acid treated natural zeolites with different concentrations were overplotted for better comparison.

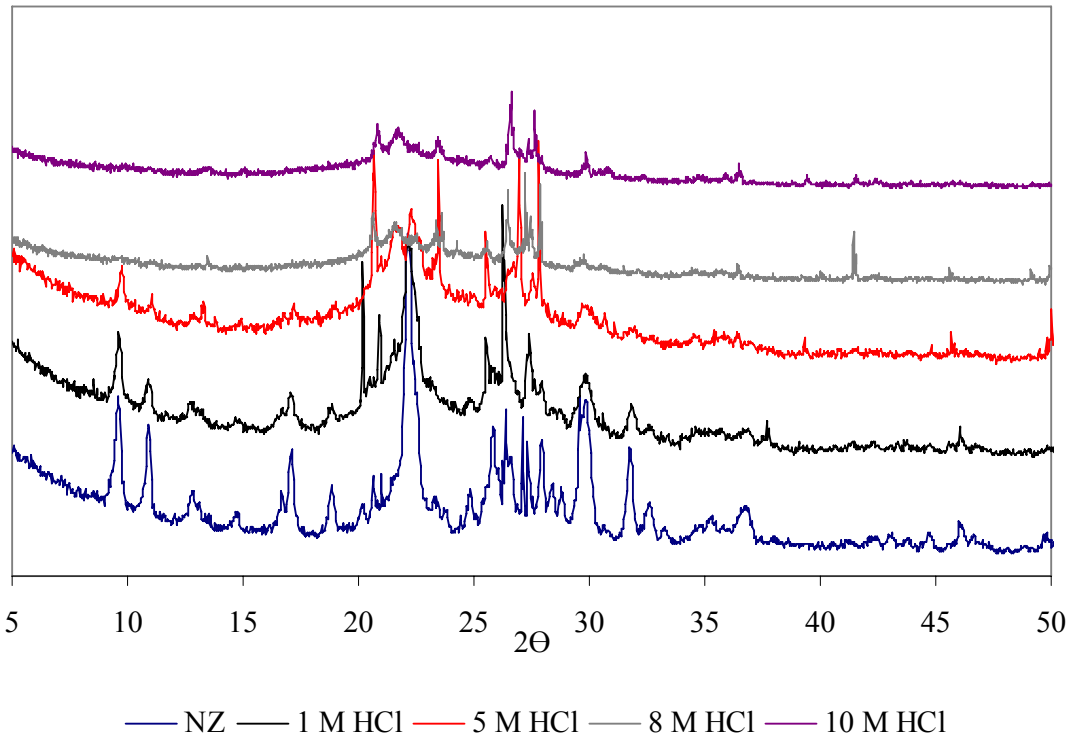

Figure 7.10. The X-Ray diffraction pattern of natural zeolite and modified zeolites.

The ICP elemental analysis results indicating the changes in elemental composition verified the effectiveness of the modification process. The chemical composition profile is summarized in Table 7.5. and Table 7.6. The results are belonging to the solid phase and liquid extract, respectively which were exposed to direct acid treatment.

Direct acid treatment enables the removal of Al^{+3}. As it is seen in Table 7.5. Al_2O_3 content of modified zeolite samples decreased to 4.91 % where the value was corresponded to 13.88 % for natural zeolite. The removal of Al^{+3} from the structure, increase the Si/Al ratio to a certain extent. The highest HCl concentration used in modification process, gave the highest Si/Al ratio. Dealumination process increases the pore volume by replacing the big Al^{+3} ions with H^{+} ions which is smaller.

Table 7.5. The elemental composition of natural and modified zeolites % by weight.

Elements	Natural Zeolite	1 M HCl treated	5 M HCl treated	8 M HCl treated	10 M HCl treated
Al_2O_3	13.88	9.15	6.87	5.10	4.91
CaO	2.24	1.04	0.79	0.59	0.49
Fe_2O_3	1.29	0.82	0.42	0.31	0.29
K_2O	7.04	4.19	3.00	2.85	2.94
MgO	0.33	0.14	0.10	0.04	0.03
MnO	0.03	0.01	0.00	0.00	0.01
Na_2O	2.20	1.39	1.26	1.27	1.32
SiO_2	72.98	84.57	87.56	90.98	91.01
Si/Al	**5.26**	**9.24**	**12.74**	**17.84**	**18.52**

Table 7.6. The elemental composition of liquid extract for modified zeolites (mg cation / g zeolite).

Elements (mg cation / g NZ)	1 M HCl	5 M HCl	8 M HCl	10 M HCl
Al	24.94	46.75	50.50	63.11
Ca	10.16	12.29	11.74	13.09
Fe	3.34	6.33	6.17	7.03
K	12.80	19.34	16.06	17.97
Mg	1.04	1.81	1.47	1.65
Mn	0.23	0.31	0.29	0.36
Na	5.81	6.19	5.94	6.70
Si	3.00	0.17	0.04	0.00

7.3. Adsorption of Proteins by Natural Zeolites

The study aims the recovery of whey proteins by NZ and production of lactic acid by using the residual supernatant containing high amounts of lactose after the

recovery of whey proteins by natural zeolite`s adsorption ability. It was not desired for lactose apt to be adsorbed onto NZ. Hence, valuable proteins could be obtained and the remainder part could be used for fermentation which is an alternative solution for the huge discharge of whey in dairy industry.

7.3.1. Bovine Serum Albumin Adsorption onto Natural Zeolites

As it is known, the net charge of proteins at their isoelectric point is zero. So, in order to see the behavior of proteins when they are treated with natural zeolites, the adsorption behaviour of standard protein, BSA at different pH values (below isoelectric point, around isoelectric point and above isoelectric point) were investigated. Considering whey and whey powder solutions which are very complex media, BSA (pI-4.9) is selected for simplicity as a reference protein to explain the process in aqueous solution. It is one of the whey proteins and its isoelectric point is very close to the other two most abundant whey proteins in whey, α- Lactalbumin (pI-4.7-5.1) and β-Lactoglobulin (pI-5.2).

In Figure 7.11 the results were plotted as BSA concentration versus time values. Besides solutions with adjusted pH values, another test was performed with the protein solution prepared with ultrapure water (UPW) which shows the change for control purposes. As it is obviously seen from Figure 7.12 the concentration of BSA depleted for solutions having adjusted pH values. Howewer, for the protein solution sample that was prepared with ultrapure water, significant change in protein concentration was not observed when the data were compared with samples pH 3.2, pH 4.9 (isoelectric point of BSA) and pH 6.6. The samples were collected for 24 hours, and the study was performed at 37^{o}. According to the initial trend of the curves, the adsorption of protein is quite rapid on local clinoptilolite rich natural zeolite.

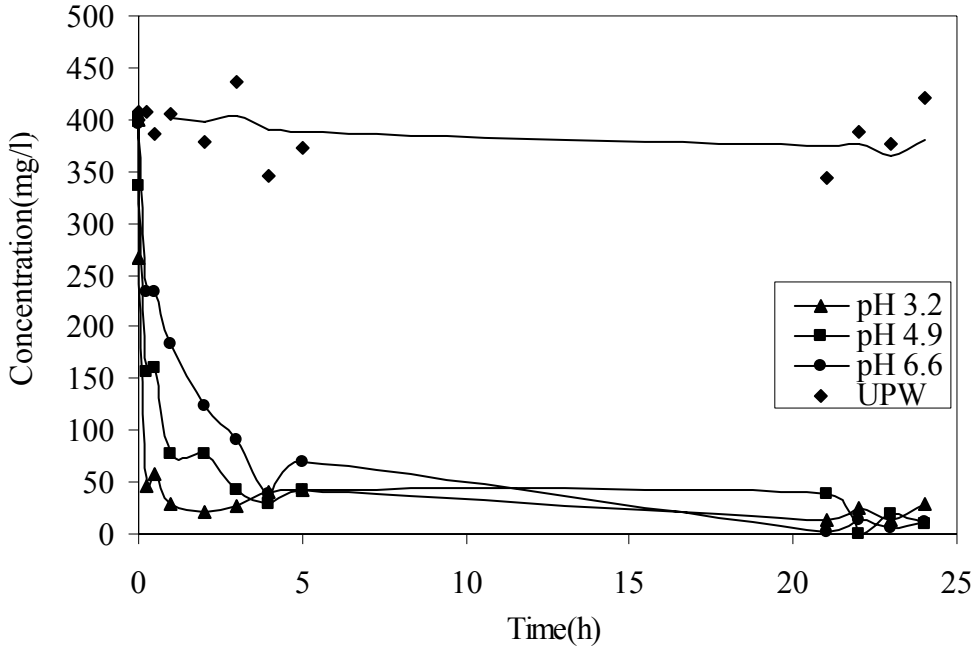

Figure 7.11. Bovine serum albumin adsorption onto natural zeolite. (Temperature: 37^{o}C, 3 g natural zeolite, 25 ml working volume, Agitation speed: 150 rpm)

The results were in good agreement with the literature. Electrostatic forces, hydrophobic interactions and van der waals forces are all effective during protein adsorption. Proteins are positively charged below their isoelectric point and are gaining a negative charge in a solution having a pH value greater than their isoelectric point. Natural zeolites carrying negative charges are susceptiple for protein adsorption. In literature most zeolites efficiently adsorbed the proteins at a pH below pI. The driving force was explained by the Coulomb's forces deriving from the positive charges of ionized proteins and the negative charge of the zeolite itself.

Although the equilibrium adsorbed amounts values for different pH values were same time period necessary for reaching the equilibrium was different. Regarding the slope for the initial part of the adsorption curve, the adsorption of proteins were greater and faster for the pH value of 3.2 than the others.

For simplicity in the experiments, the effect of standard solutions prepared with buffer solution and water on the calibration curve was investigated. The aim was determination of the absorbance consistency depending on dilutions prepared with water.

In this case, the adsorption kinetics study was performed with pH 3.2 buffer solution, the data were evaluated by using two different calibration curves. It is clearly seen that the data points were in good agreement with each other (Figure 7.12).

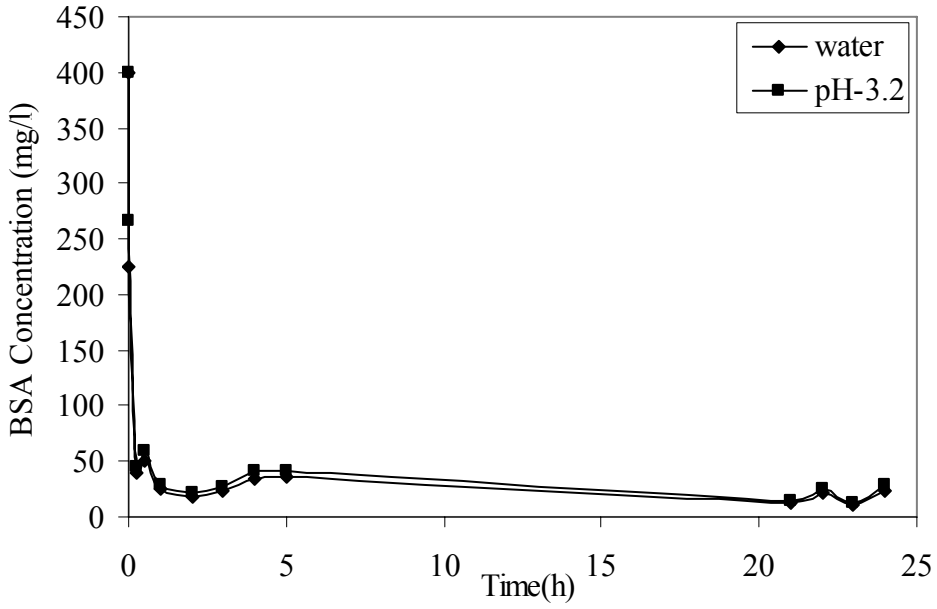

Figure 7.12. Comparison of BSA concentration adsorption data measured with standard BSA solution calibration curves prepared in buffer solution (pH:3.2) and water (Temperature:37^{o}C, agitation speed: 150 rpm, working volume: 25 ml, 3g NZ) .

During the experimental study the pH of the medium is important as it is given in Figure 7.12. On the other hand it was observed that the instrumental analysis could be performed by preparing one set of calibration standards instead of preparing standards for each liquid. In order to eliminate any differences due to instrumental or experimental effects between sets, new calibration standards before each protein analyses were prepared and each experimental set was evaluated with respect to its own calibration curve.

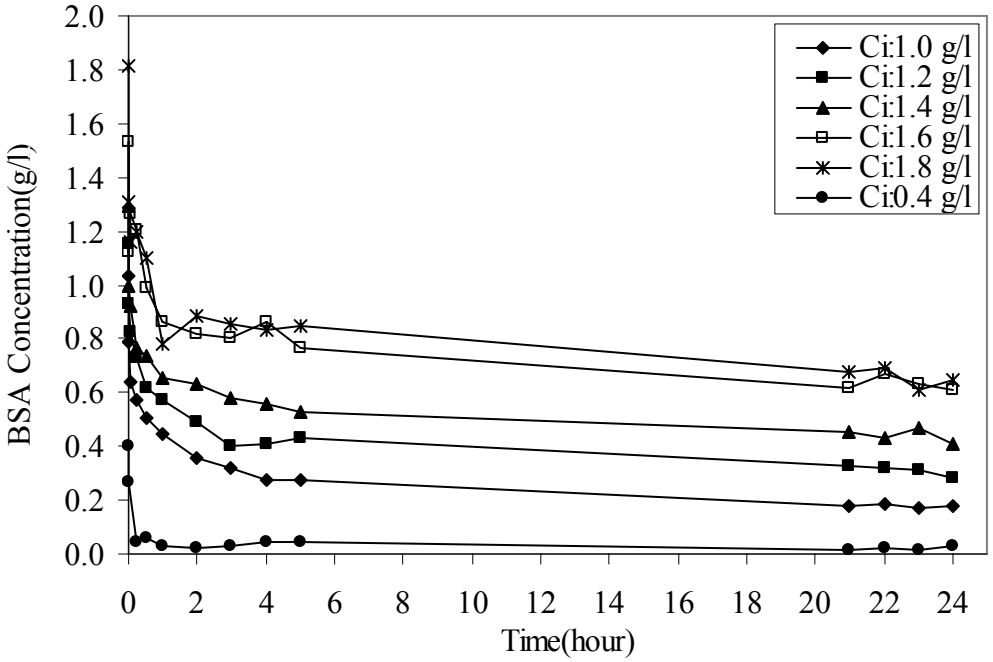

Figure 7.13. The adsorption kinetics study for BSA solution with natural zeolites for 24h (T: 37^{o}C, agitation speed: 150 rpm, working volume: 25 ml, 3 g NZ).

The equilibrium studies for BSA adsorption onto natural zeolites were performed to elucidate the best adsorption isotherm model for BSA-natural zeolite system. The adsorption study with constant natural zeolite amount but varying initial concentrations were performed. The results are represented in Figure 7.13. The experimental adsorption isotherm for BSA onto natural zeolite was plotted in Figure 7.14. For reaching equilibrium 24 h was enough.

Adsorption equilibrium for BSA onto natural zeolites were studied at two different temperatures, 15 ^{o}C and 25 ^{o}C. The aim was to make a comparison with the adsorption equilibrium study performed with whey proteins at the same temperature values. The adsorption model constants are tabulated in Table 7.7. and Table.7.8.

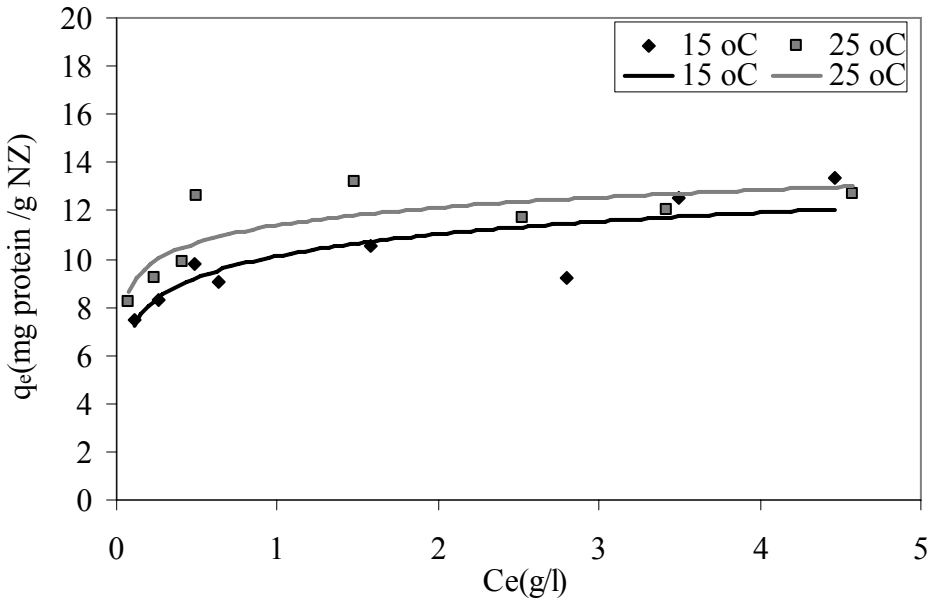

Figure 7.14. The adsorption isotherm of BSA solution with natural zeolites. (Agitation speed: 150 rpm, equilibrium time: 24 h, S/L: 1/25).

Freundlich and Langmuir adsorption models were applied for the experimental data to see which model is more suitable to explain the adsorption study. From Figure 7.15 and Figure 7.16 it is observed that Langmuir model fits better with the experimental data both at two temperatures with a regression coefficient.

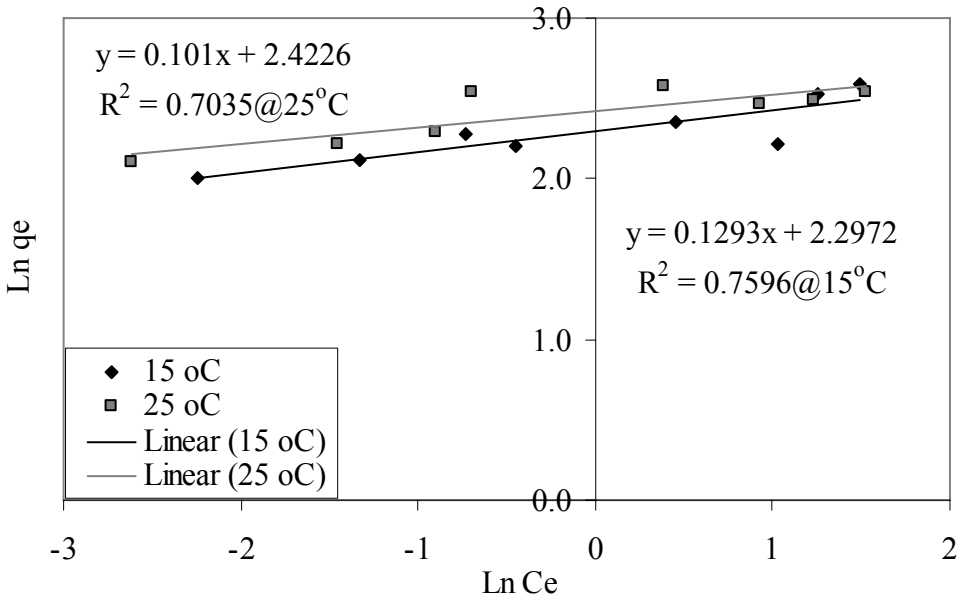

Figure 7.15. Freundlich adsorption isotherm of BSA solution with natural zeolites (Agitation speed: 150 rpm, equilibrium time: 24 h, S/L: 1/25).

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Table 7.7. Freundlich isotherm parameters for protein adsorption by natural zeolite.

Temperature (^oC)	K	1/n
15	9.95	0.13
25	11.28	0.10

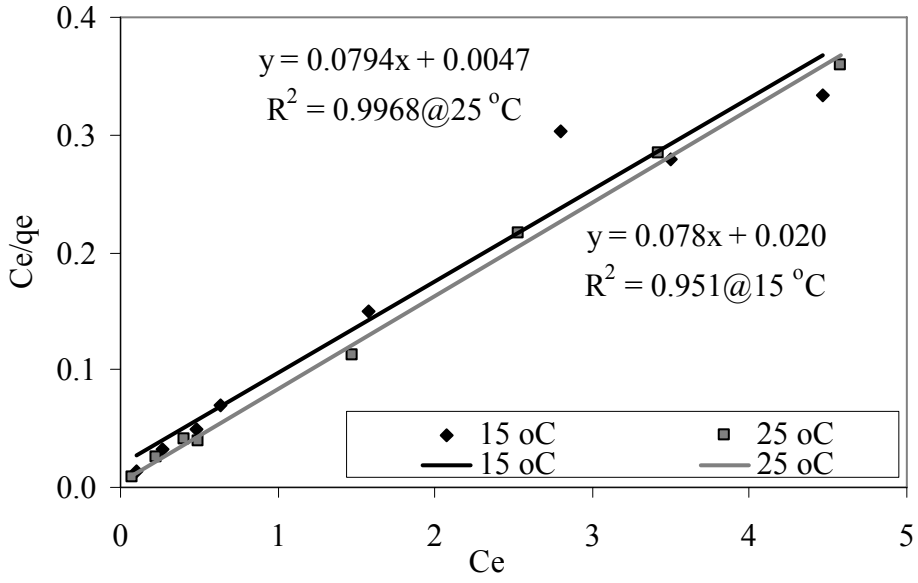

Figure 7.16. Langmuir adsorption isotherm of BSA solution with natural zeolites (Agitation speed: 150 rpm, equilibrium time: 24 h, S/L: 1/25).

Table 7.8. Langmuir isotherm parameters for protein adsorption by natural zeolite.

Temperature (^oC)	q^m (mg protein/g NZ)	K (l/g)
15	12.82	3.90
25	12.59	16.89

Maximum adsorption capacity value is calculated as q_m= 12.59 mg BSA / g natural zeolite at 25 ^oC, and q_m= 12.82 mg BSA / g natural zeolite at 15 ^oC,

Even the BSA solution was seemed to reach the equilibrium in 24 hours the time period was extended. It was examined to be sure for the time required to reach the adsorption equilibrium of aqueous BSA solution. The operating conditions were 37^oC and 150 rpm. The results obtained are in Figure 7.17.

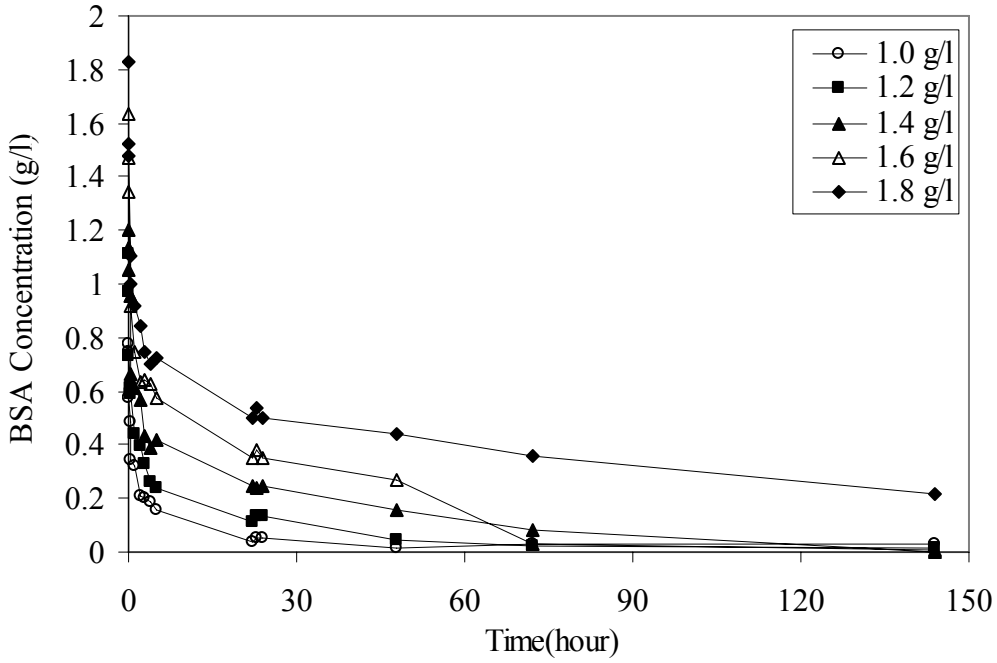

Figure 7.17. BSA Concentration variation with respect to time (T: 37^{o}C, agitation speed: 150 rpm, working volume: 25 ml, 3 g NZ).

When the time was extended it was observed that the adsorption was still continuing slowly. As all the protein amount depleted in aqueous solution, performing the study by changing the solid liquid ratio would be adequate. The idea was, performing the experiment by decreasing the amount of adsorbent and increasing the working volume so that there could be sufficient amount of protein with respect to adsorbent amount that would facilitate to obtain satisfactory results. If there is excess adsorbate, the adsorbent continues to adsorb protein up to saturation.

The study was repeated with 1/50 solid-liquid ratio. The control set which was containing BSA solution without natural zeolite was taken under observation (Figure 7.18). The variation in protein concentration after 72 hour was dramatically changed even the control set was lacking natural zeolite. In an extended time period, after than 3 days, it is not adequate to ascribe the change in protein concentration as a result of adsorption for control run.

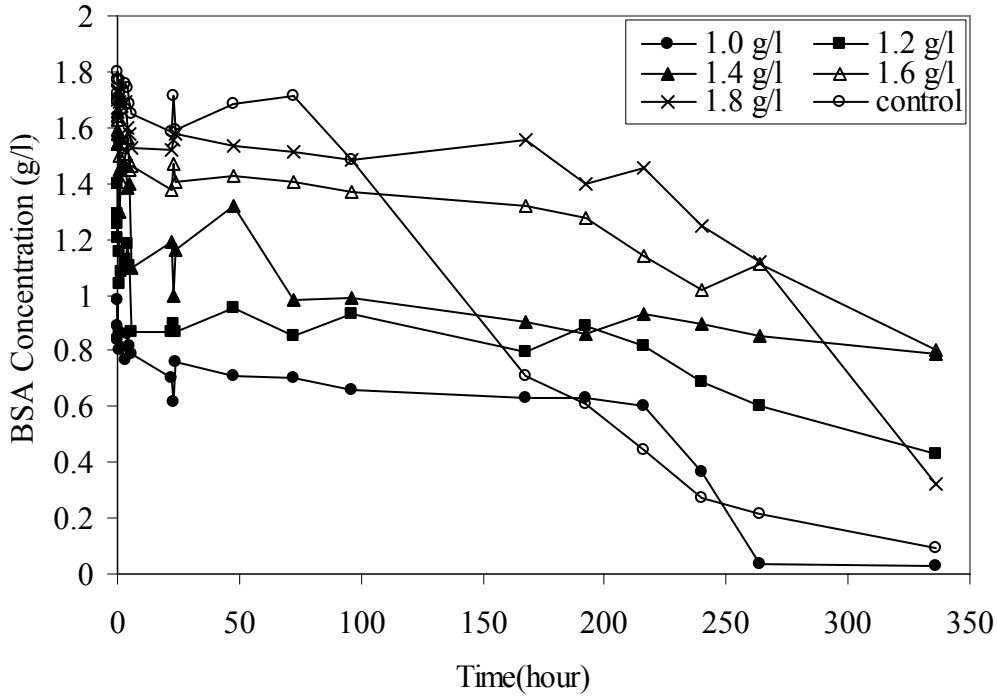

Figure 7.18. The BSA concentration change with 2 % (w/v) natural zeolites at pH 3.2 (T: 37^{o}C, agitation speed: 150 rpm, working volume: 50 ml).

However the concentration of the BSA was generally showing slight fluctuations in natural zeolite containing flasks for longer time period.. The buffering effect of natural zeolites were claimed for that behavior.

The extended time period for adsorption study was applied for other pH values and for solution prepared with ultrapure water. Figure 7.19 shows the overall results for pH 4.9. The curves of Figure were quite similar with each other at this pH value for the blank run and natural zeolite containing sets. After 24 hours, we could not talk about the stability of proteins. The experiment performed with protein solutions prepared by using buffer solution at pH 6.6 was presented in Figure 7.20. The concentration of protein in blank run and adsorbent sets started to decrease significantly after 48 hours. Up to that time there was not big deviations in concentrations. In Figure 7.19, Figure 7.20 and Figure 7.21 we have observed the variation of concentrations in blank runs. These behaviours were attributed to conformational changes of BSA.

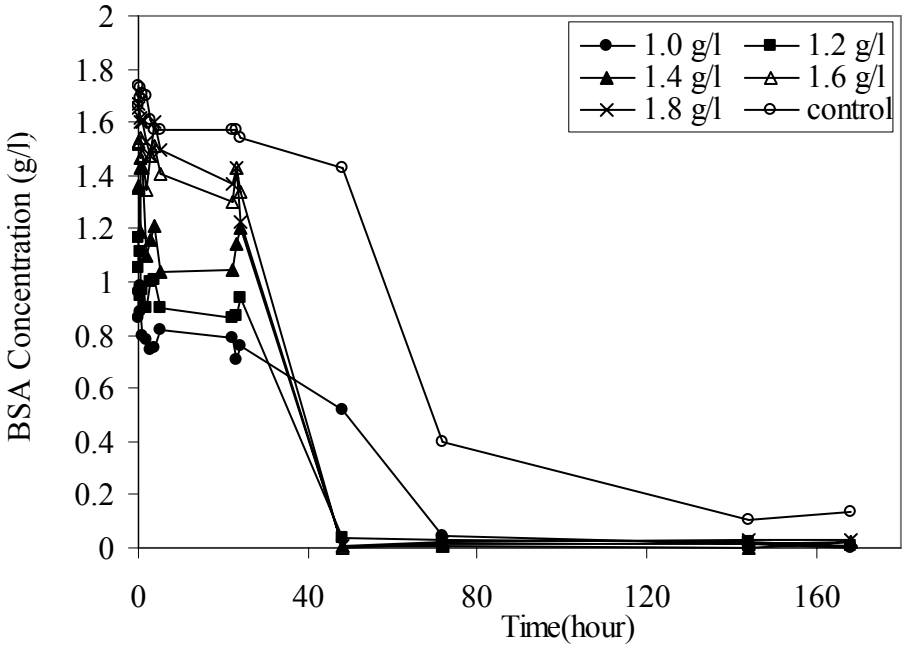

Figure 7.19. The BSA concentration change with 2 % (w/v) natural zeolites at pH 4.9 (T: 37^{o}C, agitation speed: 150 rpm, working volume: 50 ml).

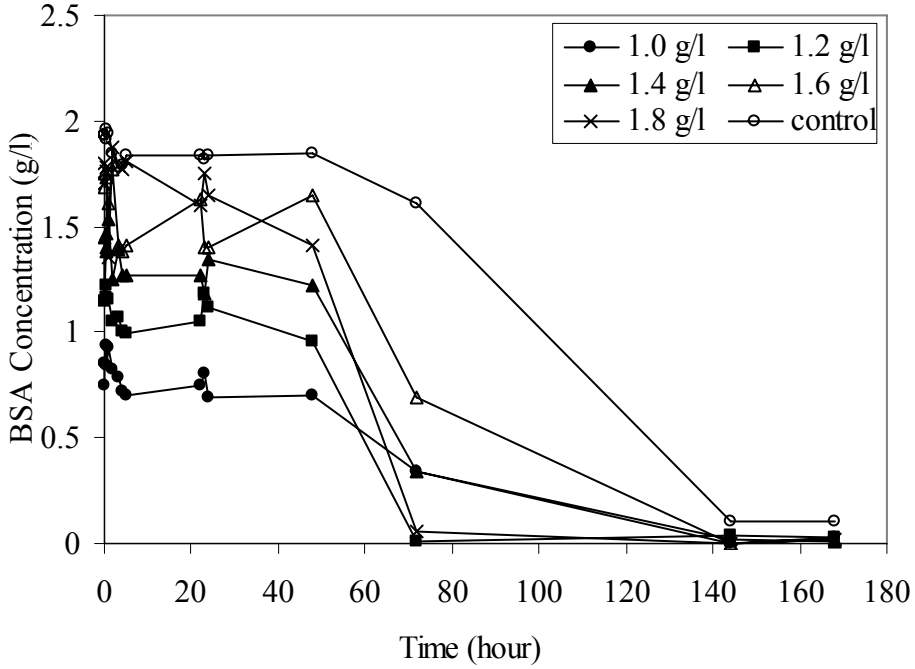

Figure 7.20. The BSA concentration change with 2 % (w/v) natural zeolites at pH 6.6 (T: 37^{o}C, agitation speed: 150 rpm, working volume: 50 ml).

The study was applied for the protein solution prepared with ultrapure water (Figure 7.21). Generally the concentrations did not change dramatically, but there was a few exceptional points for the set of initial concentration 1.0 g/l. The blank run has

showed the most consistent trend when compared with the solutions prepared with buffer solutions. The difference between the aqueous protein solutions prepared in buffer and ultrapure water tests was claimed to be due to pH.

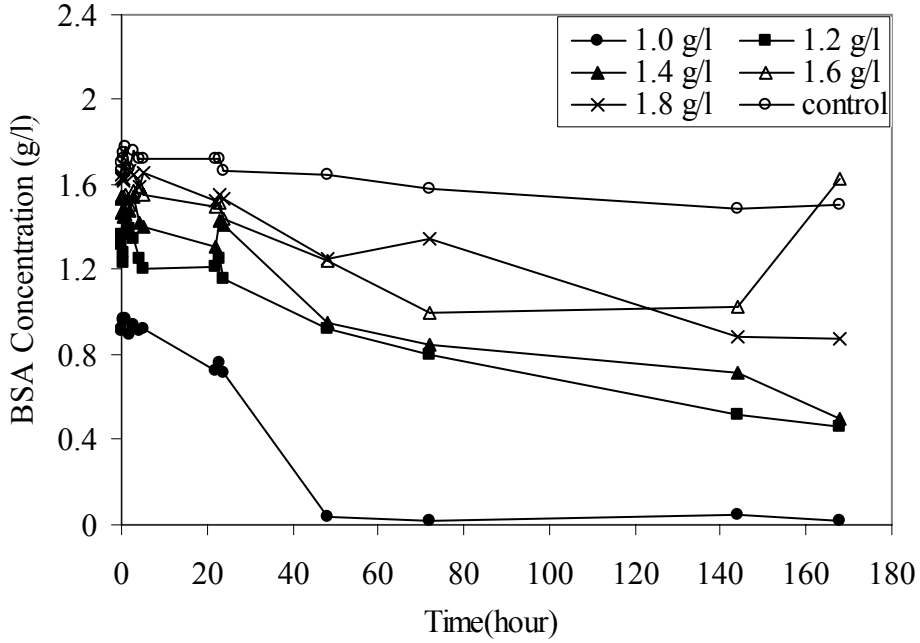

Figure 7.21. The BSA concentration change with 2 % (w/v) natural zeolites in ultrapure water (T: 37^{o}C, agitation speed: 150 rpm, working volume: 50 ml).

In order to make better comparison, the data for all control sets that did not contain natural zeolites were overplotted in Figure 7.22. When the pH was adjusted, all the control sets were following the similar trend but at different times. However protein solution in ultrapure water was preserving the structure better than the others.

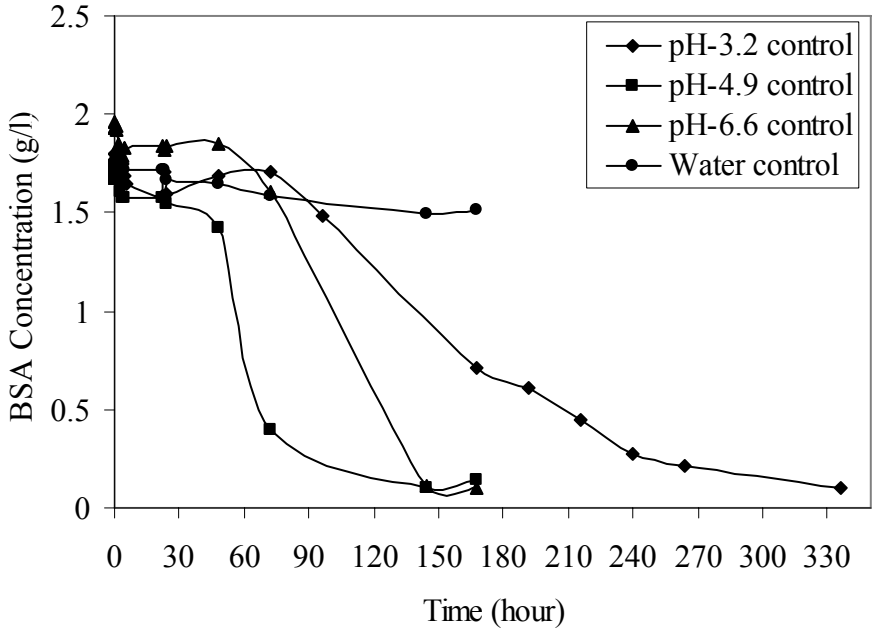

Figure 7.22. The BSA concentration change for the control sets without natural zeolite (T: 37^{o}C, agitation speed: 150 rpm, working volume: 50 ml).

7.3.1.1 Element Composition in Natural Zeolite-BSA Adsorption

When the variation of the amount of cations on the zeolite were examined after the treatment with protein solution of adjusted pH values, the elemental composition on solid part are tabulated in Table 7.9. When the results are compared with Table 7.3. the sorption mechanism taking place is proved.

Table 7.9. The elemental composition of solid part for natural zeolite-BSA adsorption .

Cations (mg cation / g NZ oxide form) % weight	**pH-3.2**	**pH-4.9**	**pH-6.6**	**Ultrapure water**
Al_2O_3	15.28	15.58	15.11	15.52
CaO	2.18	2.34	2.25	2.46
Fe_2O_3	1.29	1.49	1.91	1.31
K_2O	10.56	10.22	10.11	9.49
MgO	0.48	0.49	0.48	0.50
MnO	0.04	0.04	0.05	0.04
Na_2O	2.29	2.73	2.59	2.69
SiO_2	67.88	67.10	67.51	67.88

The results are quite similar to each other. The amount of cations for each set gives quite close values. The buffer solution and ultrapure water did not change the composition of cations existing on the structure of the natural zeolites at significant levels.

The element composition in the liquid part of natural zeolite-bovine serum albumin adsorption study is presented in Table 7.10. Besides the protein content, it was necessary if there is a cation exchange inside the medium due to the effect of buffer solutions adjusted at different pH values. The results of the analyses for the supernatant of adsorption study were compared. The element compositions prepared with buffer solutions were reflecting similar behavior. The cations Ca^{+2}, K^{+}, Na^{+} were releasing from the natural zeolite structure into the aqueous solution. However, the dissolution of cations were higher for the lowest pH value, whereas the release was decreasing as the pH value increased. In spite of that, for the solution prepared with ultrapure water, besides the cations given above, the dissolution of Al^{+3} , Fe^{+3}, Si^{+4} were observed (Table 7.10). Bearing in mind that, the values are very small, and in the exception of Ca^{+2}, K^{+}, Na^{+}, the rest of the cations are ascribed to be experimental fluctuations.

Table 7.10. Cation composition inside the supernatant of natural zeolite-BSA adsorption.

Cations (mg cation / g NZ)	**pH-3.2**	**pH-4.9**	**pH-6.6**	**Ultrapure water**
Al	0.00	0.00	0.00	0.78
Ca	5.46	2.05	1.45	0.39
Fe	0.00	0.00	0.00	0.43
K	1.34	0.54	0.53	0.25
Mg	0.21	0.07	0.05	0.01
Mn	0.03	0.01	0.01	0.00
Na	4.41	2.53	2.46	0.77
Si	0.57	0.43	0.53	2.65

7.3.1.2. Effect of Initial BSA Concentration on Adsorption

The adsorption kinetics study of BSA solution onto natural zeolites prepared with phosphate buffer at pH 3.2 is presented in Figure 7.23 and further in detail in Figure 7.24. In this case, the data of the adsorption kinetics study prepared with pH 3.2 buffer solution was performed with constant zeolite amount at different initial concentrations. The adsorption of BSA by natural zeolite was investigated to obtain a general idea about the system behaviour in model system. Thus, these preliminary empirical data were used in order to construct a case study involving the whey proteins adsorption by natural zeolite. The results showed that adsorption of BSA was completed in a few hours. Despite that, the sampling was continued for 24 hours to be sure about the equilibrium period.

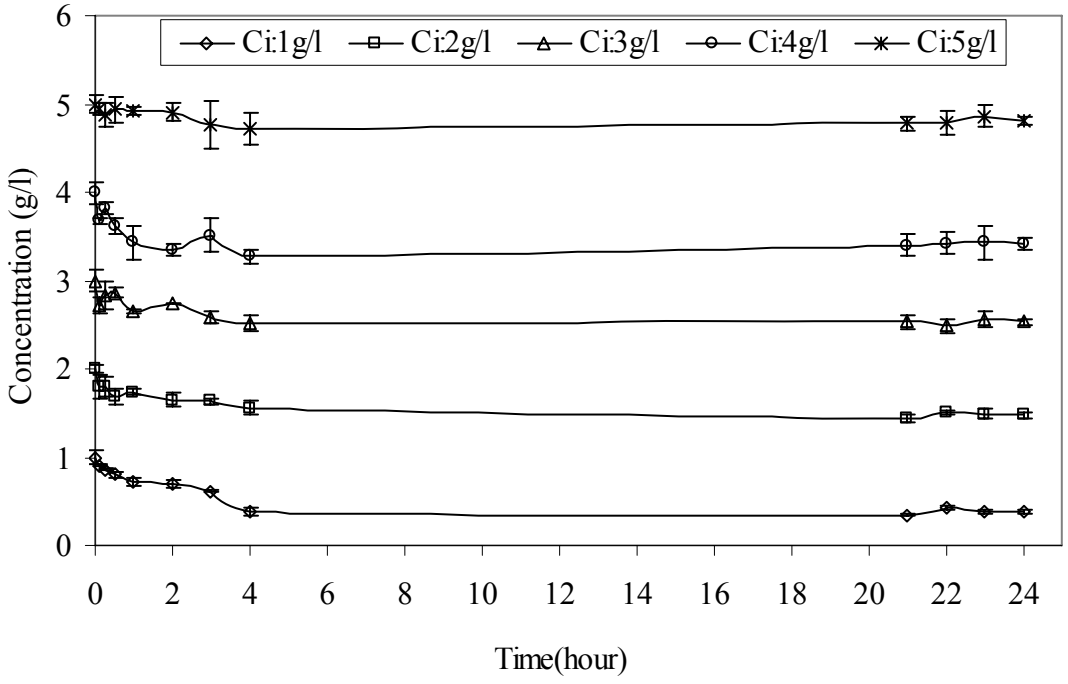

Figure 7.23. BSA concentration change with natural zeolite (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

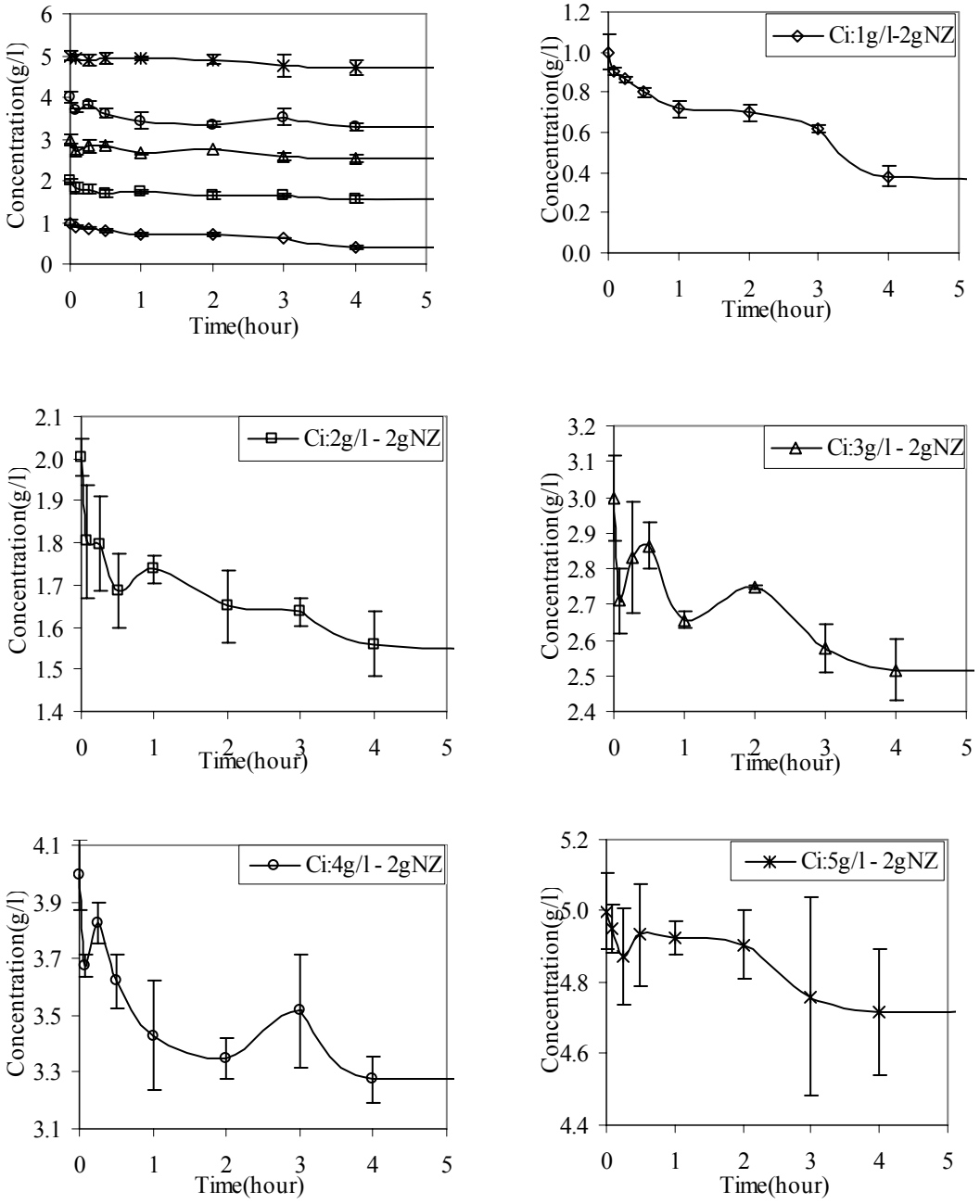

Figure 7.24. Different initial BSA concentrations (Ci) during the initial period of natural zeolite-BSA adsorption (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

The experimental medium was composed of BSA and the ions coming from the potassium phosphate buffer. In the case of higher initial concentration values such as 4g/l and 5 g/l the error bars were greater than Ci:1g/l, 2g/l and 3g/l. The main reason for that was the dilution required for the detection range of protein analysis method. As the requirement for dilution was greater, the deviations obtained were higher. Under these

circumstances, it is determined to perform the adsorption study with lower protein concentrations for BSA solution and whey powder solutions where it is possible. Especially for investigating the effects of pH, particle size, agitation speed. Besides the effect of temperature and Si/Al ratio were also studied.

In literature, IR bands called amide I, amide II and amide III have been used to analyze the protein structure. These three bands in native BSA are centered at 1650, 1539 and 1250 cm^{-1}respectively. In the following Figure 7.25 comparison of natural zeolite, BSA and BSA adsorbed natural zeolite coded as 8 and 9 are depicted in the whole range.

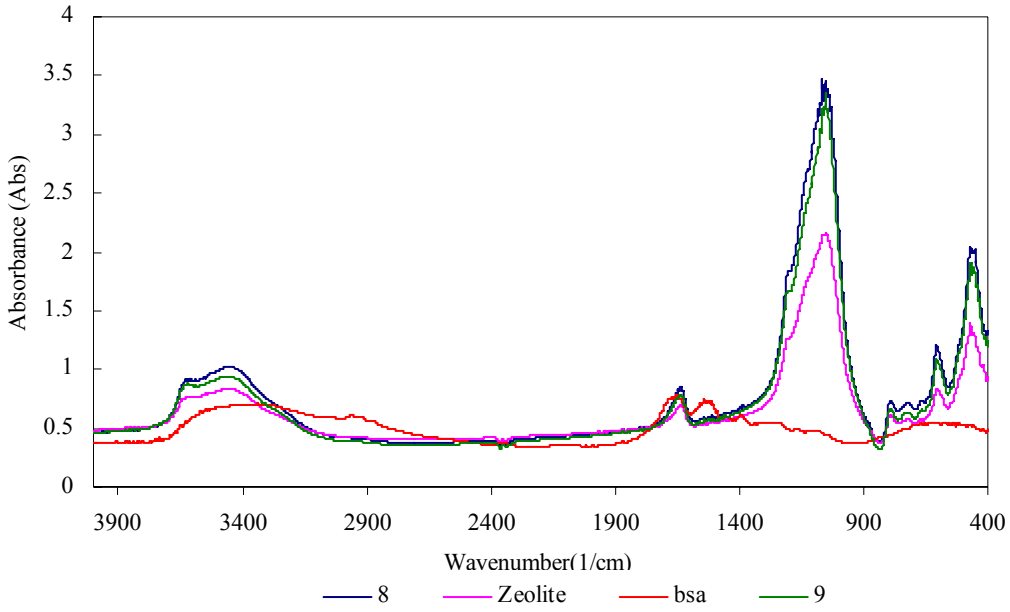

Figure 7.25. FTIR spectra of BSA and/or natural zeolite.

As the amide bands were in the extent of x-axis 1200 to 1700, they are presented in a closer view; overall and individual representation, respectively (Figure 7.26, Figure 7.27). The bands belonging to BSA were not detected by FTIR.

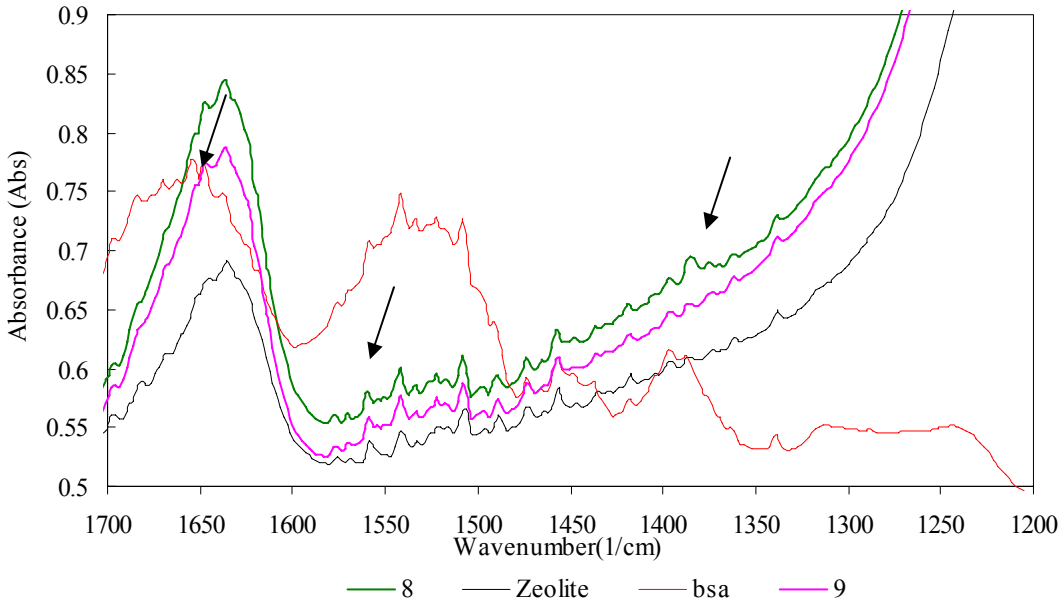

Figure 7.26. FTIR spectra of BSA and/or natural zeolite.

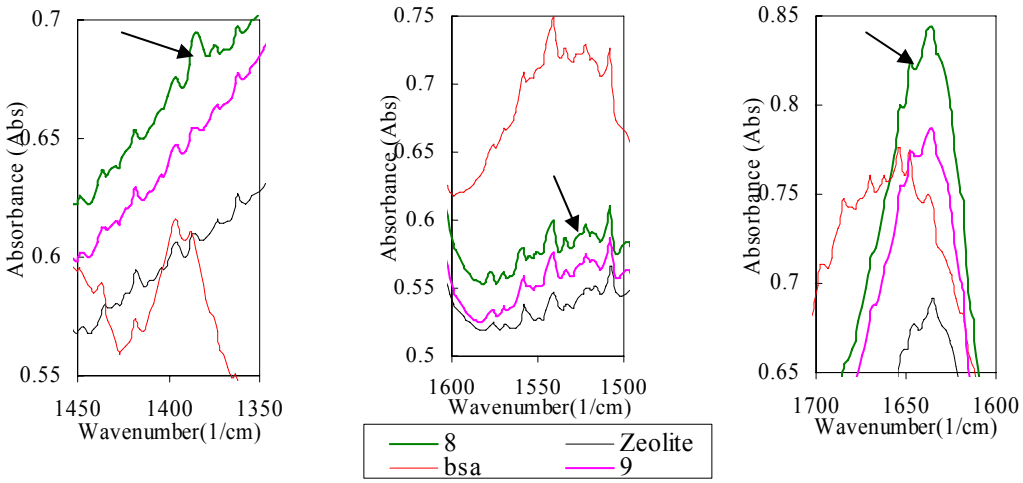

Figure 7.27. FTIR spectra of BSA and/or natural zeolite.

7.3.1.3. Effect of Solid/Liquid Ratio on Natural zeolite-BSA Adsorption

BSA adsorption with various solid liquid ratios (S/L) are given in Figure 7.28. The decrement in concentration values are in good agreement where the highest change in concentration was achieved as the S/L ratio was increased in response to the higher adsorbent mass.

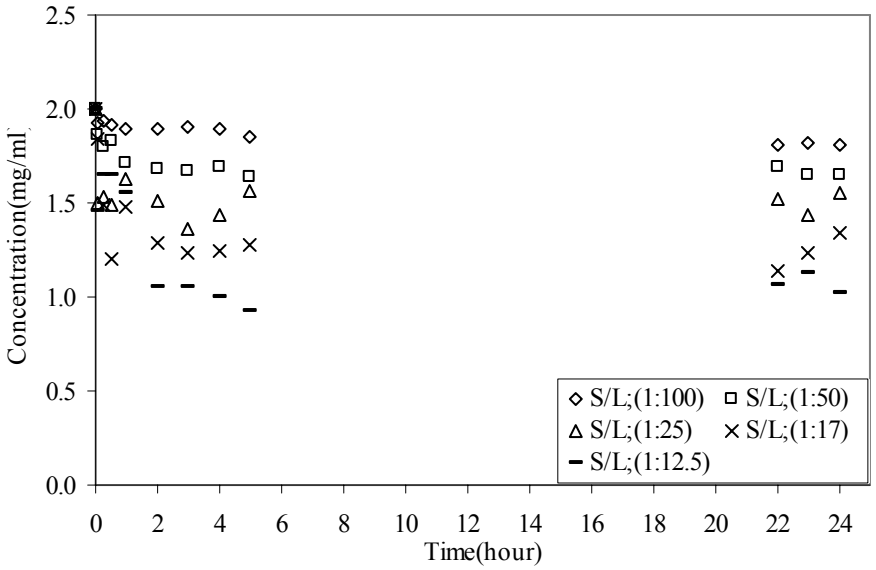

Figure 7.28. Concentration change during BSA adsorption onto natural zeolite with diferent solid/liquid ratios (T: 25°C, agitation speed: 150 rpm, working volume: 50 ml).

However, the adsorbed amount versus time plot was drawn for Figure 7.29 and it was observed that the results were similar.

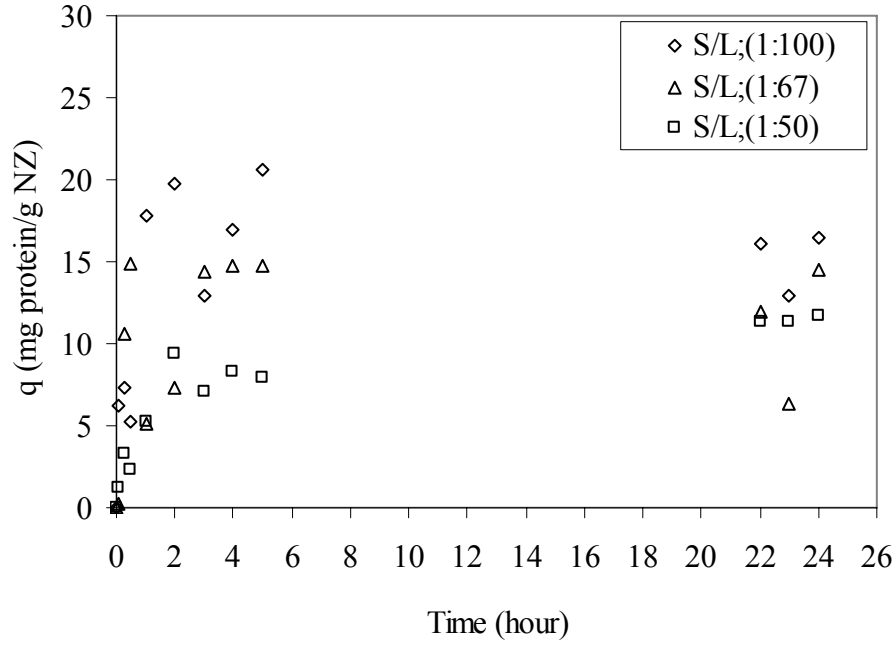

Figure 7.29. BSA adsorbed amount by natural zeolite with diferent S/L (T: 25°C, agitation speed: 150 rpm, working volume: 50 ml).

7.3.1.4 Effect of Conditioning on Natural zeolite-BSA Adsorption

In the case of protein adsorption study, researchers preferred to equilibrate the adsorbent material with the aqueous solution lacking of protein. After equilibration, they were adding the protein inside the medium and were performing the adsorption study. The effect of that conditioning step was investigated by different initial concentrations. The conditioned samples were labeled with "Eq" notation.

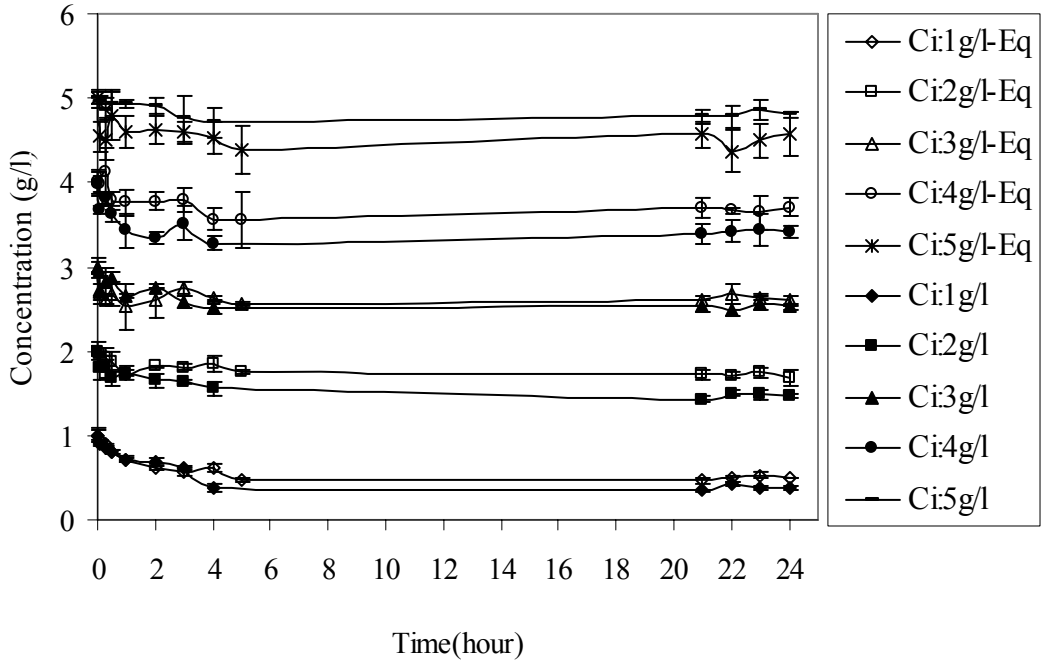

Figure 7.30. Effect of conditioning on BSA concentration change during protein adsorption onto natural zeolite (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

The results presented in Figure 7.30 and Figure 7.31 (initial period) proved that quite different concentration values were obtained during adsorption for the conditioned and unconditioned natural zeolite samples. The readily used natural zeolites adsorbed higher proteins when compared with the conditioned samples except the highest initial protein concentration 5 g/l. The error bars belonging to 5 g/l initial concentrations are higher than the others. It seems that the requirement of high dilution factors for the analyses, results higher standard deviations. When considering the bar ranges, the results of the 5 g/l initial concentration set can be accepted as identical. In conclusion, especially during the studies at lower concentrations sets, conditioning step is not

necessary. This figure helps to eliminate a pretreatment step while performing the adsorption study. Besides, higher equilibrium adsorbed amounts were obtained.

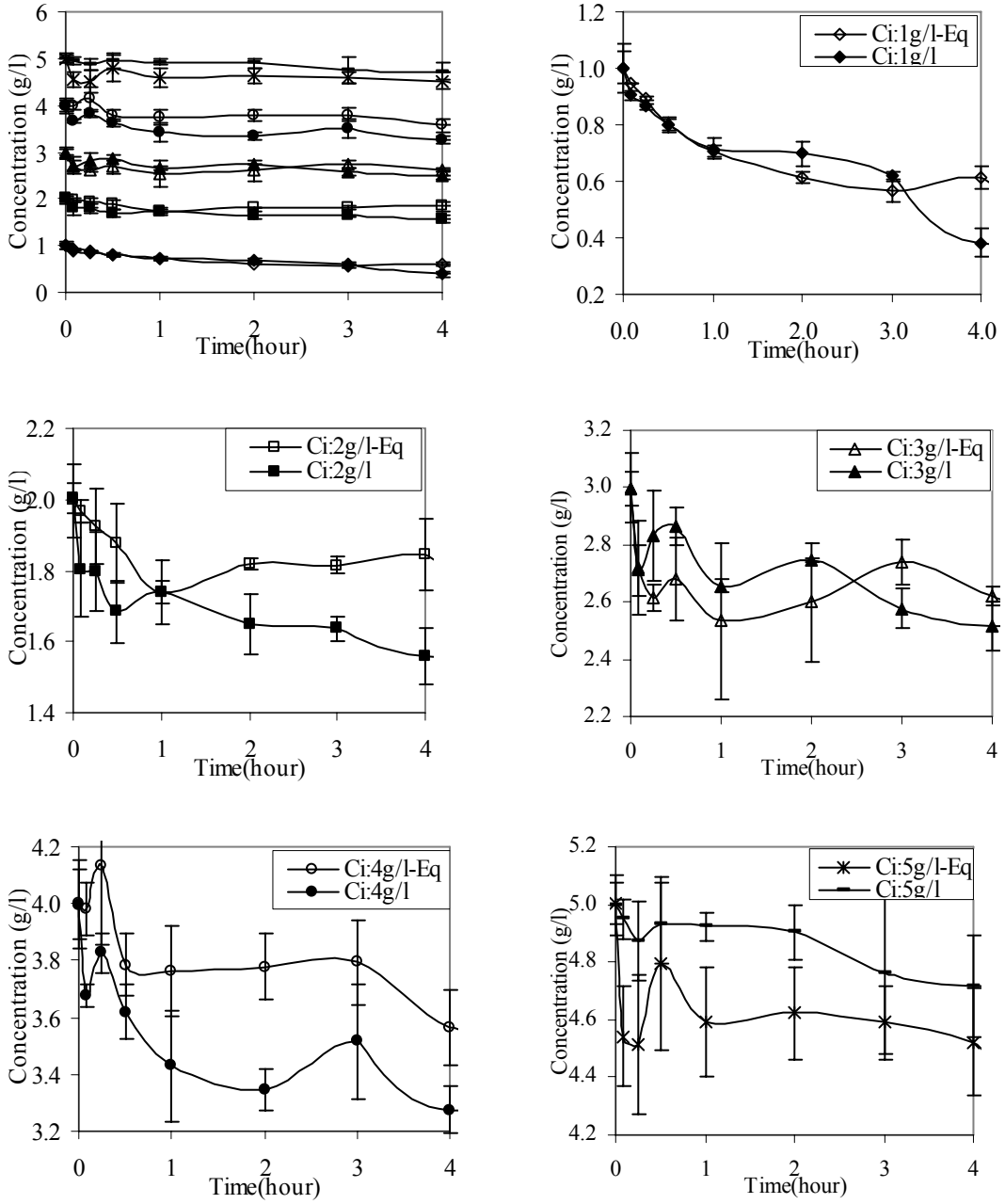

Figure 7.31. Effect of conditioning on BSA concentration change during for the initial period of protein adsorption onto natural zeolite (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

7.3.1.5 Effect of Temperature on Natural zeolite-BSA Adsorption

The effect of temperature for BSA adsorption was studied at 25 ^{o}C and 15 ^{o}C. Samples having different initial concentrations were exposed to adsorption with constant amount natural zeolite and the results were presented in Figure 7.32. The results indicated that there is a difference on the adsorbed protein amount between 25 ^{o}C and 15 ^{o}C. The differences between the adsorbed BSA amounts for two temperature values under the operating conditions are seen better for initial period in Figure 7.33.

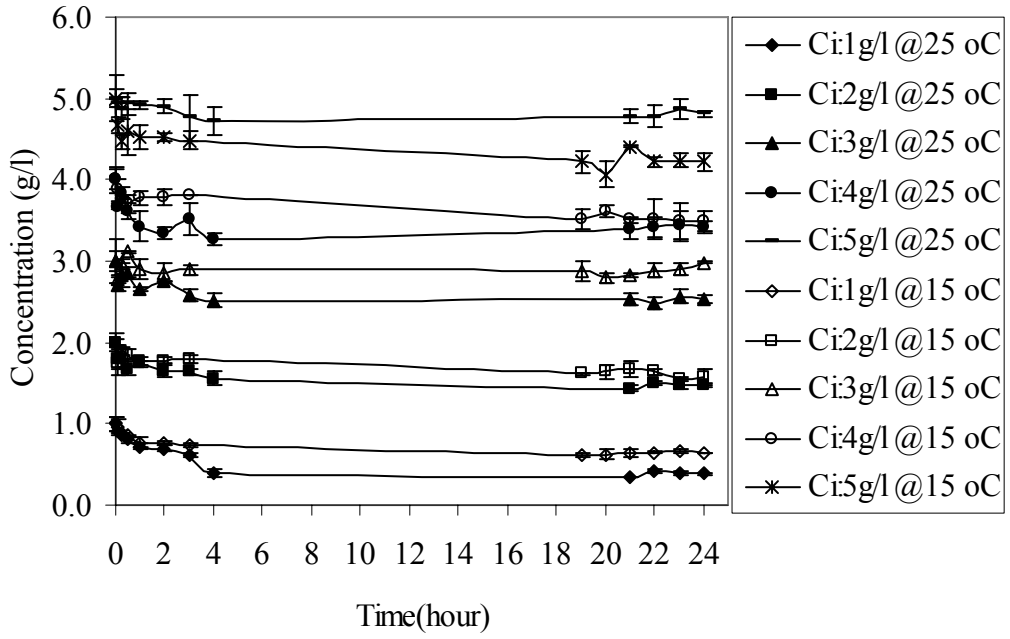

Figure 7.32. Effect of temperature on BSA adsorption by natural zeolite (Agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

Even though, the temperature difference between two sets was 10 ^{o} C, the adsorbed BSA amount was higher at higher temperature. The adsorption isotherm of BSA (Figure 7.14.) showed that the trenline of experimental data presented as q_e vs. C_e at 25 ^{o}C was greater than the temperature at 15 ^{o}C. The temperature effect may be seen better at higher temperature differences. The range of concentrations can also be changed for further information. Tavolaro reported the endothermic character of BSA adsorption onto synthetic zeolite crystals with respect to 4 ^{o}C, 18 ^{o}C and 30 ^{o}C (2007).

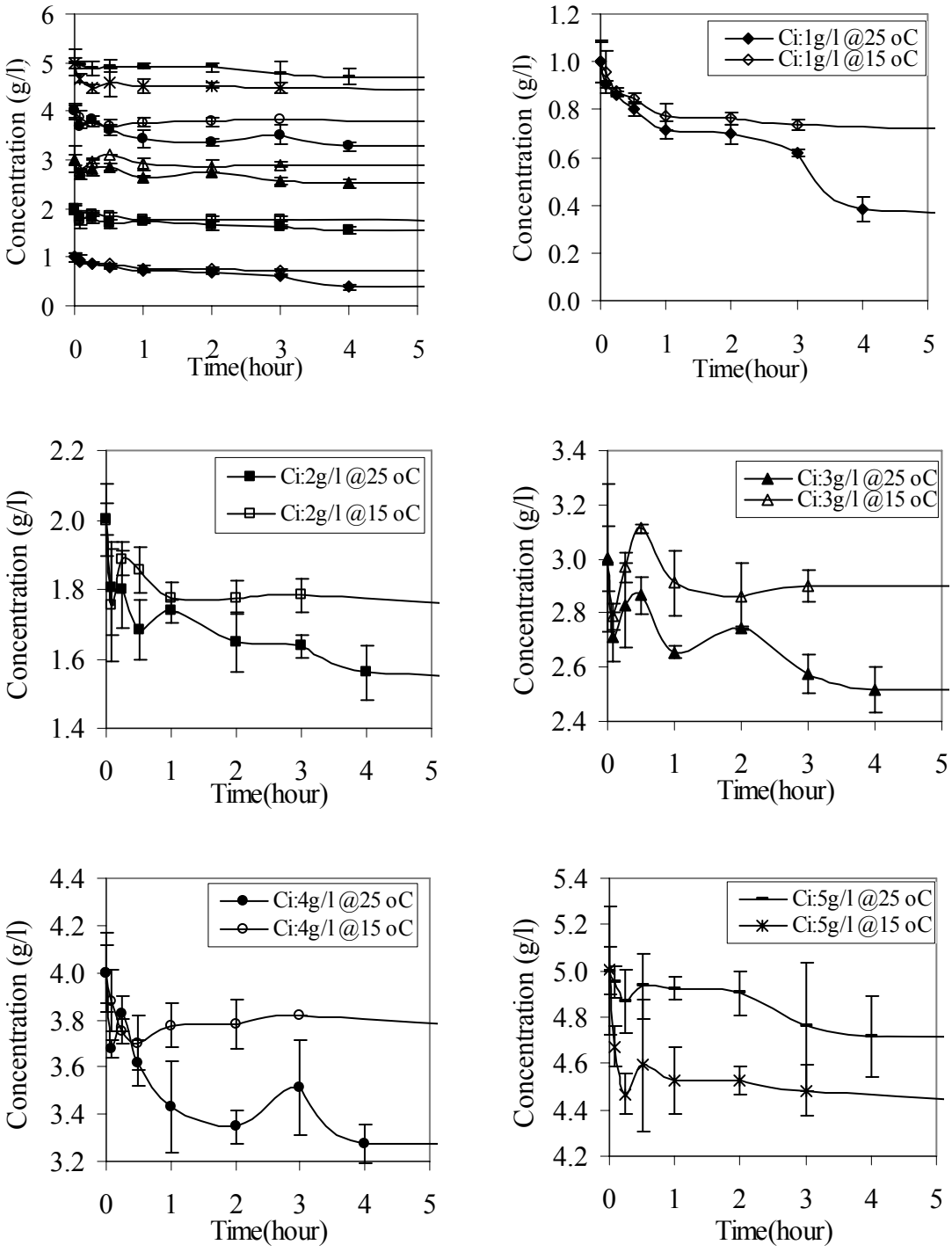

Figure 7.33. Effect of temperature on initial period of BSA adsorption by natural zeolite (Agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

7.3.2. Adsorption of Whey Proteins onto Natural Zeolites

The adsorption of whey proteins onto natural zeolites were investigated under several factors. The effect of temperature, initial concentration, S/L ratio, the effect of pH, effect of agitation sped, effect of particle size, effect of modification, effect of elemental composition, effect of purity were studied. The study was applied to whey in order to investigate the adsorption kinetics. The protein concentration values were measured as total protein for the investigation of given effects. In addition to that the tendency of natural zeolites for the adsorption of BSA, α-Lactalbumin and β-Lactoglobulin existing in whey powder solution were measured individually by a gel filtration HPLC column.

7.3.2.1 Lactose Concentration Variation in Whey Powder Solution

In the beginning of the whey protein adsorption study, the tendency of lactose adsorption by clinoptilolite rich natural zeolites (NZ) were investigated. Figure 7.34 is plotted for those conditions.

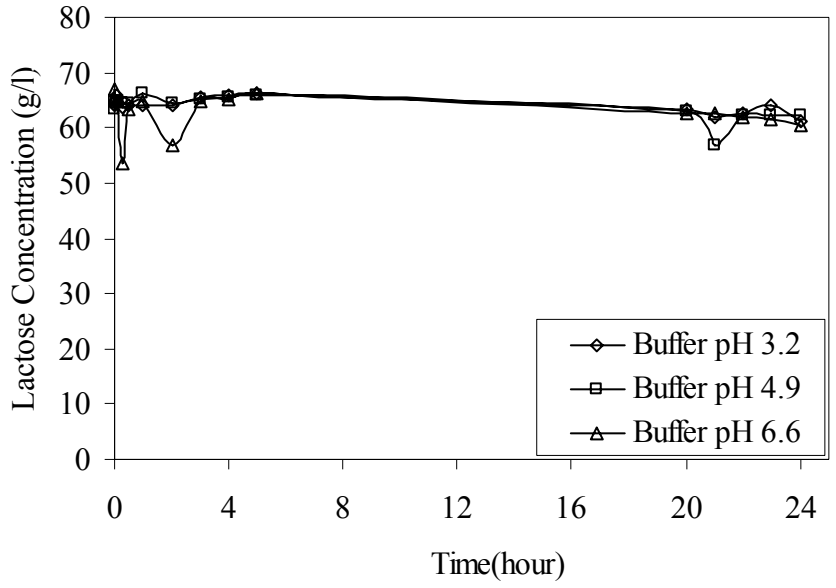

Figure 7.34. Lactose concentration variation of whey powder solution in the presence of natural zeolite (T: 37^{o}C, agitation speed: 150 rpm, working volume: 100 ml, S/L: 1/10).

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The pH effect on adsorption of proteins was significant. In that case, it was important to observe the variation of lactose concentration in whey powder solution in the presence of natural zeolites. The pH values belonging to buffer solution. Notice that, when if whey powder dissolves in buffer, the pH value of the solution is not equal to the buffer solution pH.

During the test period of 24 hour, as given in Figure 7.34 it was seen that there was not a concentration difference between the samples prepared with buffer solutions having different pH values. Consequently, natural zeolites do not adsorb lactose under the operating conditions and it is a desirable behavior for the adsorption of whey proteins. These results were similar with the tests that were performed by using deionized water.

7.3.2.2 Whey Powder Protein Adsorption by different S/L

Whey powder was used as the raw material for whey proteins adsorption. The concentration profile of whey proteins on natural zeolite are given in Figure 7.35.

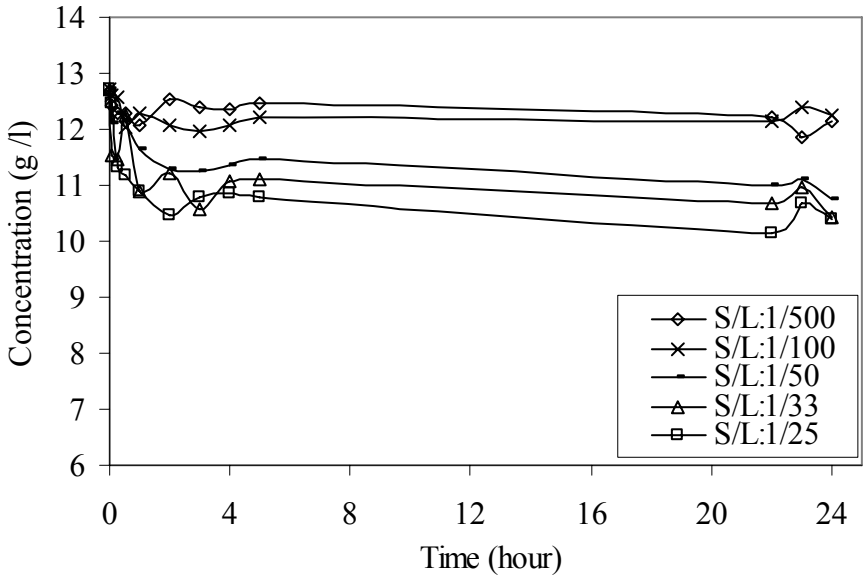

Figure 7.35. Whey powder protein concentration change for different S/L (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml).

7.3.2.3. The Effect of pH on Whey Powder Protein Adsorption

Whey proteins adsorption study, from the view point of pH effect was performed by dissolving whey powder inside the buffer solutions. The pH of buffer solution was adjusted; pH 3.2, pH 4.9 and pH 6.6. The experimental study was performed at 25 ^{o}C for 24 hours.

The results were similar with the behaviour of aqueous BSA adsorption study. In fact the adsorption of proteins were not much faster as the aqueous solution samples. It was observed that the adsorption of BSA was completed in 4 hours. On the contrary, in the case of whey proteins` adsorption it was necessary to continue the collection of samples for 24 hours as it is obviously seen in Figure 7.36. The main possible reason for that case may be related to the diffusivity limitations and the interactions of the natural zeolite with the components.

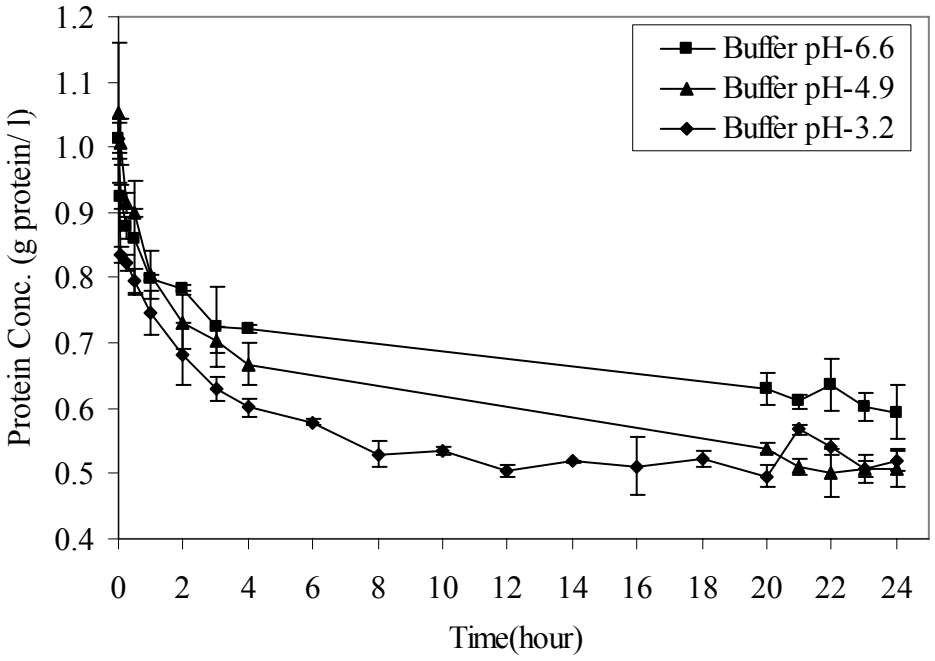

Figure 7.36. Whey powder protein concentration change at different pH (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

Figure 7.36 reveals the fact that adsorption of whey proteins is mostly favorable below the isoelectric point of BSA. The differences in adsorbed amounts at different pH values was attempted to the electrostatic interactions, Coulombic attractions and hydrophobic interactions. The whey powder proteins were mainly β-Lg, α-La,

Lactoferrin, Immunoglobulins, BSA, and Glycomacropeptides. Furthermore, each protein has its own pI which is the pH of net charge zero. The pI values of BSA, β-Lg and α-La ranges about 4.7 to 5.2. These three proteins make up of 80 % of whey proteins. So buffer pH resulting the similar interactions on whey proteins-natural zeolite system has an overall effect. Minimum adsorption was observed at buffer pH 6.6. Because negatively charged groups of natural zeolite repel the negatively charged protein molecules (above pI, negatively charged) results reduced adsorbed amount. In addition to that, natural zeolites are negatively charged between pH 2 to pH 12 and at buffer pH 3.2 the protein molecules are below their isoelectric point. Consequently they are positively charged and strong electrostatic attractions between whey proteins and natural zeolites were observed. The strength of hydrophobic interactions are said to be reduced in accordance with the charge of proteins. The surface charge of proteins are neutralized at pI, so at that pH adsorption may take place as a result of hydrophobic interactions which is Van der Waals attraction.

The configuration of the protein molecules are dependent on the pH especially for soft proteins which directly affects the adsorbed amount. On the other hand, it is difficult for hard proteins to be adsorbed on a surface having opposite charges.

7.3.2.4. Adsorption Kinetics and Effect of Elemental Composition

Adsorption study was performed by sampling small volumes from the media in order to protect the working volume. Because during the kinetics study, it is obligatory to declare the change in concentration and/or adsorbed amount at certain time intervals until the system reached the equilibrium as it is seen in Figure 7.37.

For the whole study, sampling by short time invervals was applied. The adsorption was reached equiibrium within 8 to 10 hours and the equilibrium values were verified by sampling until the 24^{th} hour. The results showed that collection of samples at the beginning and at the end of the study was sufficient to explain the kinetic behavior of the system.

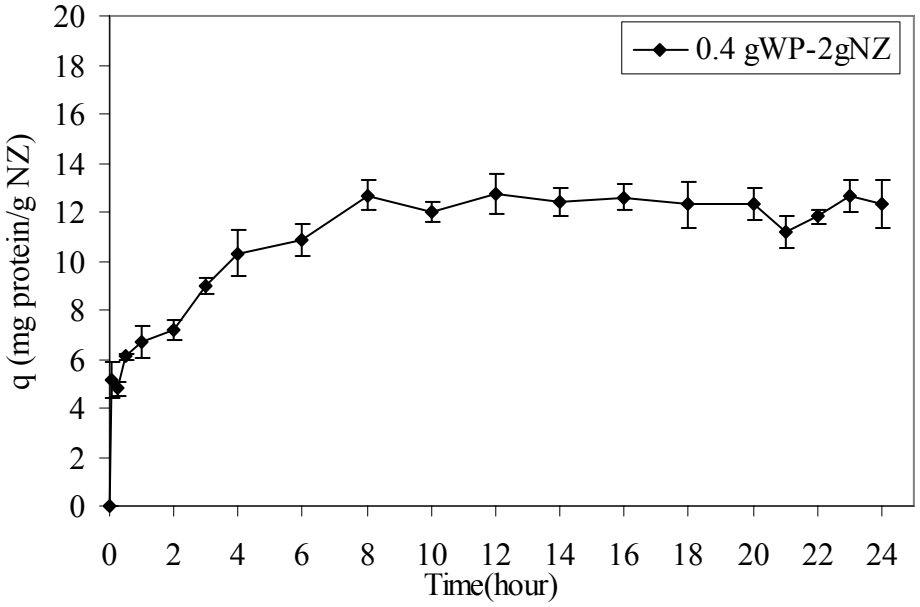

Figure 7.37. The amount of whey proteins adsorbed onto natural zeolite (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

Generally whey powder has a very rich composition from the point of trace elements. As it is seen the major trace element constituents of whey powder were, Ca, K, Mg and Na. The elemental composition in whey powder is presented in Table 7.11

Table 7.11. Elemental composition of whey powder

Elements	**Ca**	**Cu**	**K**	**Mg**	**Na**	**Zn**
mg element/g whey powder	5.64	0.01	18.26	1.60	9.47	0.03

The elemental composition of whey powder that was prepared with buffer solution, was investigated. The major and minor elements were taken into consideration and the profiles were represented in the following figures. The kinetics of total elements` concentration in zeolite-whey powder solution in buffer was presented in Figure 7.38.

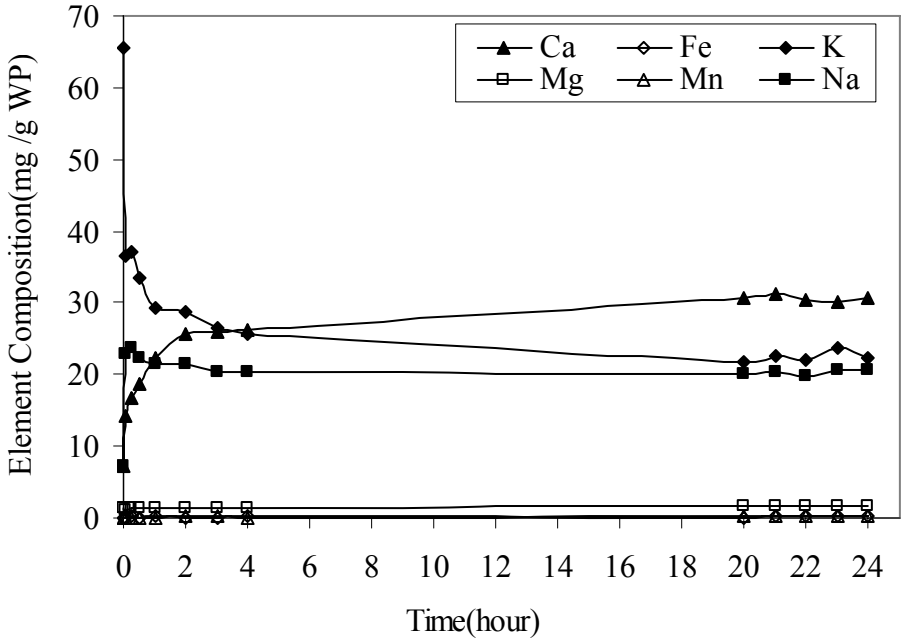

Figure 7.38. Element composition change during protein adsorption (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

Minor elements separately given to see the profiles better (Figure 7.39). The major elements profile was indicating the cation exchange of Ca and Na elements from natural zeolite into the solution as time goes by. Besides for minor elements similar behaviour was observed for Fe,Mg and Mn. On the contrary of dissolution, there was also an adsorption behaviour of K ions from solution onto natural zeolite. The amount of K ions present inside medium was not only coming from the whey powder source but also from the buffer composition as well.

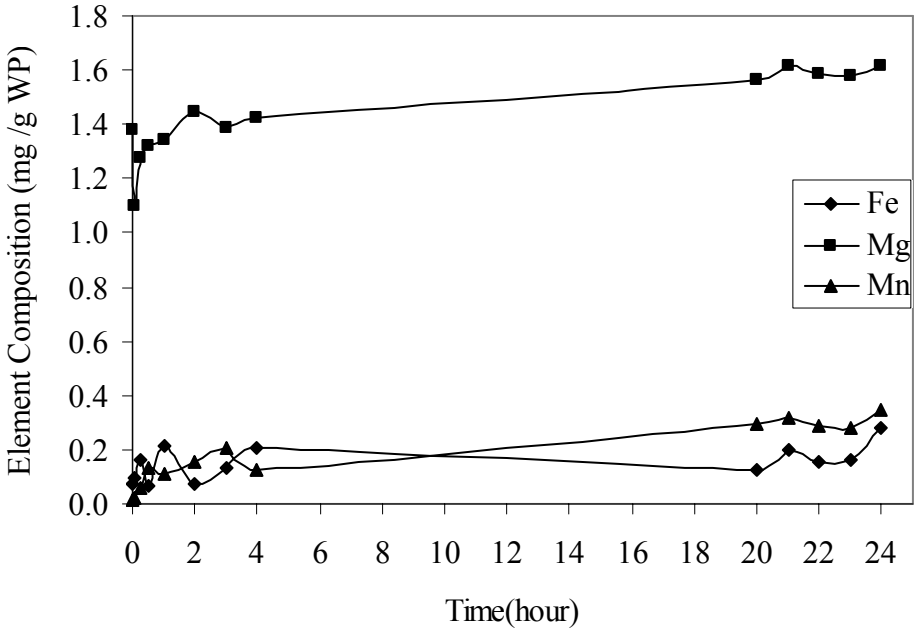

Figure 7.39. Minor elements composition during protein adsorption (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

7.3.2.5. Effect of Particle Size

The surface area is a very important parameter in adsorption studies. It has a significant effect on the adsorption capacity of the adsorbents due to the presence of binding sites. The proteins are large molecules, as an example BSA is known as an oval globule with a long radius of 7 nm (Sakaguchi, 2005). The protein molecule can not go through the pores of natural zeolites which are at A^{o} level. So adsorption occurs at the outer surface of the natural zeolites. From this standpoint it was decided to perform experiments with natural zeolite having comparable particle sizes. The results are given in following figures. Figure 7.40 represents the whole equilibrium period where Figure 7.41 is the representation of the initial period of adsorption. As the particle size decreases, the system reaches the equilibrium plateau faster and the adsorbed amount increases.

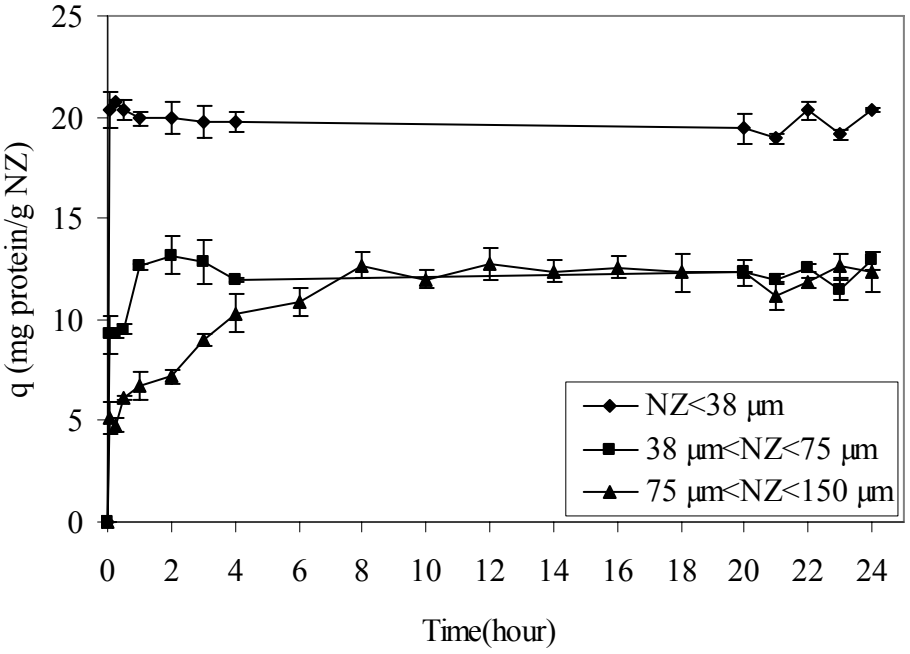

Figure 7.40. The amount of whey proteins adsorbed onto natural zeolite (T: 25°C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

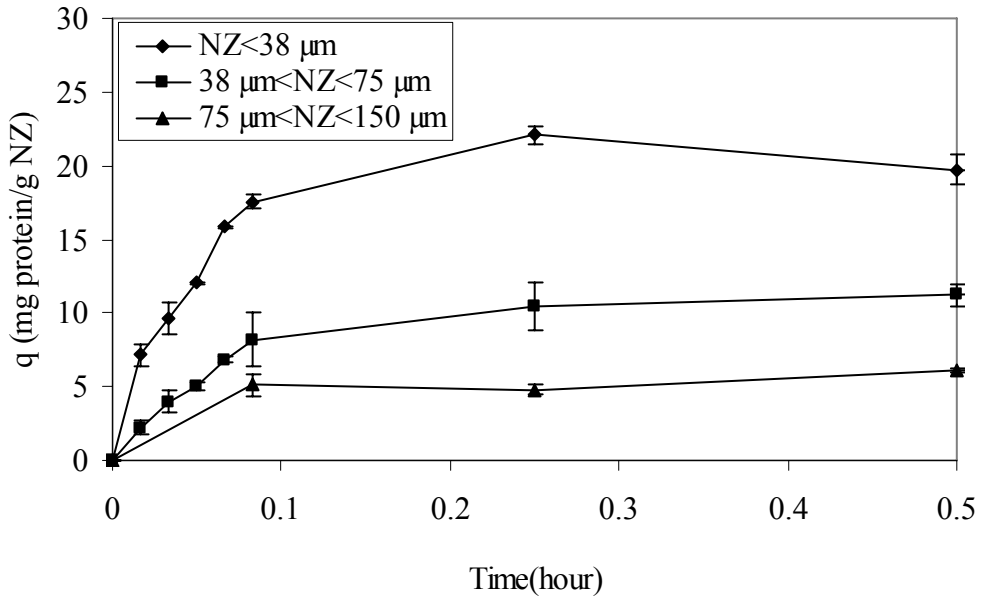

Figure 7.41. The amount of whey proteins adsorbed onto natural zeolite during initial period (T: 25°C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

The change in adsorbent particle size is a required system variable that should be studied to understand the controlling mechanism for mass transfer. Because, the higher interfacial areas, as a result of smaller natural zeolite particles provide increased binding

sites. In particular, when external film diffusion is the rate limiting step, the mathematical model is supported with particle size effect. The main assumption for external film diffusion is the nonporous structure of the adsorbent or similarly with skin resistance the limitation of the mouths of pores. Both assumption is valid for natural zeolite having pore diameters around 5 A^o where proteins can not enter the pores. It is observed experimentally that higher adsorbed amounts were obtained for lower particle size of natural zeolite which is smller than 38 μm.

7.3.2.6. Effect of Dealumination

The dealumination process reduces the electrostatic charge in the structure of the zeolite and their adsorption properties become hydrophobic. The following study was performed to investigate the effect of Si/Al ratio on the adsorption of proteins on the dealuminated natural zeolite.

The effect of framework composition was investigated by Tavolaro et al., to see the acid-base properties playing a role during protein adsorption. The zeolite structure has acid sites on its crystal surface which can transform the protons from the solid to the adsorbed molecules called Brönsted acid sites. In chemical adsorption the adsorbed protein amount increases as the Brönsted acidity of the crystals increase. They are the silanol groups (Si-OH) having mobile protons. The Brönsted acidity depends on the structure of the zeolite framework and the chemical composition of the zeolite. The acid silanol groups can be seen as Si-O-H group and its acidity can be enhanced by the Al^{+3} which is is known as strong Lewis center. As the acidity increase, the protein adsorbed amount increase (Tavolaro et al., 2007).

The Si/Al ratio of the natural zeolite (NZ) was: 5.26. After the dealumination process the Si/Al ratio of the exchanged natural zeolites were: 9.24 for natural zeolite exchanged with 1M HCl; 12.74 for natural zeolite exchanged with 5 M HCl; 17.84 for natural zeolite exchanged with 8 M HCl; 18.52 for natural zeolite exchanged with 10 M HCl (Table 7.5). In Figure 7.42 the equilibrium adsorbed amounts for natural zeolite and 1M HCl exchanged zeolite were same. However, the equilibrium adsorbed amounts for the other samples were quite different. If focusing on the initial time period of the Figure 7.42 it is seen that the adsorption takes place faster for exchanged samples which is better seen in Figure 7.43. The effect of dealumination increases the speed of

adsorption for all exchanged samples however the equilibrium adsorbed amount is lower for all exchanged samples except 1 M HCl exchanged natural zeolite.

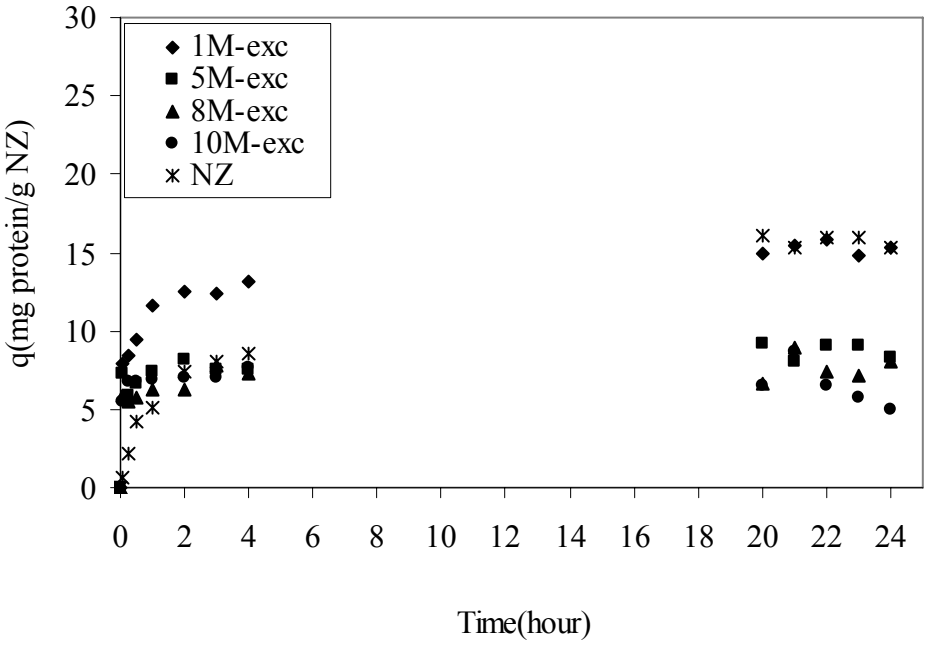

Figure 7.42. The amount of whey proteins adsorbed onto modified zeolite (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

The decrease in the adsorbed amount is attributed to the low Al^{+3} content. Because the number and the strength of Si-OH groups related with Brönsted acidity are highly affected by Al^{+3} ions. Their presence play a significant role on the adsorbed amount increase.

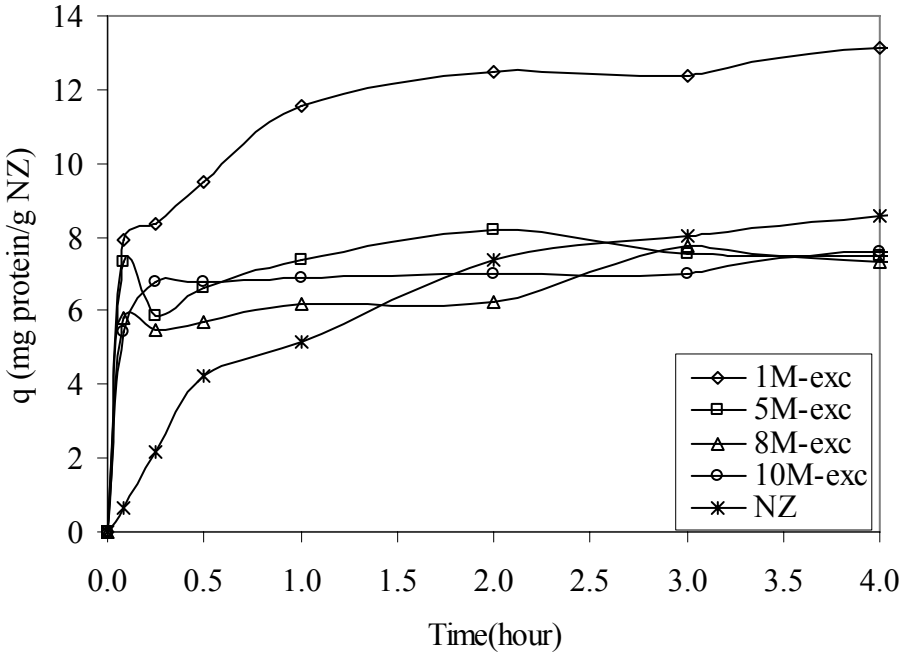

Figure 7.43. The amount of whey proteins adsorbed onto dealuminated natural zeolite during initial period of adsorption (T: 25^{o}C, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

As the hydrophobicity related with the acid concentration in this case, increase, time to reach constant concentration for whey proteins decrease. The adsorption was apt to be faster for highly dealuminated zeolites. Howewer, from the point of proteins` adsorbed amount, lower values were achieved by highly dealuminated zeolites when compared with the results of natural form.

7.3.2.7. Effect of Agitation Speed

Adsorption of proteins was investigated under the effect of agitation speed. Four different agitation speeds were selected. The results of the kinetic study until the system reached equilibrium are plotted in Figure 7.44. The experiment was performed at room temperature.

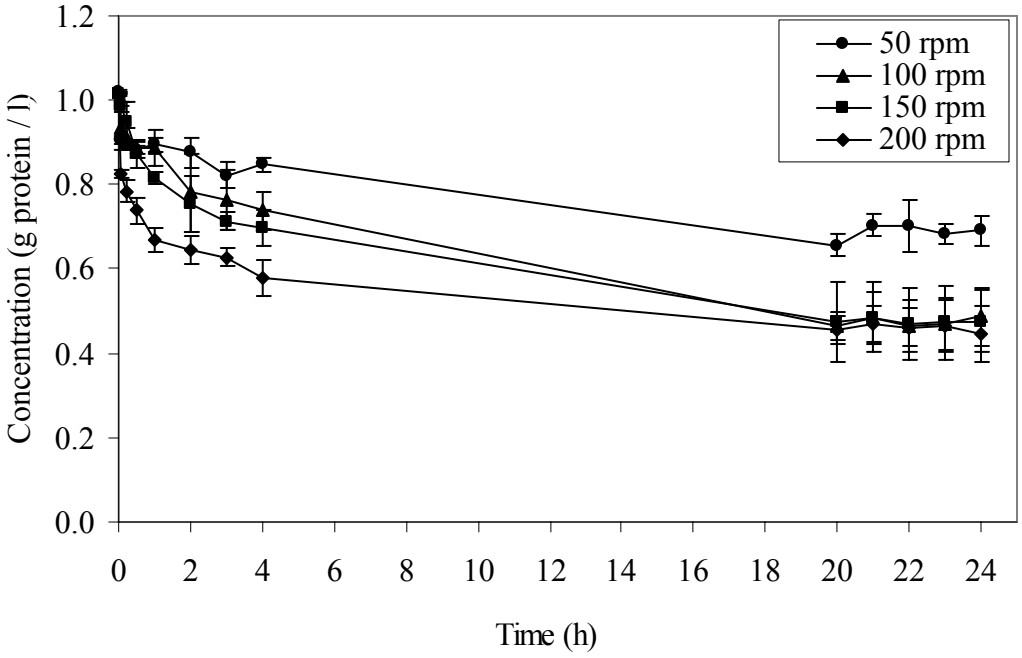

Figure 7.44. The concentration change of whey proteins adsorbed onto natural zeolite at different agitation speeds (T: 25^{o}C, working volume: 50 ml, S/L: 1/25).

The adsorption rate was higher for the highest agitation speed where the speed was lowest for 50 rpm. Besides that, when a comparison between the adsorbed protein amount was done the adsorbed amounts were identical at the end of 24 hours for 100 rpm, 150 rpm and 200 rpm. In addition to that, the adsorbed amount for the experimental set of 50 rpm had the lowest quantity.during the experimental period for 50 rpm we did not observe a homogenous mixing. The agitation speed was not sufficient to initiate a mobility on natural zeolite inside the flasks. On the contrary for the experimental sets of 100 rpm, 150 rpm and 200 rpm, the movement of media could be seen very easily. In conclusion, agitation speed did not effect the equilibrium adsorbed amount if agitation was sufficiently provided but significantly effect the adsorption rate.

The agitation speed implies the variation of process conditions can affect the rate limiting steps. It is reported that the effect of mass transfer control dominated by external film diffusion is reduced at higher agitation speeds (Gerente et al., 2007). Because the solute transfer more rapidly from the external boundary layer and reaches the subsequent available sites. Consequently, the mechanism can be explained by few controlling steps.

7.3.2.8. Effect of Temperature

To see better figures are plotted separately for nine concentrations in Figure 7.45 and Figure 7.46. The data for comparing adsorption at 15 ^{o}C and 25 ^{o}C showed that differences between these two temperature values for all nine sets seems to be small in concentration vs. time figures. Under working conditions whey proteins were adsorbed in higher amount as the temperature was increased to 25 ^{o}C.

The possible reason for that behavior can be depicted as, the rate of diffusion for protein molecules passes through the external boundary layer and reaches the internal pores of natural zeolites. So, the maximum adsorption capacity increases.

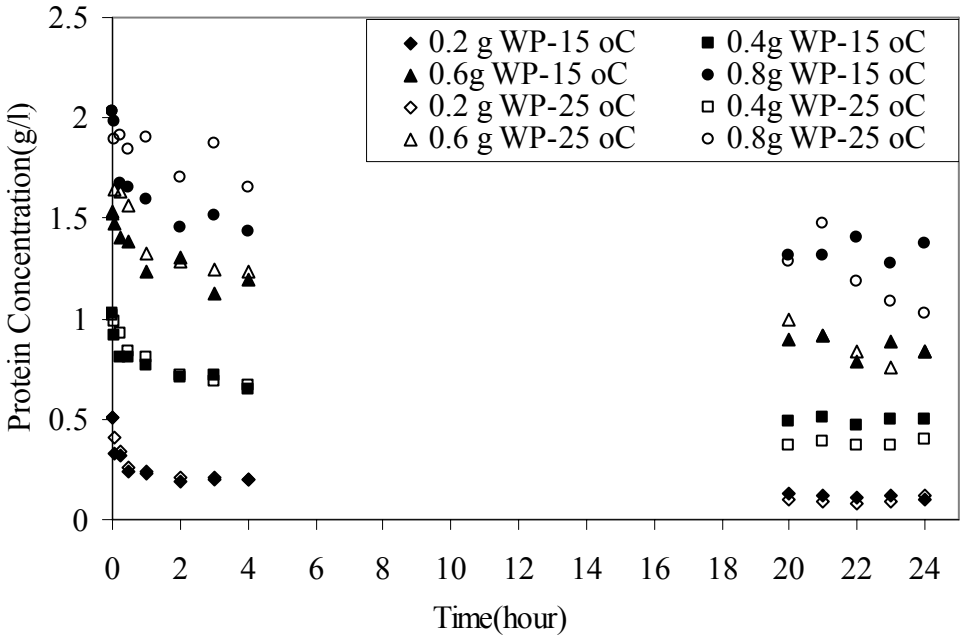

Figure 7.45. The concentration change of whey proteins adsorbed onto natural zeolite (initial whey powder amount: 0.2g to 0.8 g, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

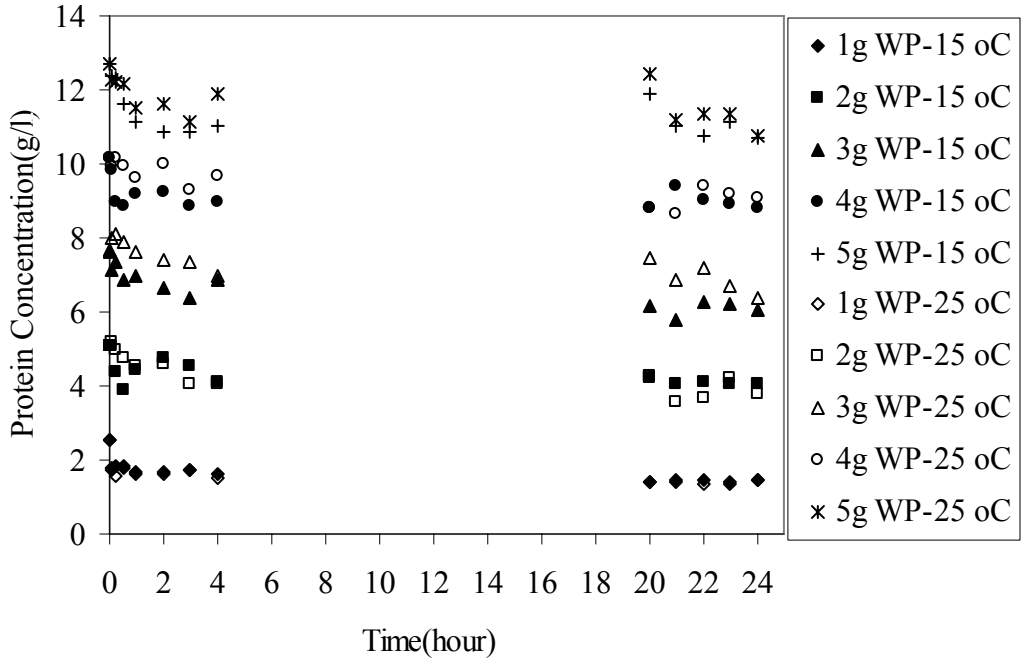

Figure 7.46. The concentration change of whey proteins adsorbed onto natural zeolite during adsorption (initial whey powder amount: 1.0 g to 5.0 g, agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).)

The effect of temperature is a beneficial tool to understand the strength of the interactions between zeolite crystals and the protein molecule. Tavolaro et al., studied the effect of incubation temperature for 4^{o}C, 18^{o}C and 30^{o}C on synthetic zeolites. They obtained that as the temperature increased, the equilibrium adsorbed amount increased. This behavior was explained as chemical adsorption for some zeolite types.

7.3.2.9. Equilibrium and Kinetics Studies

Adsorption equilibrium for two temperature values were investigated. Two widely used adsorption isotherm models, Langmuir isotherm and Freundlich isotherm models were correlated with the experimental data in order to see which isotherm model explains the system better. The model constants are tabulated in Table 7.12 and table 7.13. Adsorption for Langmuir isotherm model is monolayer, where as for Freundlich isotherm model is described as multilayer adsorption.

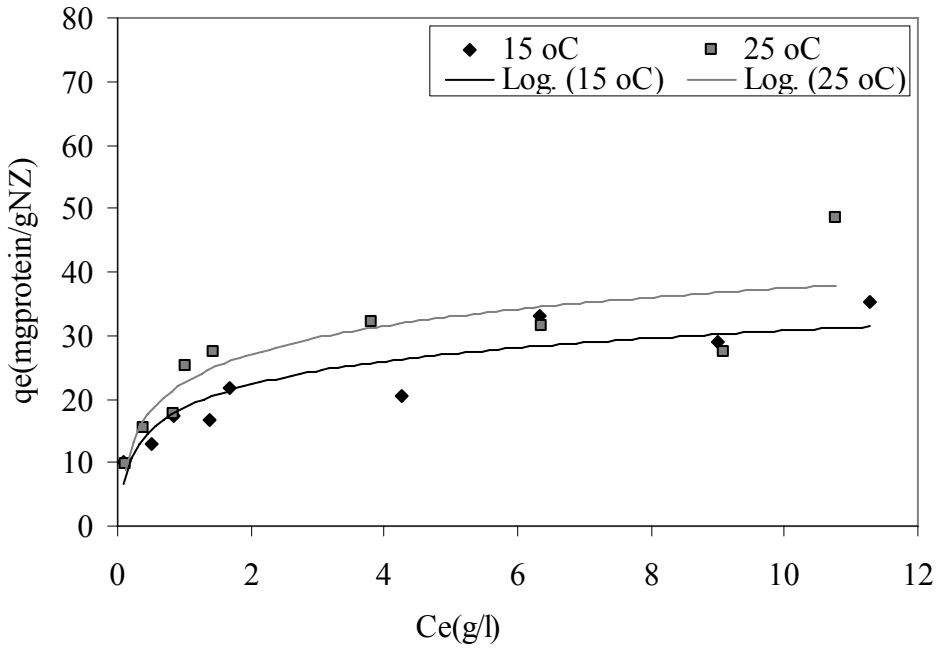

Figure 7.47. Adsorption isotherm of whey proteins (Agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

The Langmuir adsorption equation can be derived in the form. The experimental results of the q_{eq}, C_{eq} values were plotted in Figure 7.47.

$$(C_{eq}/q)= (C_{eq}/q^{m}_{eq}) +(1/(K.\ q^{m}_{eq})) \quad (7.1)$$

This equation may be represented in the y=mx+n form

$$(C_{eq}/q_{eq})=y \quad (7.2)$$

$$(1/\ q^{m}_{eq})=\text{slope}=m \quad (7.3)$$

$$1/(K.\ q^{m}_{eq})=\text{intercept}=n \quad (7.4)$$

K denotes rate of adsorption, q^{m}_{eq} denotes maximum adsorption capacity.

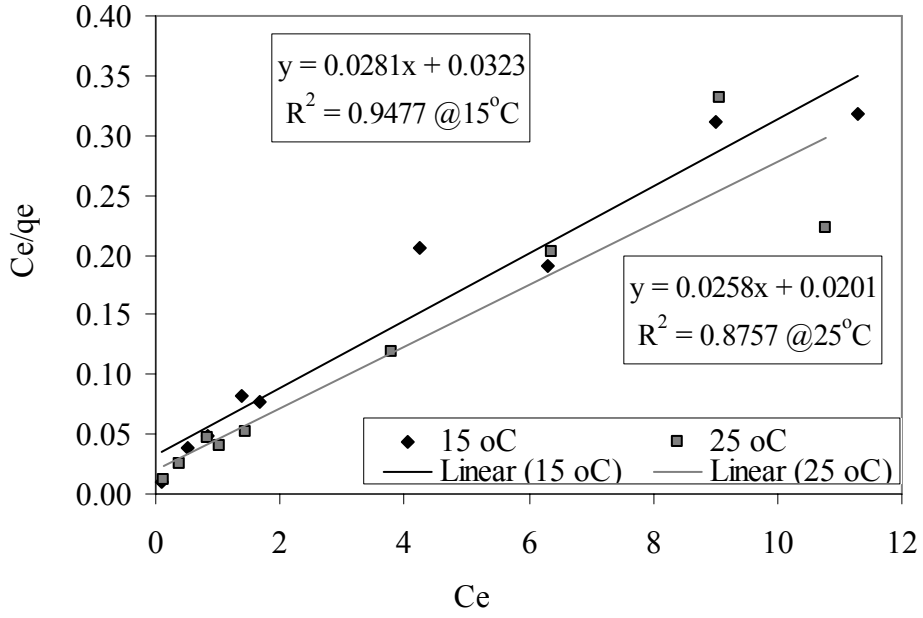

Figure 7.48. The Langmuir adsorption model applied for experimental data (Agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

Table 7.12. Langmuir isotherm parameters for protein adsorption by natural zeolite.

Temperature (^oC)	**q^m (mg protein/g NZ)**	**K (l/g)**
15	35.59	0.87
25	38.76	1.28

In order to apply Freundlich adsorption model to the experimental data and find its constants the basic equation is linearized.

$$q_{eq}= n^a/m^s=K.(C_{eq})^{1/n} \quad (7.5)$$

$$\ln q_{eq}=\ln K+(1/n)\ln C_{eq} \quad (7.6)$$

Freundlich constants K, related with adsorption capacity in equilibrium and 1/n related with adsorption intensity.

Table 7.13. Freundlich isotherm parameters for protein adsorption by natural zeolite

Temperature (^oC)	K	1/n
15	17.21	0.26
25	20.39	0.28

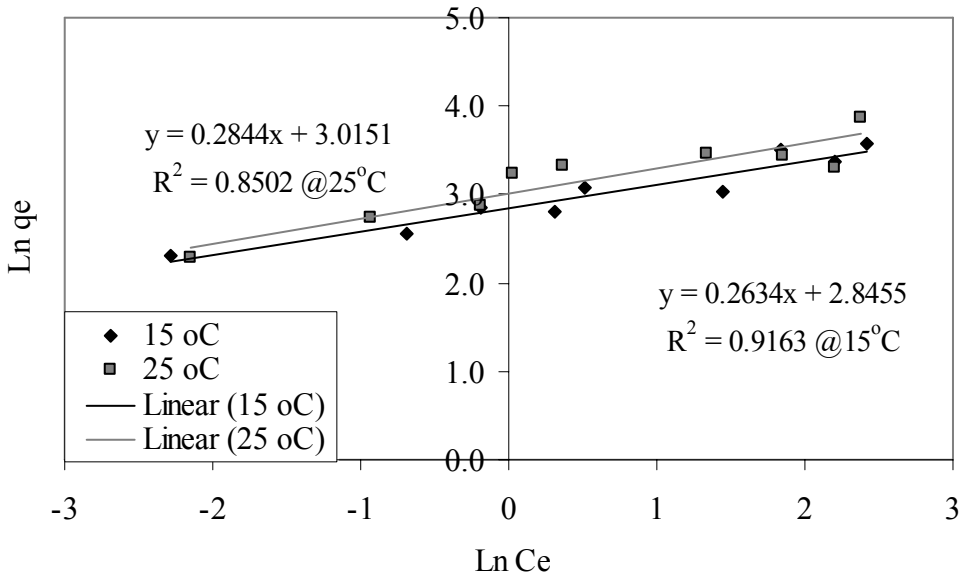

Figure 7.49. The Freundlich adsorption model applied for experimental data (Agitation speed: 150 rpm, working volume: 50 ml, S/L: 1/25).

The maximum adsorption capacity for natural zeolites at two different temperatures were compared. The values are very close to each other. Depending on the values given in Table 7.10, the temperatures studied do not have a significant effect on the adsorption of proteins by natural zeolite.

According to the constants of Freundlich and Langmuir isotherm models, q_{eq} was calculated. The experimental data and the isotherm model data were represented in Figure 7.48 and Figure 7.49. The linearized form of the Langmuir adsorption model was fitted better than the Freundlich model with respect to their regression coefficient values.

The kinetics study of the whey proteins onto natural zeolites adsorption system were analysed by several mathematical models. Those model equations were explained in Chapter 5. The pseudo first order, pseudo second order, Elovich`s equation and intraparticle diffusion models were applied to the experimental data for 24 hour time

period (Figure 7.50 to Figure 7.53). The correlation coefficient, rate constants and the corresponding linear equations are given in Table 7.14.

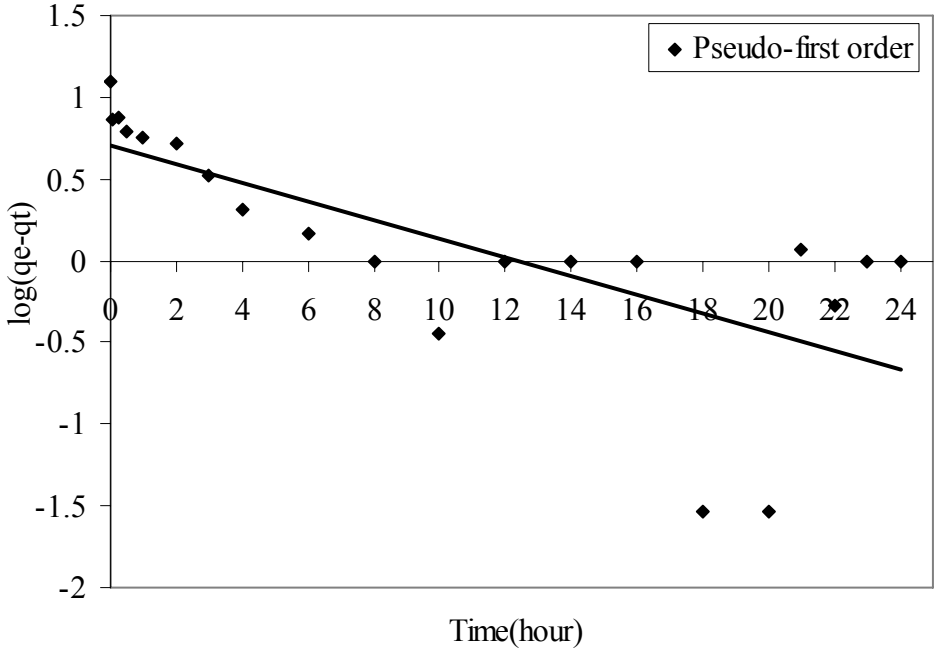

Figure 7.50. Pseudo-first order kinetics plot for the adsorption of whey proteins from whey powder solution onto natural zeolites (S/L: 1/25, T=25^{o}C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

The linear equations are used to calculate the rate constants. The adsorption models were compared with respect to the mathematical models fitting the experimental data. The correlation coefficients (R^2) are the conventional comparison tools to evaluate the suitability of the reaction or diffusion models. Because the linearity of the plots indicates the applicability of the models.

The best fit for whey powder solution adsorbed onto natural zeolite was obtained with the pseudo-second order kinetic model with a R^2 value of 0.9940. Besides, it is seen in Figure 7.53 that the straight line of intraparticle diffusion model does not pass through the origin which indicates that the mechanism is controlled by external film diffusion.

The value of intercept denotes the boundary layer diffusion effects. In addition to that, the plot of intraparticle diffusion model shows deviation from linearity. It is regarding that the system is controlled by more than one mechanism. Figure 7.53 seems to be made up of three linear region; the initial period which ends within 4 hours, the second period belonging to the following 5 hours and the last period of the final 15

hours. Depending on the studies performed with the system variables; effect of particle size and effect of agitation and considering the results of intraparticle diffusion model, the adsorption mechanism controlling the adsorption of whey proteins onto natural zeolites is external film diffusion.

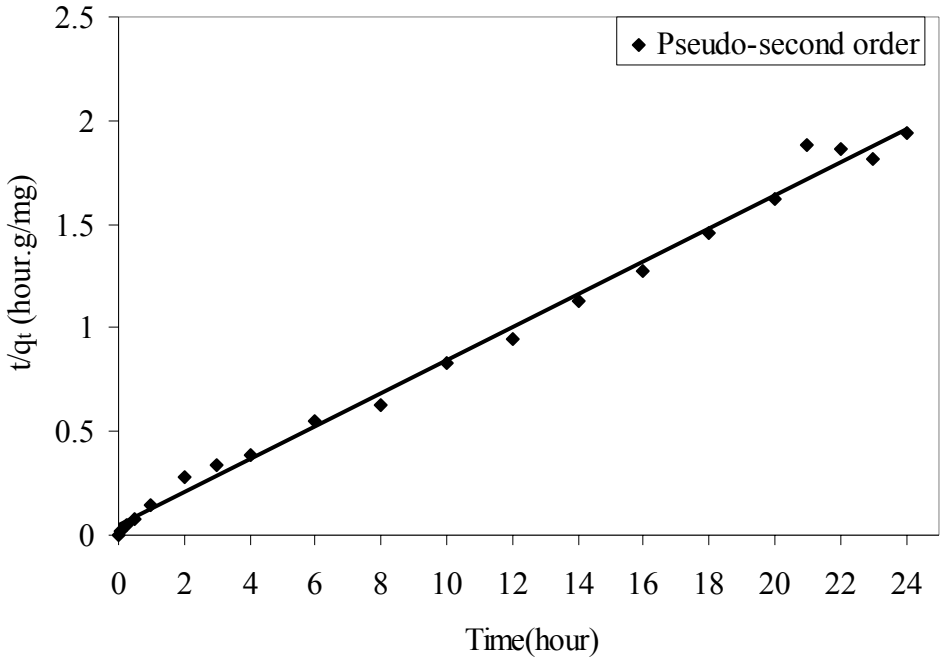

Figure 7.51. Pseudo-second order kinetics plot for the adsorption of whey proteins from whey powder solution onto natural zeolites (S/L: 1/25, T=25^{o}C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

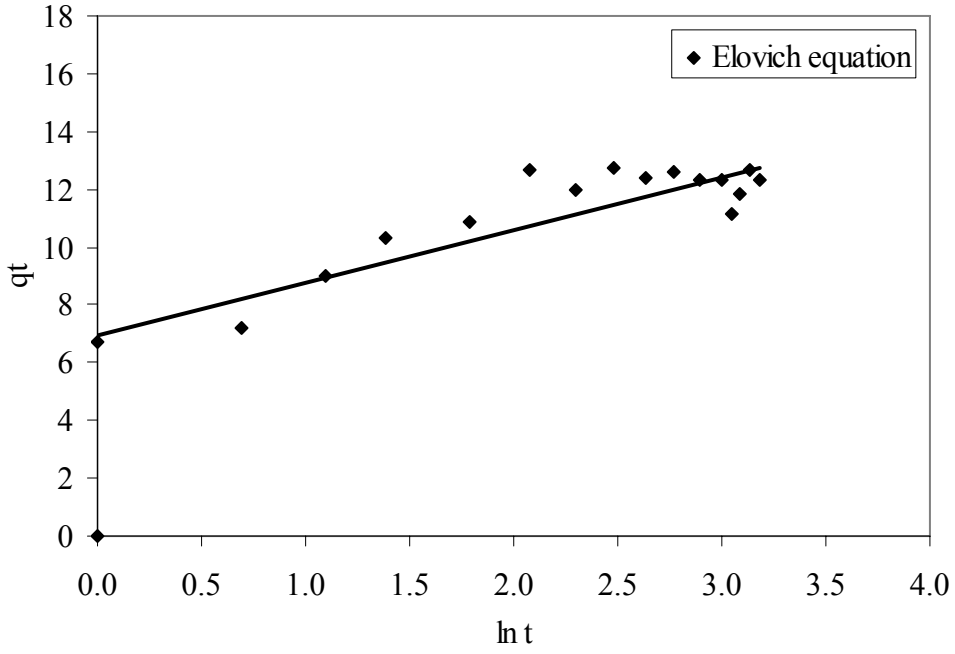

Figure 7.52. Kinetics plot of Elovich`s quation for the adsorption of whey proteins from whey powder solution onto natural zeolites (S/L: 1/25, T=25°C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

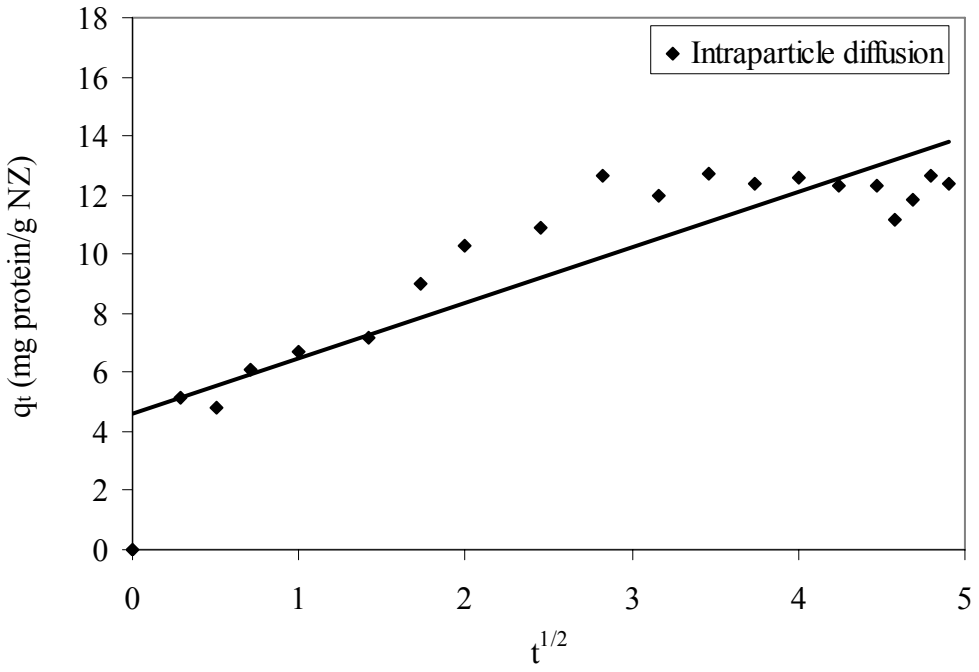

Figure 7.53. Intraparticle diffusion plot for the adsorption of whey proteins from whey powder solution onto natural zeolites (S/L: 1/25, T=25°C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

Table 7.14. Parameters of protein adsorption kinetic models for natural zeolites.

Model	Equation	Correlation Coefficient	Rate constant
Pseudo-first order	y = -0.0572x + 0.7052	R^2 = 0.5118	k_1=0.132 (h^{-1})
Pseudo-second order	y = 0.0799x + 0.0437	R^2 = 0.9940	k_2=0.146 (g/mg.h)
Elovich model	y = 1.8123x + 6.9461	R^2 = 0.7235	α=6.649 (mg/g.h)
Intraparticle diffusion	y = 1.886x + 4.5733	R^2 = 0.7823	k_d=1.886 (mg/g.$h^{1/2}$)

Adsorption kinetics study was also performed for dealuminated natural zeolites. As the Al^{+3} content of the natural zeolites decreased the experimental data was poorly fitting the related mathematical model which is seen in Figure 7.54 to Figure 7.57. The similar trend was observed through the whole adsorption kinetics model applied. The pseudo first order, pseudo second order, Elovich`s equation and intraparticle diffusion models were applied. In Table 7.15 the value of correlation coefficients corresponding with the applied mathematical models are given. The effect of dealumination process related with the HCl concentration on the adsorption kinetics mechanism of whey proteins are investigated. As the HCl concentration was increased the data fitting the applied model was deviating from the straight line. Consequenlty, lower R^2 values were obtained as the concentration of acid was increased. However, similarly with the natural zeolite results, the mathematical models applied for the dealuminated zeolites were compared and the best fit was again found as the pseudo-second order kinetics model. The results belonging to the dealuminated zeolites are not passing through the origin (Figure 7.57). The intercepts of the plots for all dealuminated zeolites are greater than the intercept value obtained for natural zeolites. The boundary layer effects are increased for acid treated natural zeolites. The adsorption mechanism is controlled by external film diffusion identically within the case of natural zeolites for whey proteins.

Table 7.15. Parameters of protein adsorption kinetic models for natural zeolites.

Adsorption Model	**(R^2) 1 M HCl**	**(R^2) 5 M HCl**	**(R^2) 8 M HCl**	**(R^2) 10 M HCl**
Pseudo-first order	R^2 = 0.8045	R^2 = 0.2567	R^2 = 0.1997	R^2 = 0.0700
Pseudo-second order	R^2 = 0.9986	R^2 = 0.9943	R^2 = 0.9826	R^2 = 0.9482
Elovich model	R^2 =0.5291	R^2 =0.2341	R^2 =0.2581	R^2 =0.0348
Intraparticle diffusion	R^2 = 0.6707	R^2 = 0.4224	R^2 = 0.4122	R^2 = 0.0841

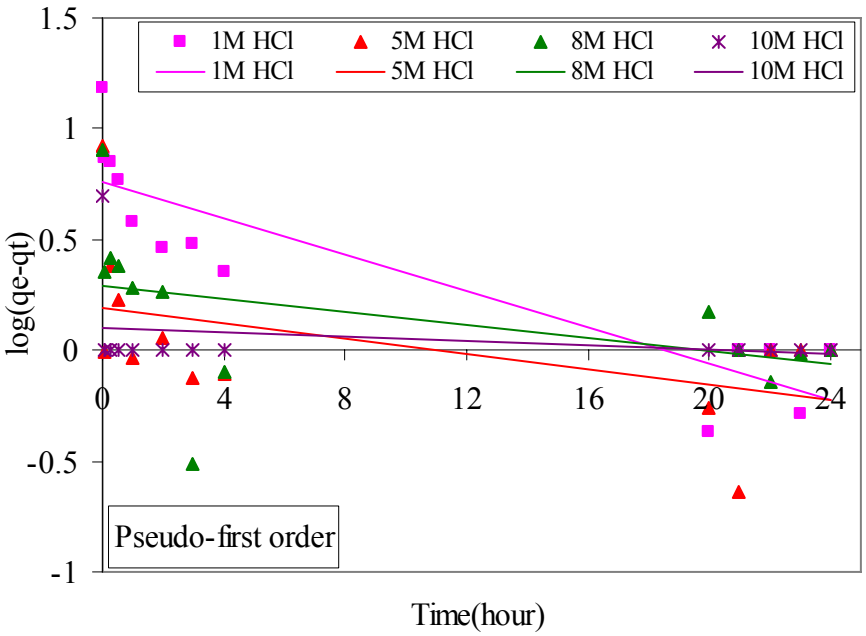

Figure 7.54. Pseudo-first order kinetics plot for the adsorption of whey proteins from whey powder solution onto dealuminated natural zeolites (S/L: 1/25, T=25°C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

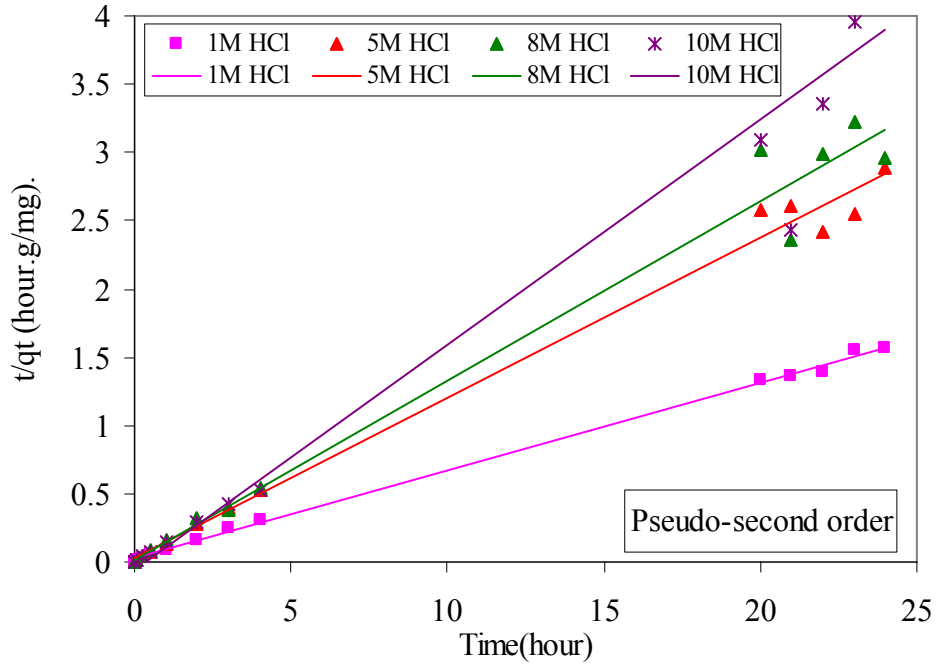

Figure 7.55. Pseudo-second order kinetics plot for the adsorption of whey proteins from whey powder solution onto dealuminated natural zeolites (S/L: 1/25, T=25°C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

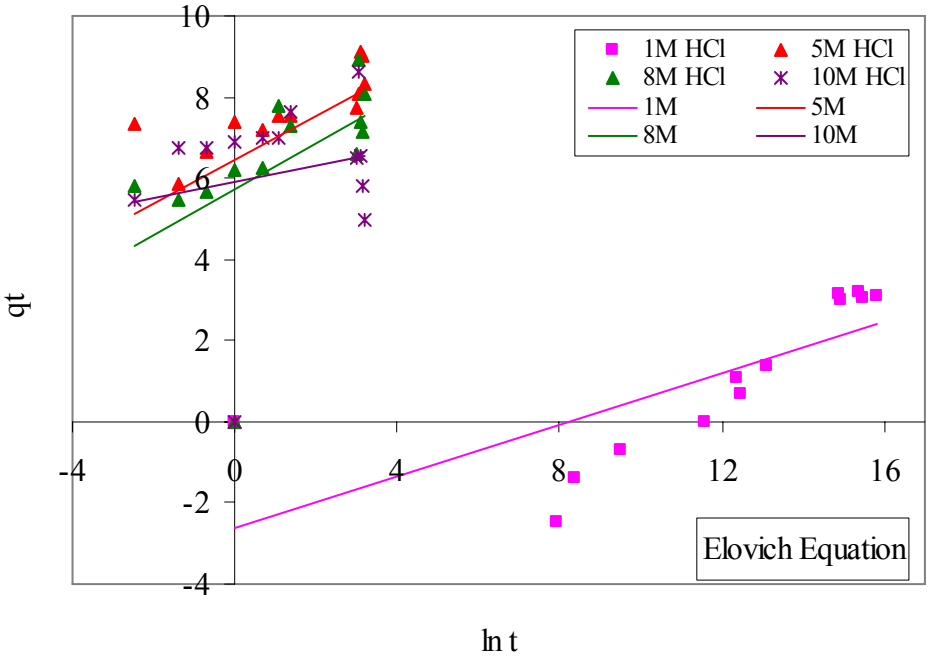

Figure 7.56. Kinetics plot of Elovich`s quation for the adsorption of whey proteins from whey powder solution onto dealuminated natural zeolites (S/L: 1/25, T=25°C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

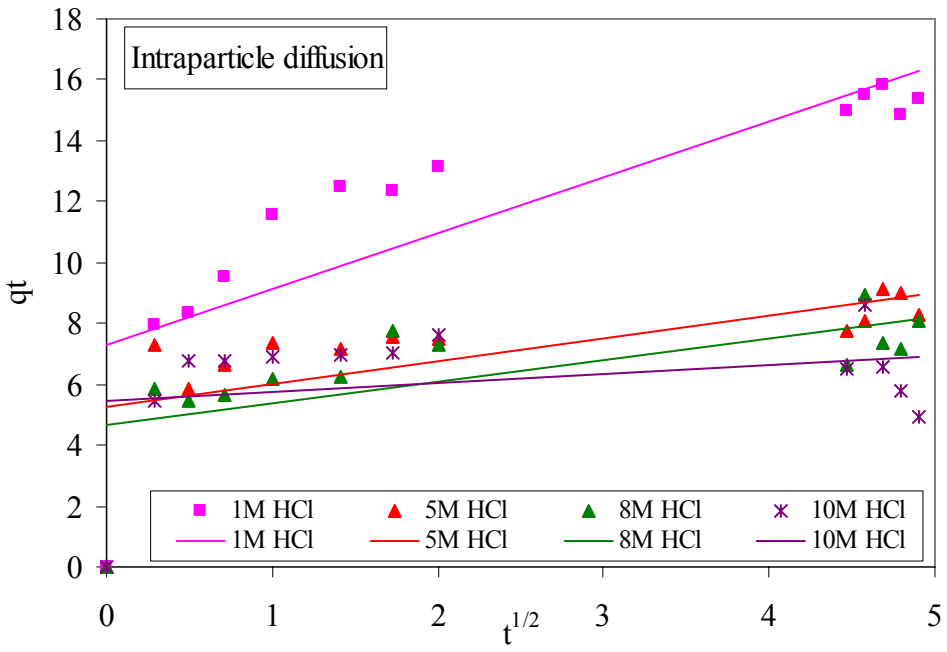

Figure 7.57. Weber and Morris intraparticle diffusion plot for the adsorption of whey proteins from whey powder solution onto dealuminated natural zeolites (S/L: 1/25, T=25°C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

7.3.2.10. The Effect of Purity

The experimental results of the whey powder protein adsorption with pure natural zeolite which was kindly supported by Narin (2009) are plotted in Figure 7.58. The results were given in comparison with different particle size experiment set. The adsorbent capacity and the time to reach constant values are strongly affected.

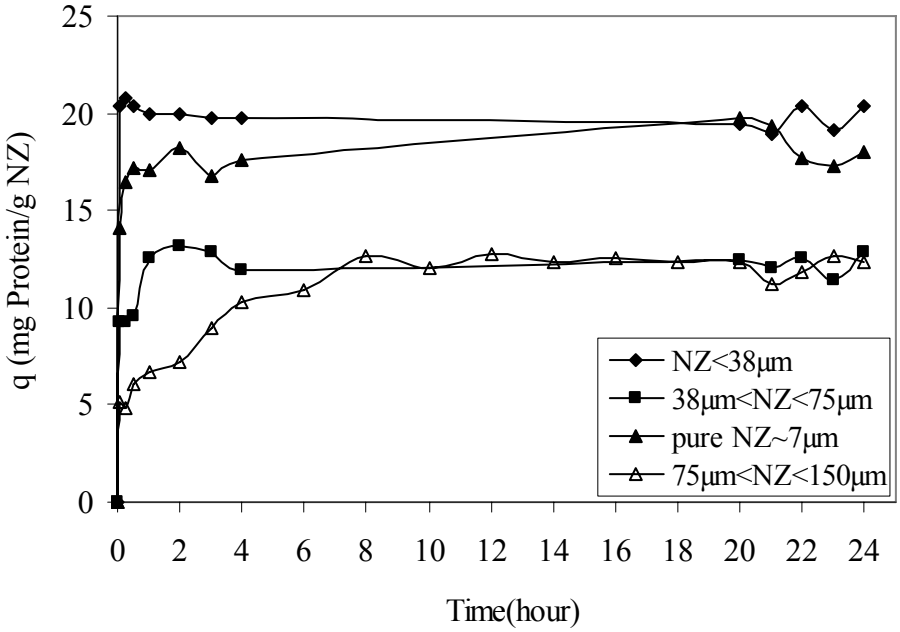

Figure 7.58. Adsorption of whey proteins from whey powder solution onto natural zeolites (S/L: 1/25, T=25°C, Ci: 1.016 g/l protein, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

In order to come to a conclusion about the equilibrium adsorbed amount and the time to reach the equilibrium value, between the samples having different purity, the particle sizes should have the same value. Only one parameter should change while the others must be kept constant. Noticing that fact, to make a clear comparison is not easy in this case.

7.3.2.11. Adsorption of Whey Proteins

Adsorption of whey proteins were studied at different temperatures to make a comparison with the whey powder protein adsorption studies. Adsorbed amounts of proteinsfrom whey are given in Figure 7.59.

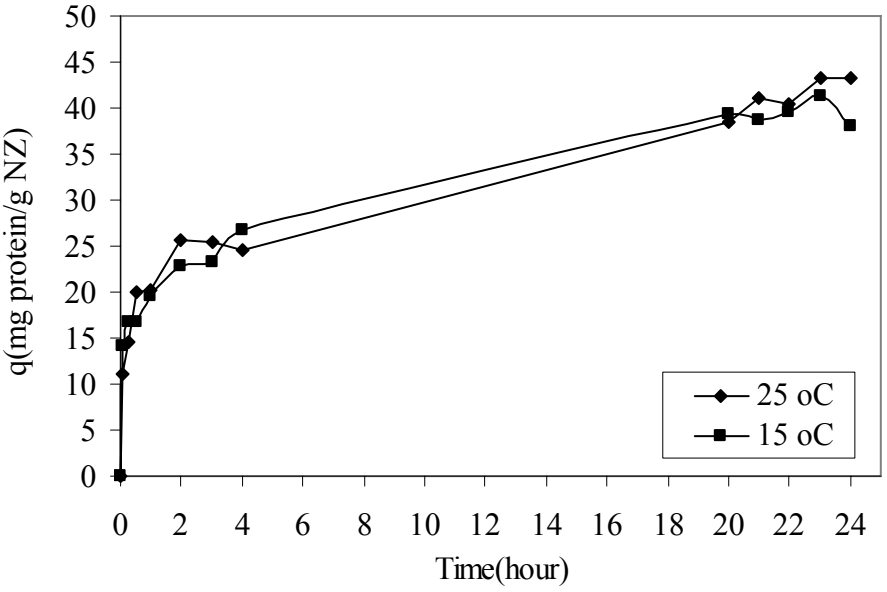

Figure 7.59. Whey proteins adsorbed amount from whey (S/L: 1/25, T=25°C, adsorbent dose: 2 g NZ, agitation speed: 150 rpm).

The control sets for whey, whey powder and BSA at 15°C and 25°C were examined to see whether the protein amount changes without the addition of natural zeolite, due to aglomeration, protein precipitation etc. (Figure 7.60 to 7.65). In order to declare that the change in protein content is ascribed due to the existence of natural zeolite, the control sets should possess constant concentrations.

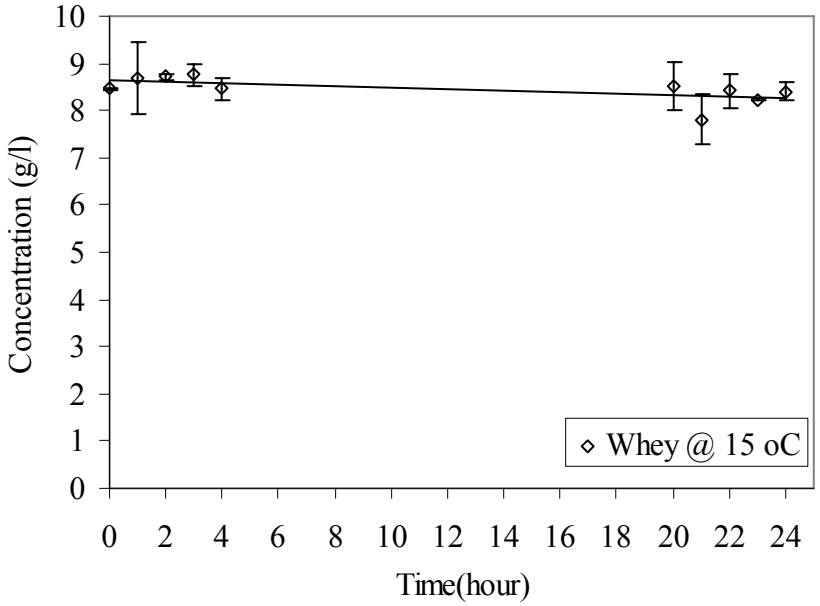

Figure 7.60. Control concentration without natural zeolites for whey at 15 °C (Working volume: 50 ml, agitation speed: 150 rpm).

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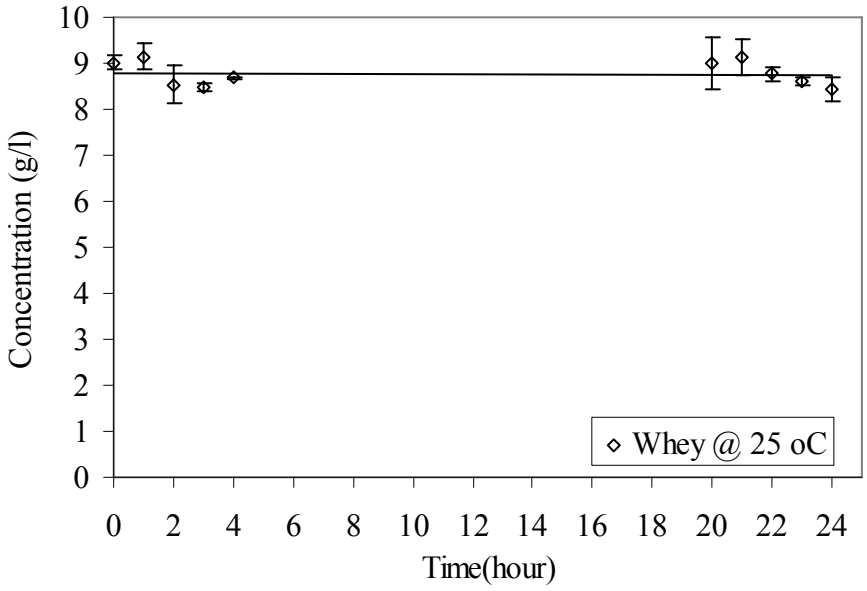

Figure 7.61. Control concentration without natural zeolites for whey at 25 ^{o}C (Working volume: 50 ml, agitation speed: 150 rpm).

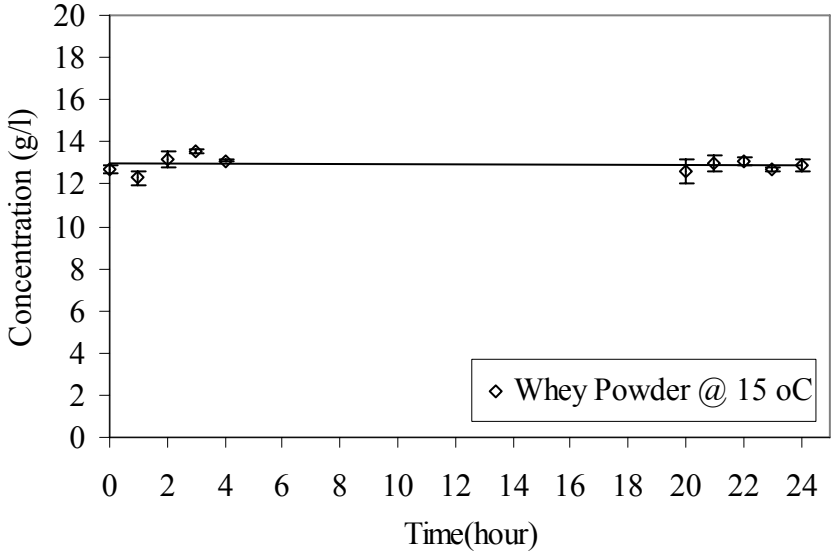

Figure 7.62. Control concentration without natural zeolites for whey powder at 15 ^{o}C (Working volume: 50 ml, agitation speed: 150 rpm).

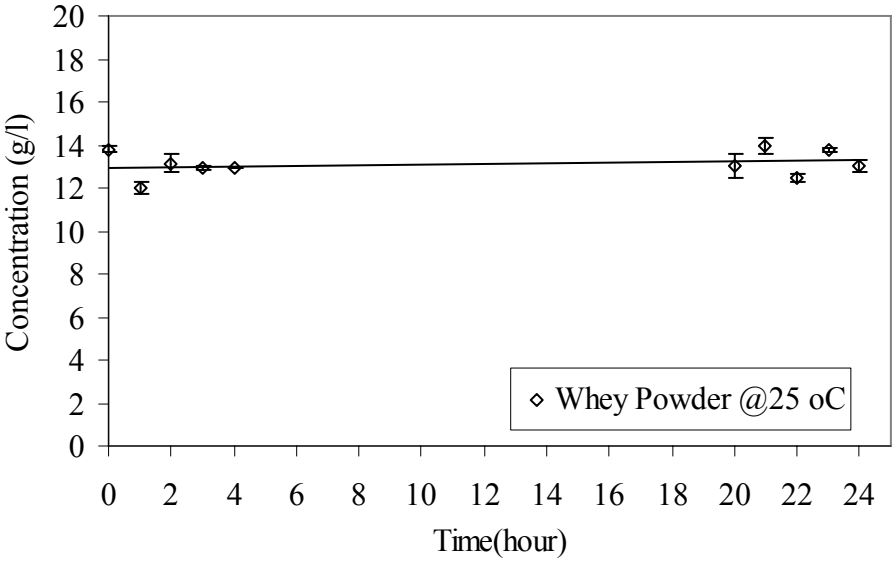

Figure 7.63. Control concentration without natural zeolites for whey powder at 25 ^{o}C (Working volume: 50 ml, agitation speed: 150 rpm).

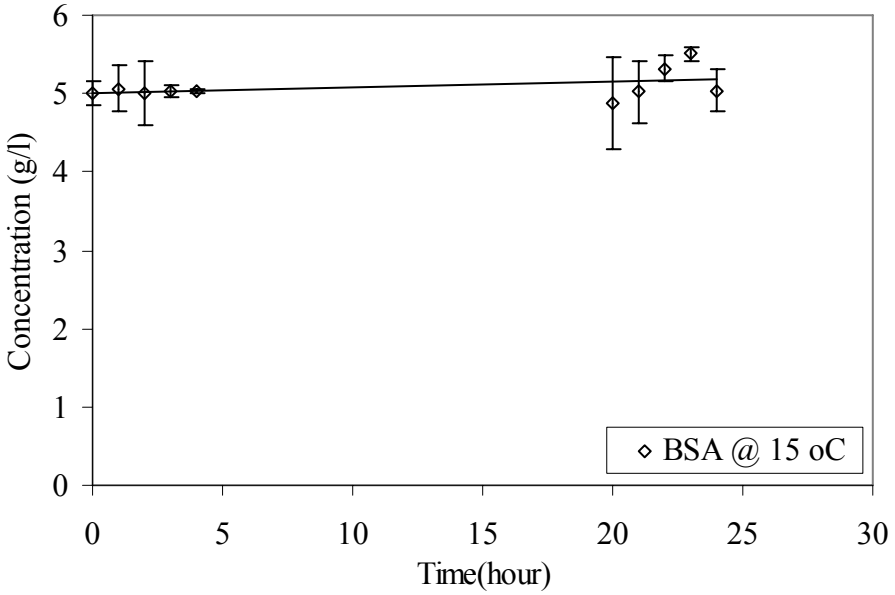

Figure 7.64. Control concentration without natural zeolites for BSA at 15 ^{o}C(Working volume: 50 ml, agitation speed: 150 rpm).

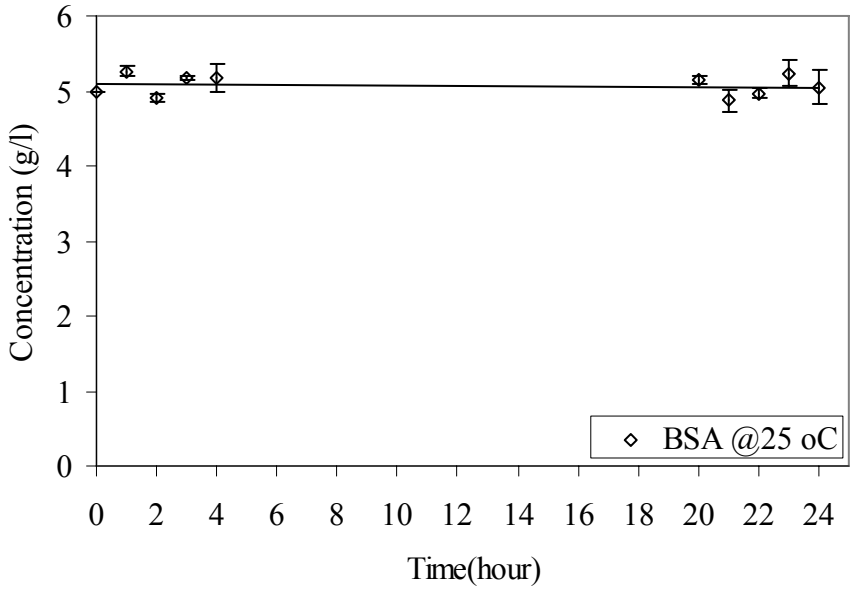

Figure 7.65. Control concentration without natural zeolites for BSA at 25 ^{o}C (Working volume: 50 ml, agitation speed: 150 rpm).

7.4. Desorption of Proteins

The desorption ability of the proteins adsorbed on the surfce of the natural zeolites were investigated. Few studies concerning the desorption of proteins from zeolites but not natural zeolites indicate that, polyethylene glycol is a good candidate as a desorption eluent for proteins (Sakaguchi et al,2005; Chiku et al, 2003). However, the analytical method is a critical point when studying with polyethylene glycol (PEG). Polyethylene glycol is not working well with the reagents of Lowry. The samples turn into a turbid light yellow structure. On the other hand, in literature the studies about the investigation of polyethylene glycol as eluent are using Bradford method for protein quantification. Unfortunately, Bradford method is not an appropriate method for whey-whey powder solutions. The complex nature of protein mixture prevents the sensitive detection of real protein concentration inside the medium. Thus, eluents other than PEG were studied and analyzed with Lowry method. Desorption of proteins from zeolites is a new area attracting the attention in protein engineering studies. The possible new eluents are investigated so as to desorb whey proteins from the adsorbed surface of natural zeolites.

7.4.1. BSA Desorption From Natural Zeolite

Desorption study of BSA with eluents having different pH values were applied to BSA adsorbed natural zeolite samples. Desorption study was performed at 25 ^{o}C , by 150 rpm agitation. It was observed that the desorbed BSA amount was constant after 60 minutes time period. In order to see the desorption profile better, time to reach equilibrium period was plotted and is presented in Figure 7.66.

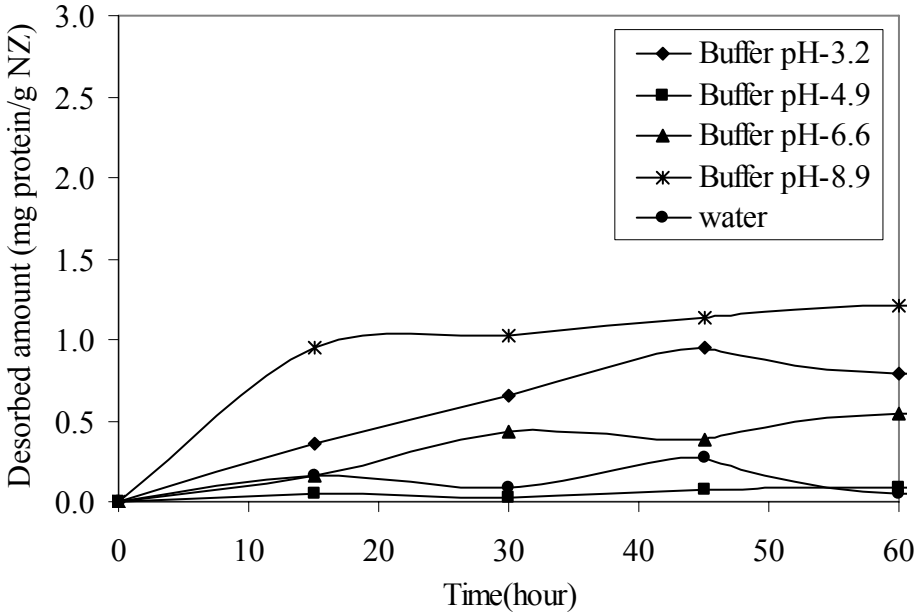

Figure 7.66. Desorbed BSA amount at different pH values (S/L: 1/25, T=25^{o}C, eluent volume: 50 ml, agitation speed: 150 rpm).

7.4.2. Whey Protein Desorption From Natural Zeolite

Desorption of whey proteins from the adsorption of whey and whey powder solutions were investigated. The constant concentration was reached within 60 minutes for desorption of whey proteins similar with the desorption of BSA from natural zeolite. As the desorption curve reached the constant concentration within 60 minutes, the study was mainly focused for 60 minutes time period. The pH of the buffer solution is important for the desorption efficiency. As the pH values were higher better results were obtained. Figure 7.67 summarizes the desorption time period even the duration of the process was extended for 16 hours. In Figure 7.67 and Figure 7.68, desorption was applied to the samples that were exposed to adsorption study with whey powder

solution and whey. The labels given as buffer and water are belonging to whey powder adsorption set whereas whey is representing the adsorption medium. The same explanation is valid for the following desorption studies.

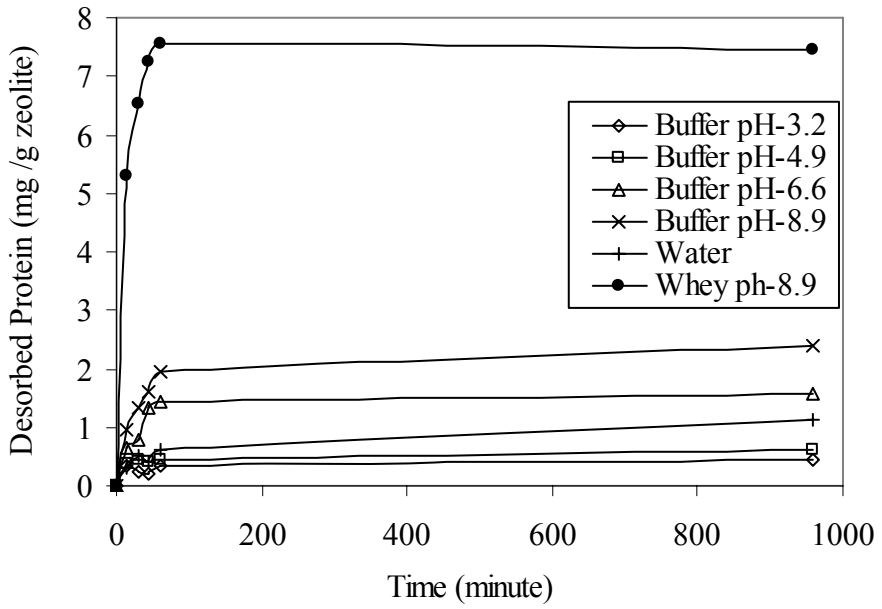

Figure 7.67. Desorbed whey proteins amount from whey powder and whey adsorbed natural zeolites at different pH values (S/L: 1/25, T=25^{o}C, eluent volume: 50 ml, agitation speed: 150 rpm).

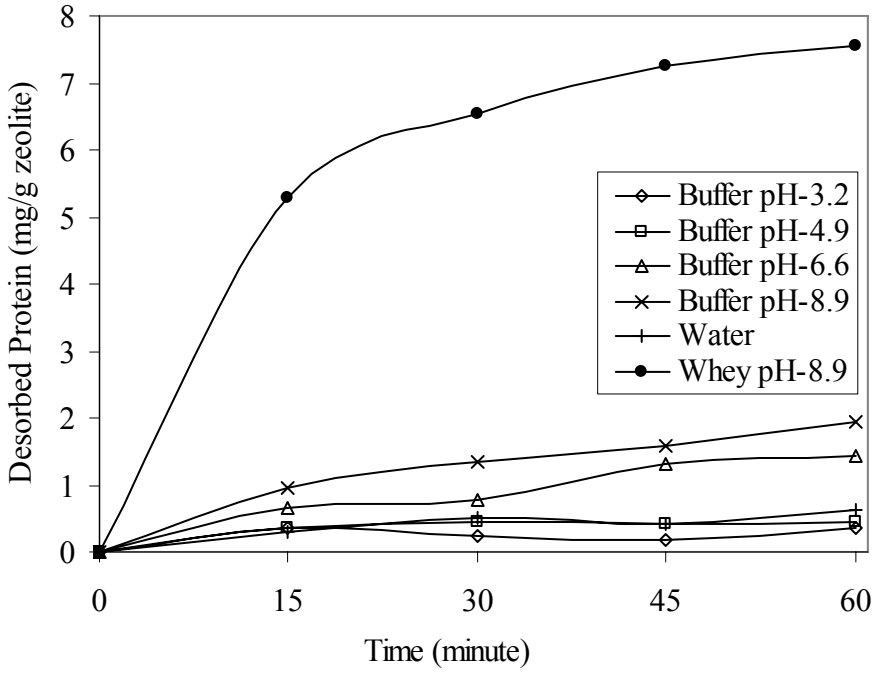

Figure 7.68. Initial period for desorbed whey proteins amount from whey powder and whey adsorbed natural zeolites at different pH values (S/L: 1/25, T=25^{o}C, eluent volume: 50 ml, agitation speed: 150 rpm).

Desorption study was performed to elucidate the adsorption mechanism better. By applying desorption, one can evaluate the reversibility and irreversibility. In literature especially β-lactoglobulin which has high internal stability mostly prefer to be adsorbed on oppositely charged surfaces reversibly (Nakanishi et al, 2001). In the following Figure 7.69, similar desorption study and the conditions were applied after the whey and whey powder adsorption study. The desorption results which is ascribed to whey-natural zeolite medium were given in Figure 7.70.

Another desorption study concerning the whey-natural zeolite system is shown in Figure 7.70. It was observed that desorption values of proteins from whey is higher than the desorption values of proteins from whey powder as it is seen both in Figure 7.68 and Figure 7.69.

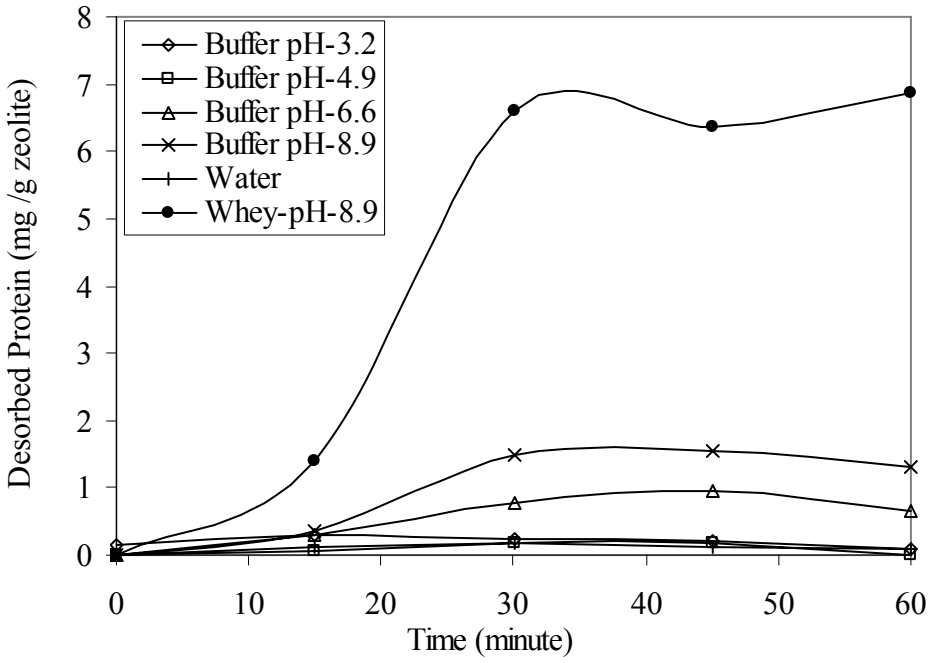

Figure 7.69. Desorbed whey proteins from whey powder and whey adsorbed natural zeolites at different pH values (S/L: 1/25, T=25°C, eluent volume: 50 ml, agitation speed: 150 rpm).

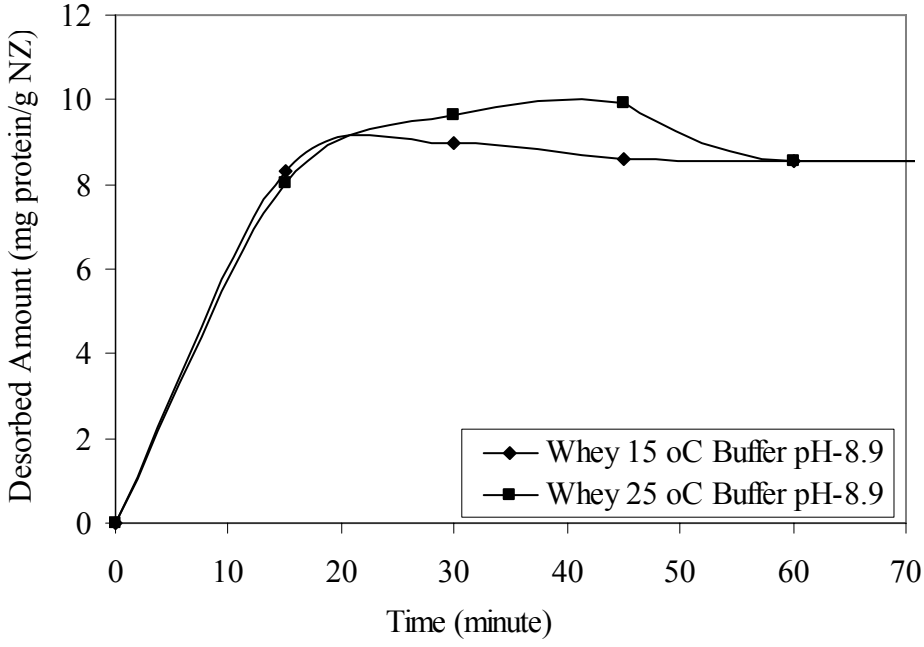

Figure 7.70. Desorption of proteins from whey adsorbed at 15 °C and 25°C (S/L: 1/25, T=25°C, eluent volume: 50 ml, agitation speed: 150 rpm).

In Figure 7.70 the desorption study was applied to whey-natural zeolite adsorbed system. Adsorption study was done at 15 ^{o}C and at 25 ^{o}C. All the desorption studies were performed at room temperature. There is not much difference in the desorbed amount of proteins.

The effect of washing step on the desorption of whey proteins adsorbed onto natural zeolites was investigated. The experimental set of adsorption study was doubled for same whey powder content and same adsorbent dose. One of the set was exposed to washing step with buffer solution while the second was not washed before desorption study. All the other parameters and conditions were kept constant. The results are given in Figure 7.71 which is not washed and Figure 7.72 that is washed after adsorption.

Considering all the desorption stuty it is obtained that, desorption of whey proteins adsorbed onto natural zeolite from whey gives higher amount than the desorbed samples obtained from whey powder adsorbed natural zeolites. Additionally, if washing step is applied the protein desorbed amounts are much lower than the samples where washing step was not performed.

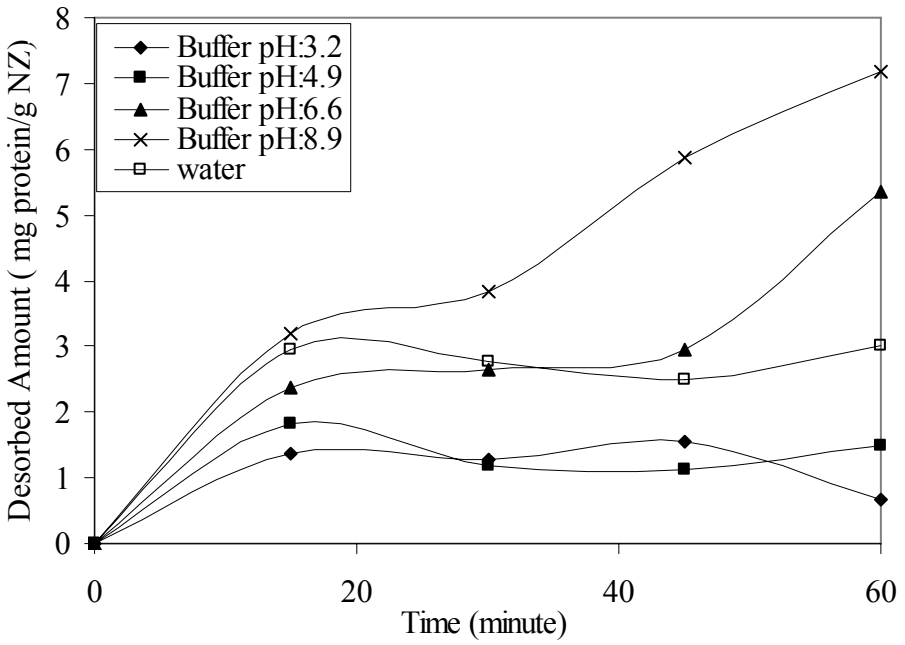

Figure 7.71. Desorbed whey proteins from whey powder adsorbed onto natural zeolites eluted with different pH values without washing step (S/L: 1/25, T=25^{o}C, eluent volume: 50 ml, agitation speed: 150 rpm).

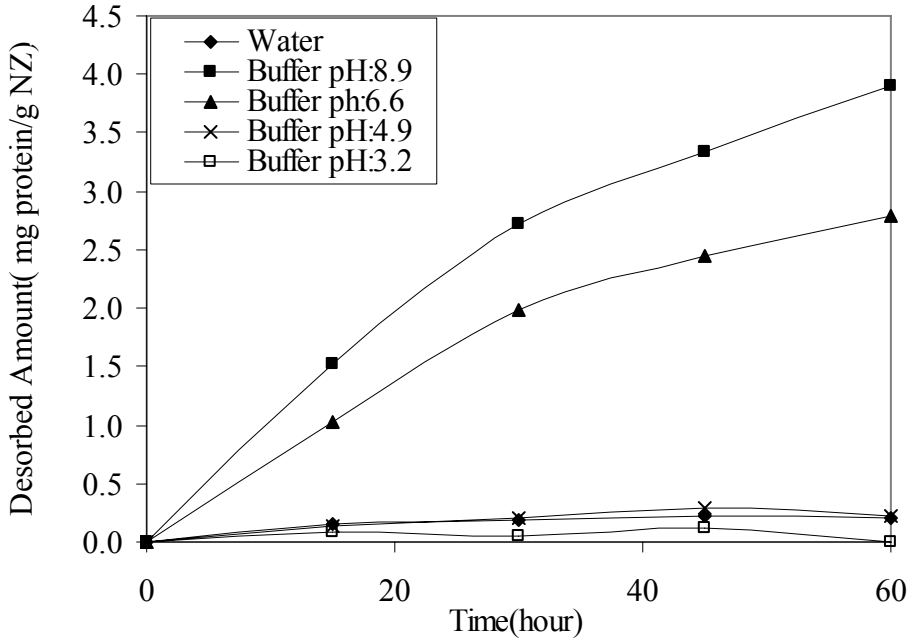

Figure 7.72. Desorbed whey proteins from whey powder adsorbed onto natural zeolites eluted with different pH values with washing step (S/L: 1/25, T=25^{o}C, eluent volume: 50 ml, agitation speed: 150 rpm).

7.4.3. Residual Protein in Fermentation

Following the protein adsorption studies in the previous section, the combined protein adsorption with lactic acid fermentation was studied. The first stage of the process was the recovery of whey proteins from whey powder solution prepared with pH 3.2 phosphate buffer solution. The results of adsorption study are shown in Figure 7.73. The temperature was 37 ^{o}C with agitation speed of 150 rpm. The solid part was obtained by centrifugation and the supernatant was sterilized for fermentation study.

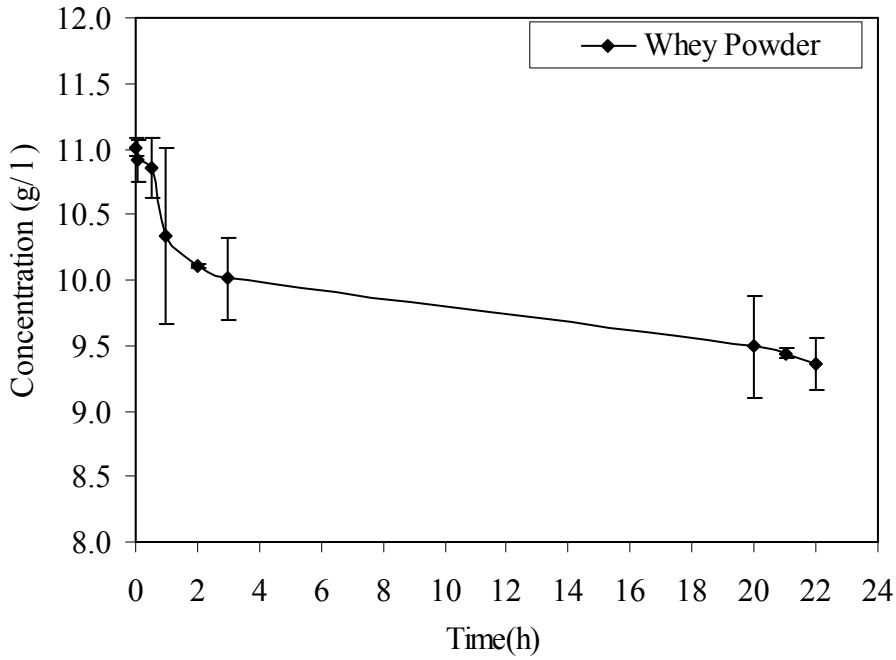

Figure 7.73. Adsorption of whey proteins onto natural zeolite (S/L: 1/20, T=37^{o}C, adsorbent dose: 5 g, agitation speed: 150 rpm).

Proteins in whey and whey powder were analysed individually by HPLC-Zorbax GF-250 column. The comparison of different mobile phase compositions were illustrated as chromatograms in Figure 7.74. Best resolution was obtained by 2000mM NaCl + 300mM Na-Phosphate. Figure 7.75 is the result of a similar study where as the usage of two HPLC columns in series is observed (Merina et al., 2001). In Figure 7.76, standard β-Lg, and α-La and whey powder were presented as an overlay view. Big protein molecules elute first from the column.

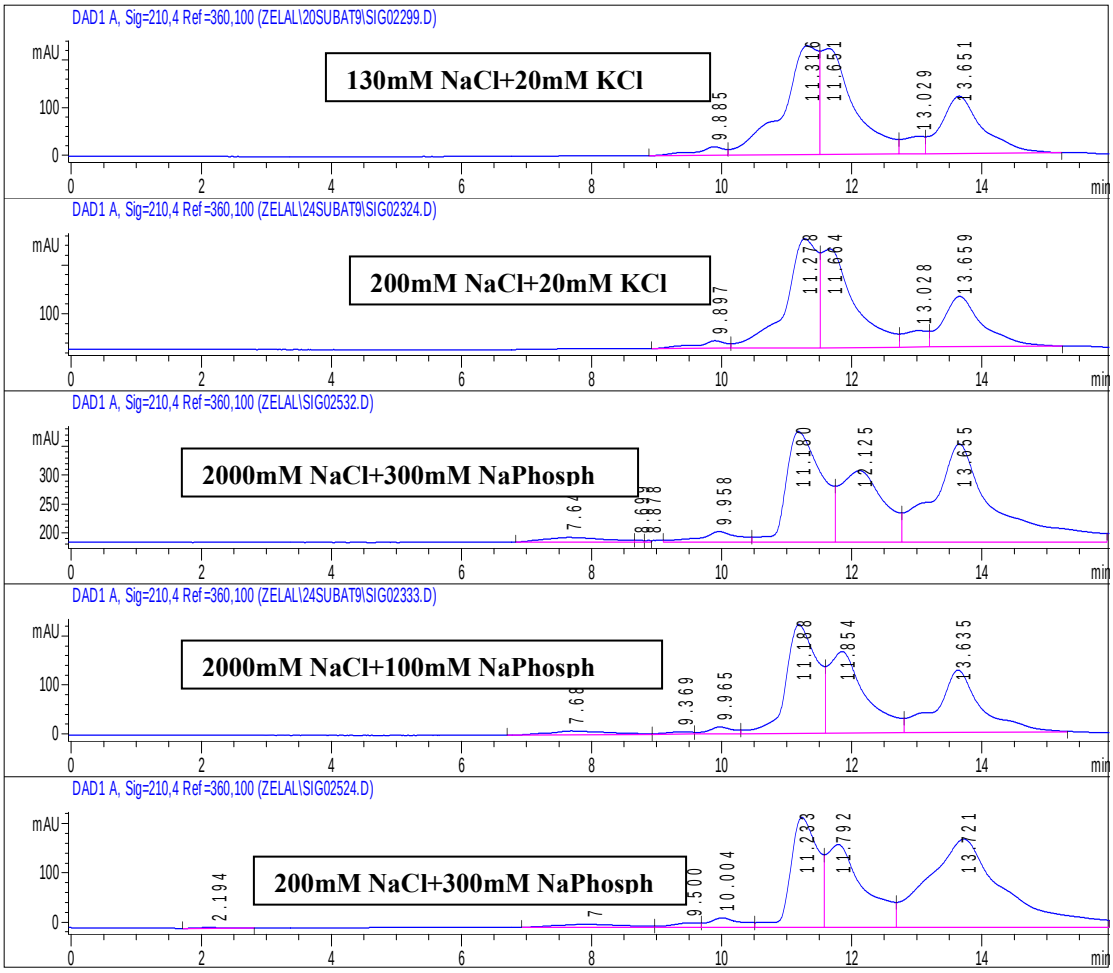

Figure 7.74. Comparison of mobile phases on chromatographic separation

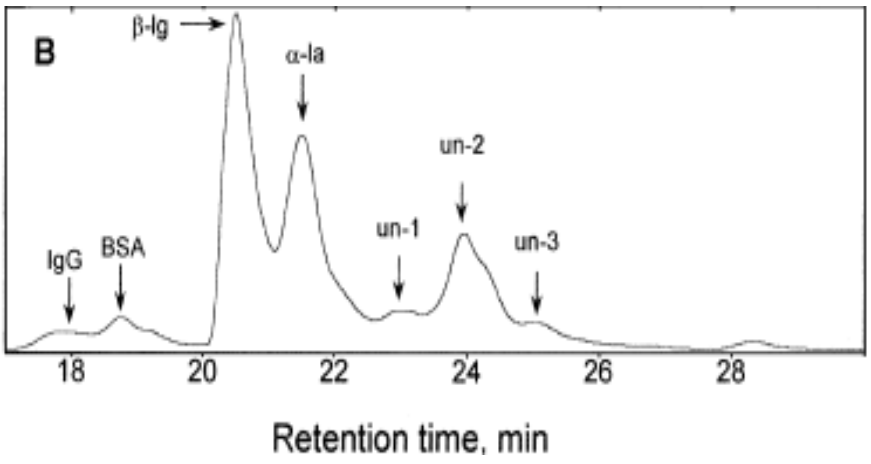

Figure 7.75. HPLC chromatogram of bovine milk in literature
(Source: Merina et al., 2001).

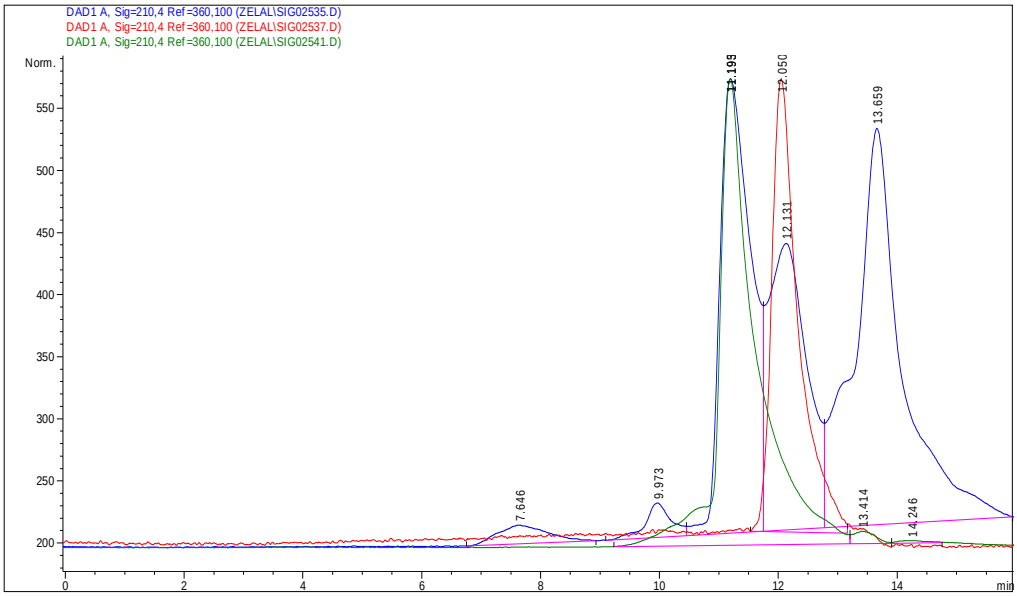

Figure 7.76. HPLC Chromatograms; Blue: Whey powder, Green: β-Lactoglobulin, Red: α-Lactalbumin.

During sterilization proteins in whey powder undergoes change. Comparison of whey powder proteins before and after sterilization is investigated and the results are given in Figure 7.77. The peliminary blue peaks in Figure 7.77 belongs to BSA, β-Lg, α-La. In red peak it was observed that the characteristic peaks of protein in whey powder disappear. The exposure of temperature and its duration are important parameters for whey proteins as given in literature.

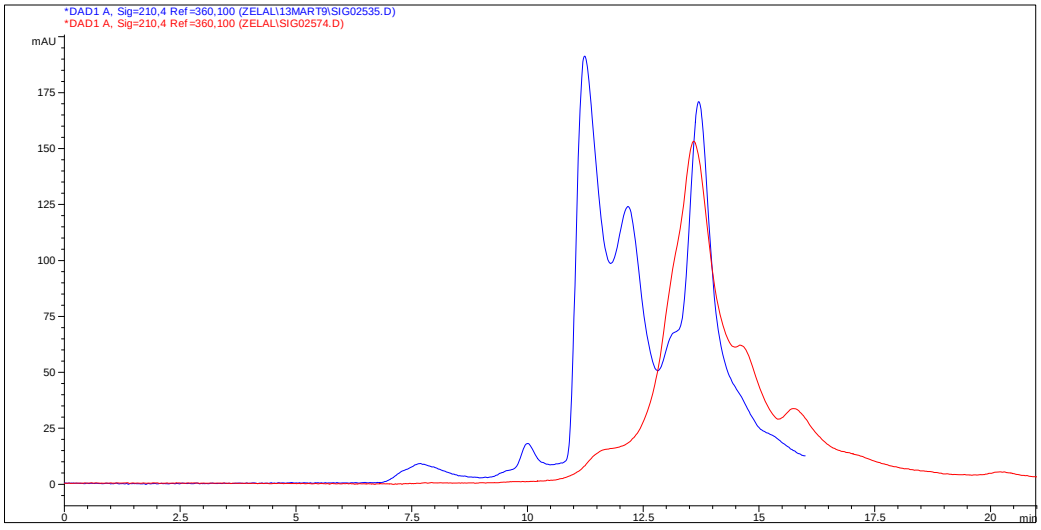

Figure 7.77. HPLC Chromatogram of whey powder solution; before sterilization (blue), after sterilization (red).

The composition of whey proteins during adsorption study is illustrated in Figure 7.78. Depending on the protein composition it was measured as β-Lg 56 %, α-La 20 %, and BSA 4 %. The values are in good aggreement with the literature even BSA is smaller than the literature data which is given as 10 %. The difference may be due to the difference in composition of whey used to produce whey powder. Because the composition is directly affected by seasonal changes and lactation period.

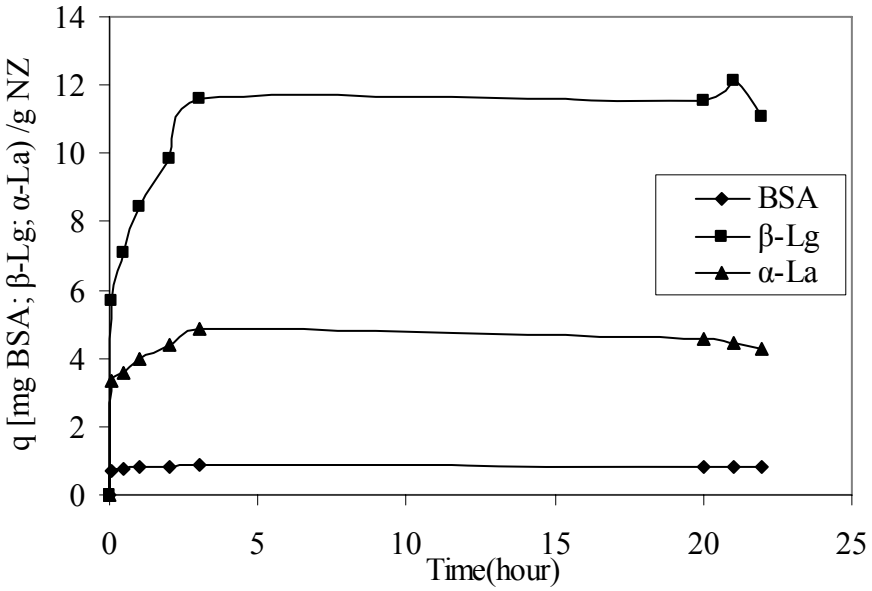

Figure 7.78. Adsorption profile for whey proteins (S/L: 1/20, T=37°C, adsorbent dose: 5g, agitation speed: 150 rpm).

Fermentation was performed with whey powder which was pretreated with natural zeolite in order to recover whey proteins, and subsequently to see the protein requirements of the medium. Figure 7.79 shows the lactose consumption and Figure 7.80 is presenting the lactic acid production. It is obvious that yeast extract is obligatory for fermentation medium, otherwise if the medium does not contain yeast extract (Z_3), lactose conversion to lactic acid decrease significantly when compared with the control fermentation (Z_4). Besides if the results for Z_1 indicating no mineral addition are compared with control fermentation, it is clear the results are close to each other. Because during adsorption of proteins before fermentation, the natural zeolite enriched the medium by releasing Mn^{+2} and K^{+} ions which are required by the microorganism. In this case mineral requirement can be provided by natural zeolite and supernatant can be used for the lactic acid fermentation. And Z_2 coded samples are compared with the

control set (Z_4) better results are obtained. These samples were treated with natural zeolite in protein adsorption part. As it is seen from figures, after the recovering some of the whey proteins the residual amount is sufficient to proceed fermentation when compared with the control set.

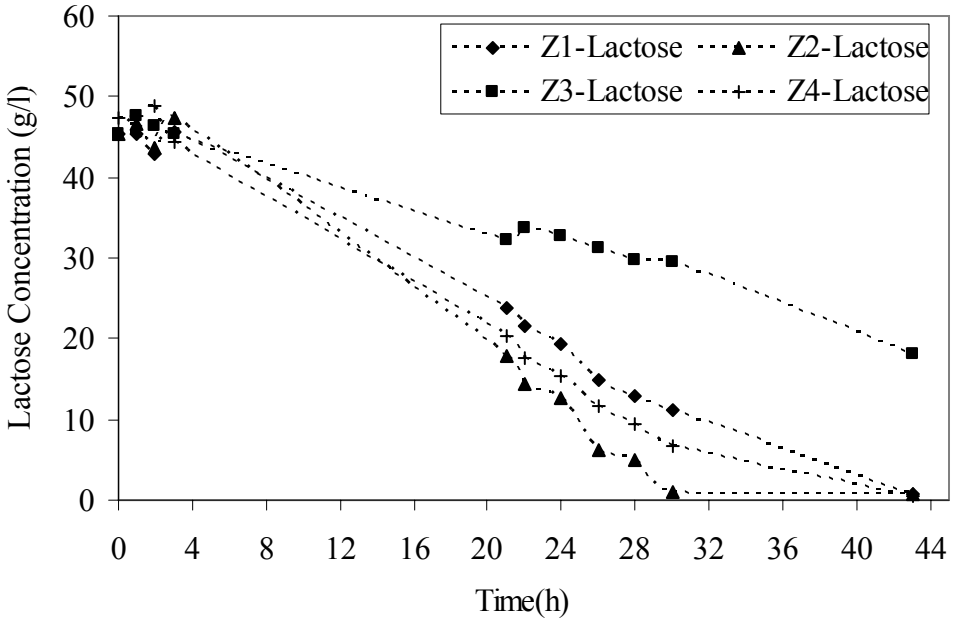

Figure 7.79. Lactose concentration with/without mineral sources and protein sources (Temperature: 37°C, agitation speed: 150 rpm).

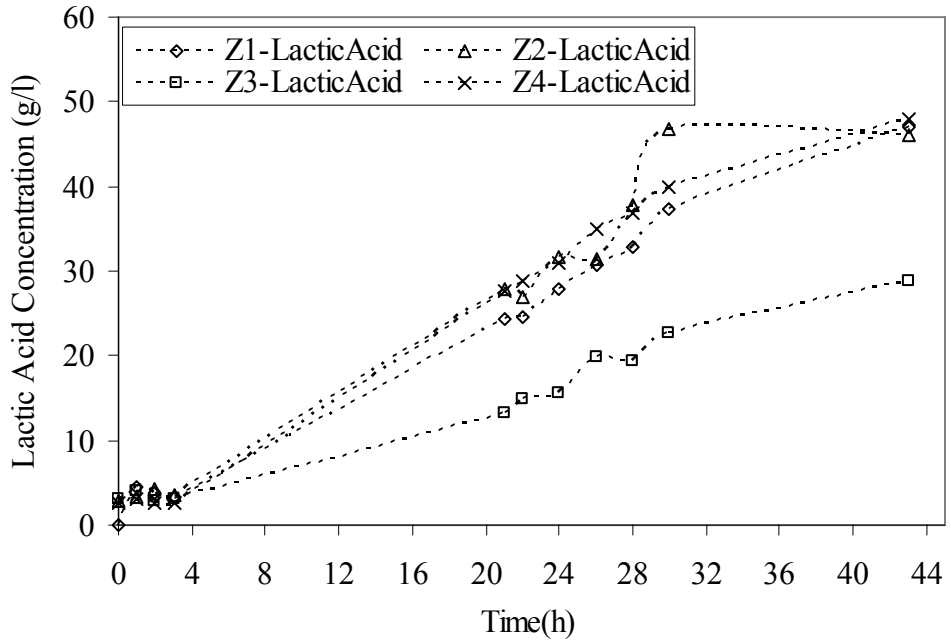

Figure 7.80. Lactic Acid concentration profile with/without mineral and protein sources (Temperature: 37°C, agitation speed: 150 rpm).

7.5. Natural Zeolites in Fermentation

In this study, natural zeolites were used both in aqueous solutions and in fermentation media. Initially, the comparison between the lactose consumption of the lyophilized culture of *Lactobacillus casei* NRRL B441, was made with the stock culture stored in litmus milk at 4°C (Figure 7.81). The lactose consumption and lactic acid production showed similar concentration values. The storage and the propagation procedure for the microorganism was adequate. A bacterial count, both with litmus milk and MRS Broth was performed (Table 7.16). It was seen that the litmus milk was adequate media for microbial growth.

Table 7.16. Bacterial Count (Poured Plate Method).

Dilution factor	MRS Broth	Litmus Milk
10^{-6}	More than 300	More than 300
10^{-7}	77	147
10^{-8}	8	15

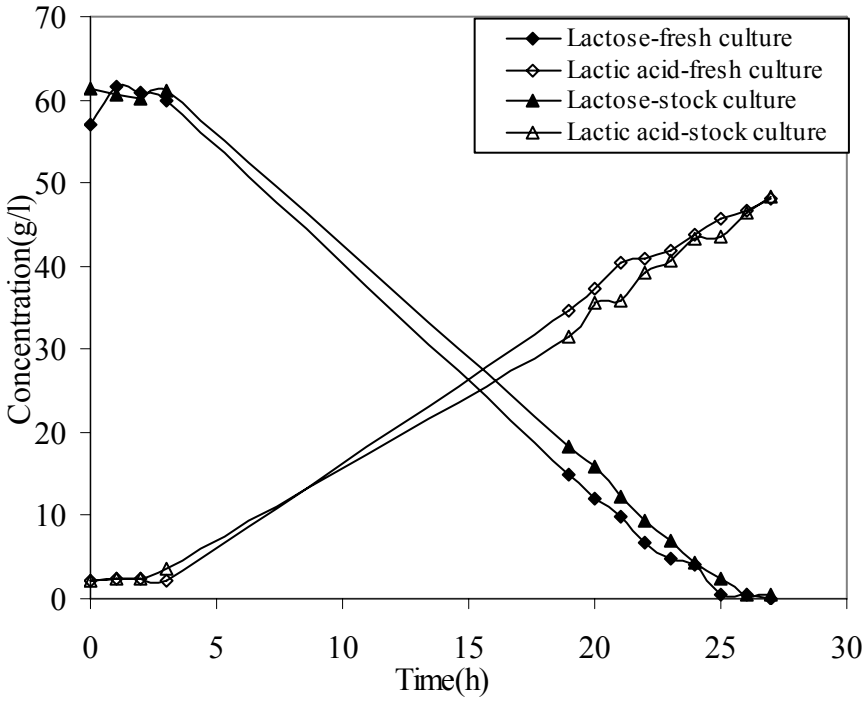

Figure 7.81. Comparison of lyophilized fresh culture with litmus milk stock culture on lactic acid fermentation (Temperature: 37°C, agitation speed: 150 rpm).

The effect of clinoptilolite rich mineral amounts in aqueous lactic acid solution and fermentation broth on the uptake of lactic acid by different zeolites were investigated. The pH change in aqueous lactic acid solution-zeolite samples and fermentation broth-zeolite samples are monitored.

When natural zeolites were transferred into ultrapure water having pH around 6.4, the pH increase was observed up to around the value of 7.4. In Figure 7.82, the results of pH profile up to the equilibration time were presented. Sampling was done by 5 minutes intervals and it was observed that natural zeolite samples reached equilibrium within 5 minutes. The explanation of this increase in pH, was explained by either the release of OH^- ions into water by natural zeolites or the exchange of H^+ ions in solution with some of the cations in natural zeolite which decreased the acidity. It was observed that the natural zeolites possessed a buffering effect inside aqueous medium.

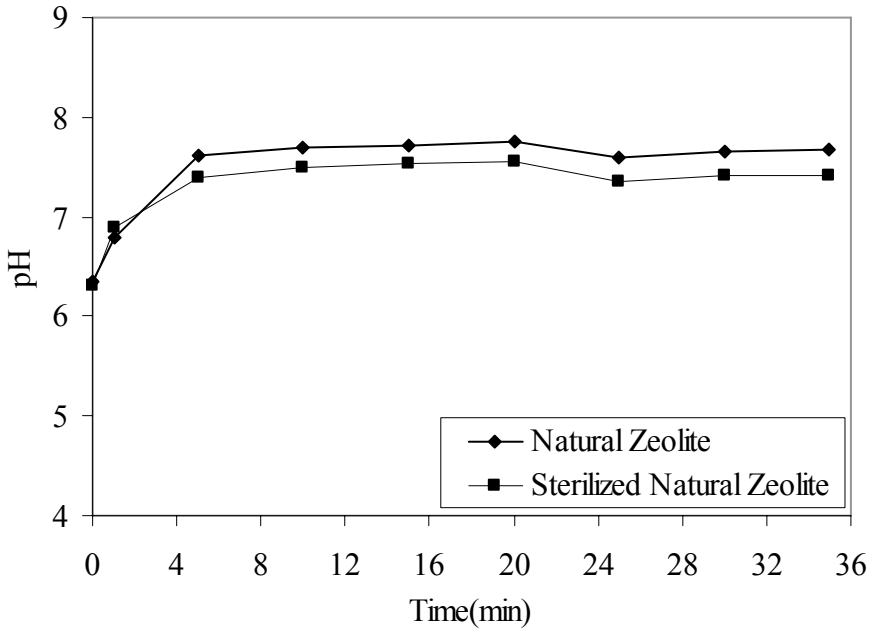

Figure 7.82. Variation of UPW pH by addition of natural zeolite and sterilized form.

7.5.1. Effect of Natural Zeolites on Fermentation pH

As a matter of fact, lactic acid production results acidic medium where the microorganism could not grow and could not produce desired product. Figure 7.83 and Figure 7.84 are given as standard operation results for lactose utilization, lactic acid production and pH variation of control fermentation experiments.

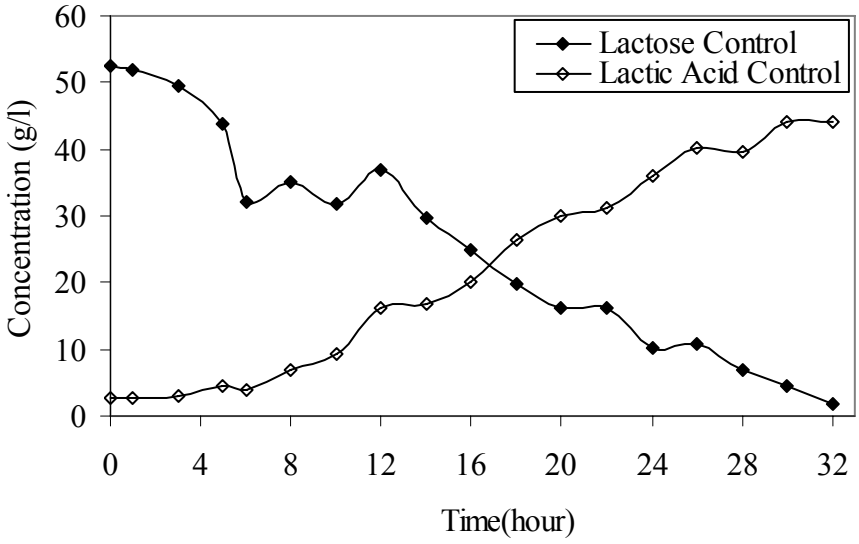

Figure 7.83. Control experiment set of lactic acid production from lactose (Temperature: 37^{o}C, agitation speed: 150 rpm).

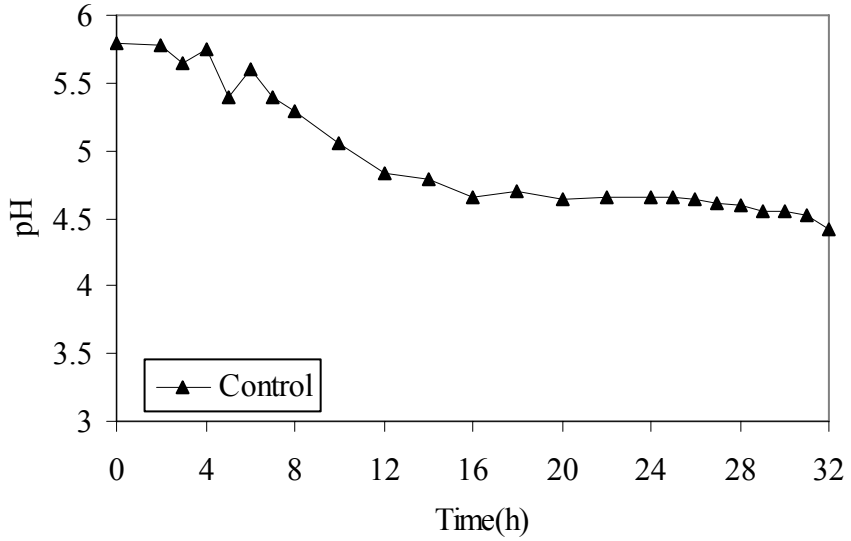

Figure 7.84. The pH profile for control experiment set of lactic acid production from lactose (Temperature: 37^{o}C, agitation speed: 150 rpm).

The utilization of natural zeolites as a pH regulator in fermentation media was studied by the treatment with different amounts of natural zeolites with and without $CaCO_3$. To see the buffering effect of natural zeolites, the use of natural zeolites during fermentation was studied. The aim was whether zeolite could act similarly as $CaCO_3$ and could provide a suitable pH range for the growth of microorganism which leads to produce lactic acid. Natural zeolites were added at different amounts, in fermentation media . All the fermentation studies were performed at 37^{o}C and 150 rpm. The pH

profiles related with the corresponding studies were given in Figure 7.86, Figure 7.88, Figure 7.90, Figure 7.92.

The results were compared with the experimental set without $CaCO_3$. The figures represented the lactose and lactic acid concentration profiles in natural zeolite containing fermentation media with and without $CaCO_3$. The data are plotted with the results of control fermentation. 3g. natural zeolite(Figure 7.85), 5g. natural zeolite (Figure 7.87), 8g. natural zeolite (Figure 7.89) and 15g. natural zeolite (Figure 7.91) additions were given. When a comparison was made between the control fermentation (Figure 7.83) and natural zeolite added fermentations, the results proved that natural zeolites did not have a negative effect on fermentation. The lactic acid production was a growth associated product. Natural zeolites did not inhibit the growth of microorganism and did not make a retardation on the production of lactic acid. The fermentation has ended in 32 hours.

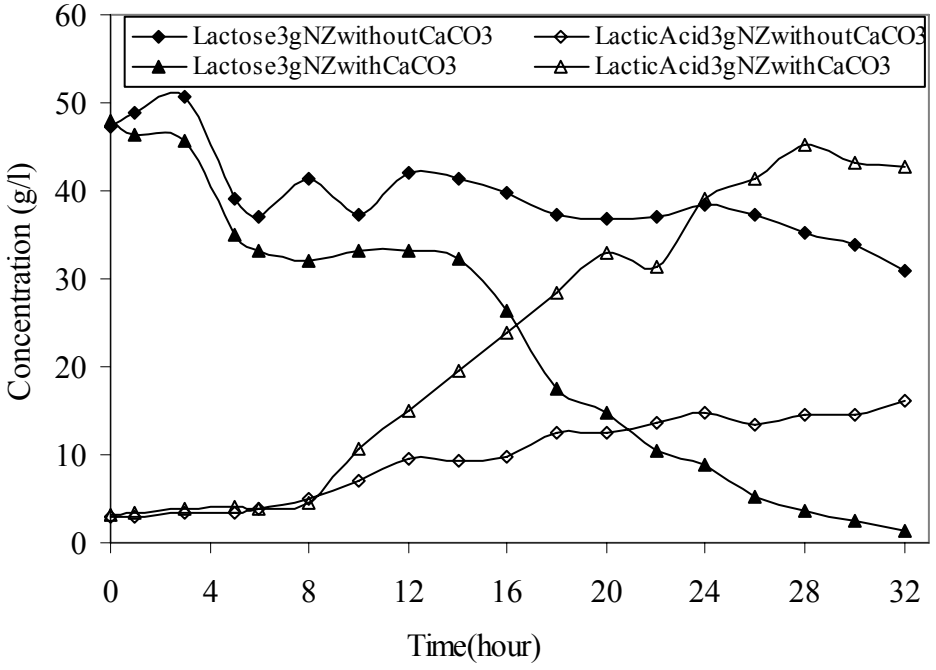

Figure 7.85. Lactic acid fermentation with and without $CaCO_3$ under the effect of natural zeolite addition (3g-NZ) (Temperature: 37^{o}C, agitation speed: 150 rpm).

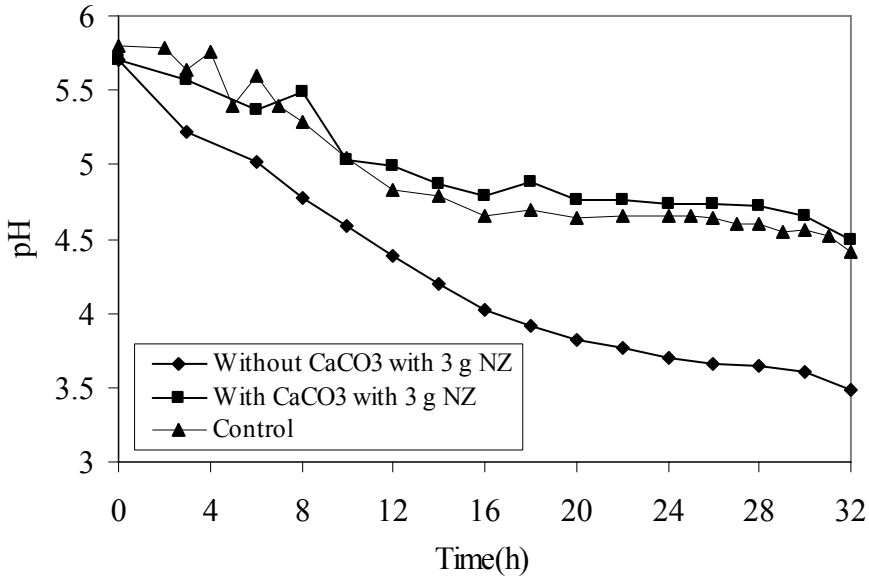

Figure 7.86. Lactic acid fermentation pH with and without $CaCO_3$ under the effect of natural zeolite addition (3g-NZ) (Temperature: 37^{o}C, agitation speed: 150 rpm).

The variation of pH during fermentation with the addition of 3g. natural zeolites in the presence and absence of $CaCO_3$ are given in Figure 7.86. It was clearly seen that when the $CaCO_3$ was not added inside the medium the pH was decreased up to pH 3.4. And the added amount of natural zeolites were insufficient to provide the similar pH value of the control set. The results showed that conversion of lactose into lactic acid without $CaCO_3$ were quite less than the results for the set of natural zeolite addition in the presence of $CaCO_3$. The difference in concentrations for the two sets was mainly attributed due to the pH of the medium. The lactic acid fermentation could be performed around pH 5 and below that value the reaction was pH limited. On the other hand the pH value could be maintained for ethanol production by yeast fermentation around 3.7-3.8 which led the increase in productivity. The lower pH tolerance of yeast fermentation was the main reason between the two processes.

The subsequent figures, from Figure 7.87 to Figure 7.92 were belonging the studies with higher amounts of natural zeolite addition. Even the amount was increased, conversion of lactose to lactic acid was not improved.

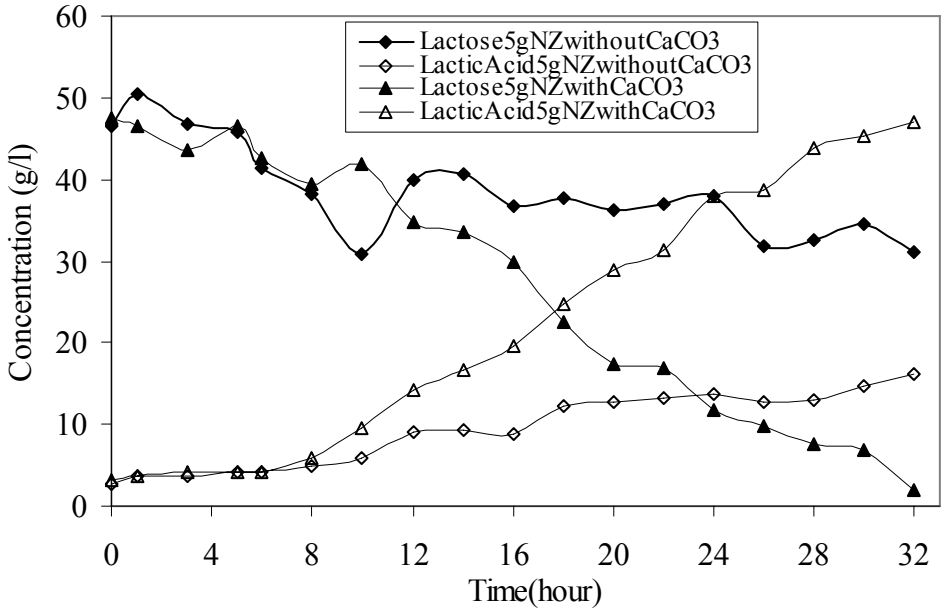

Figure 7.87 Lactic acid fermentation with and without $CaCO_3$ under the effect of natural zeolite addition (5g-NZ) (Temperature: 37^{o}C, agitation speed: 150 rpm).

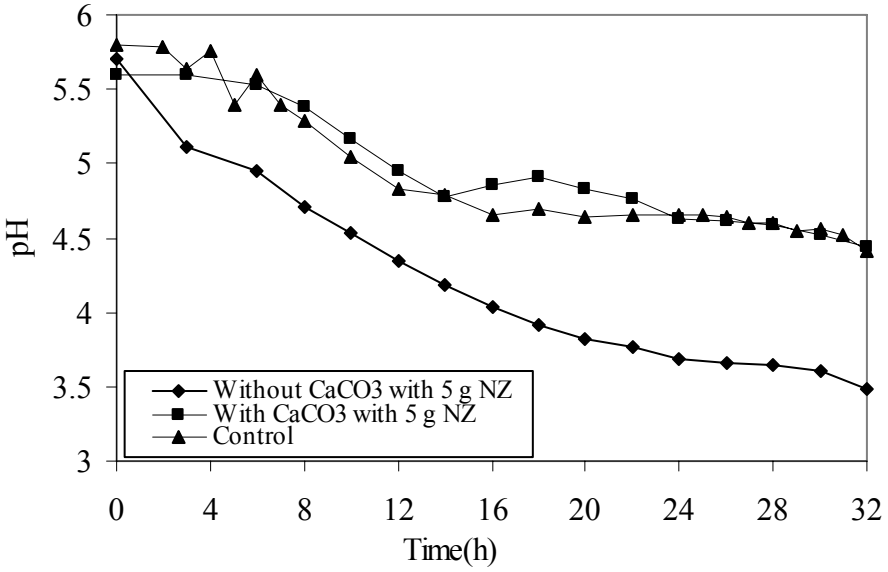

Figure 7.88. Lactic acid fermentation pH with and without $CaCO_3$ under the effect of natural zeolite addition (5g-NZ) (Temperature: 37^{o}C, agitation speed: 150 rpm).

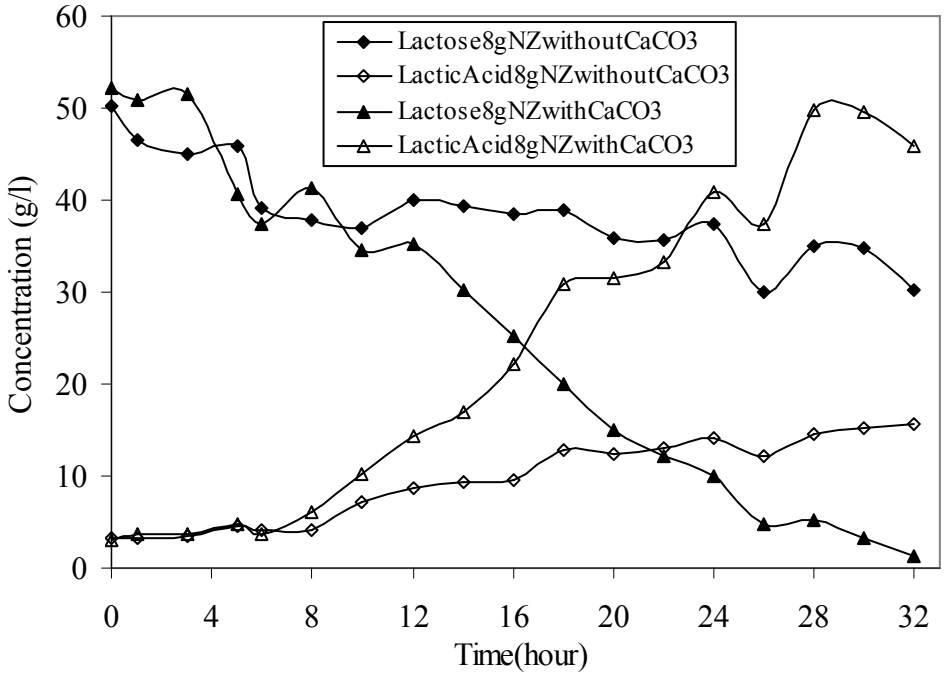

Figure 7.89. Lactic acid fermentation with and without $CaCO_3$ under the effect of natural zeolite addition (8g-NZ) (Temperature: 37°C, agitation speed: 150 rpm).

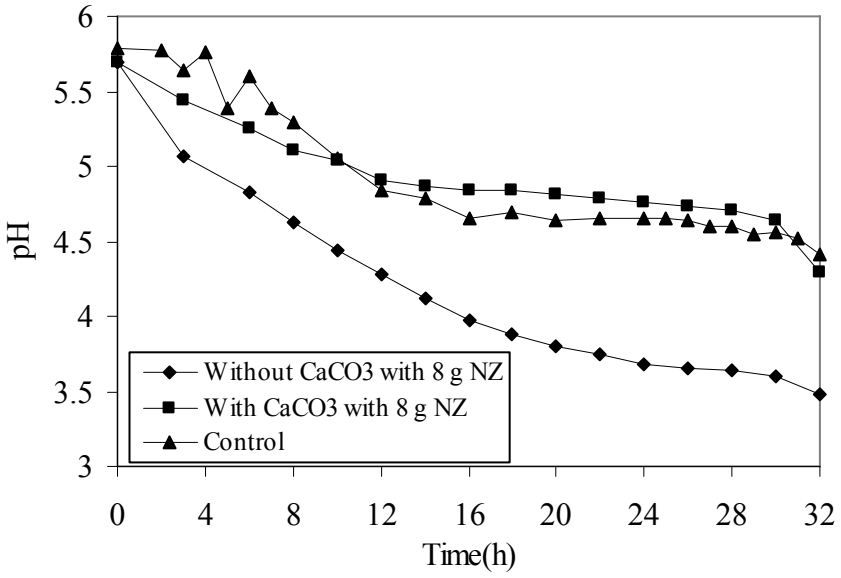

Figure 7.90. Lactic acid fermentation pH with and without $CaCO_3$ under the effect of natural zeolite addition (8g-NZ) (Temperature: 37°C, agitation speed: 150 rpm).

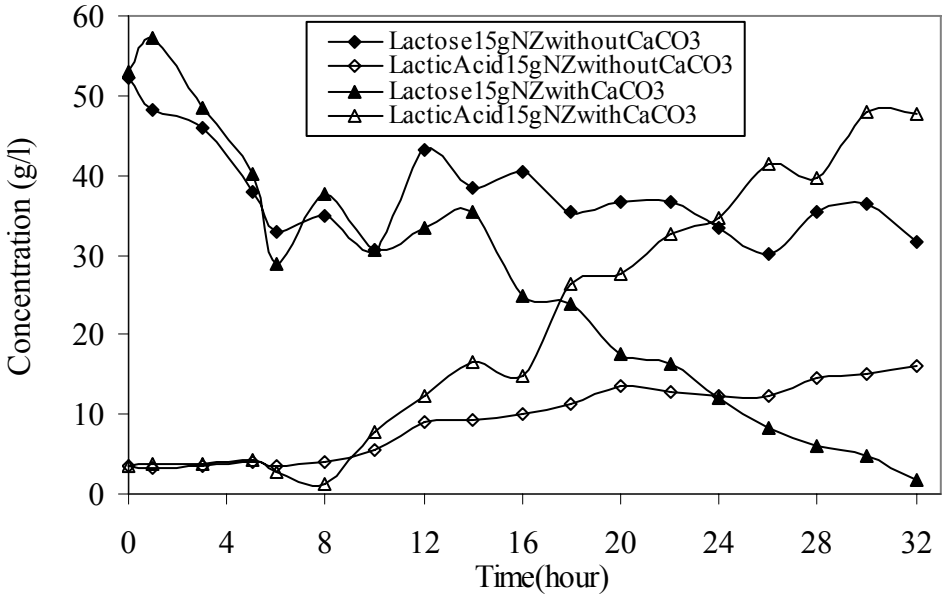

Figure 7.91 Lactic acid fermentation with and without $CaCO_3$ under the effect of natural zeolite addition (15g-NZ) (Temperature: 37°C, agitation speed: 150 rpm).

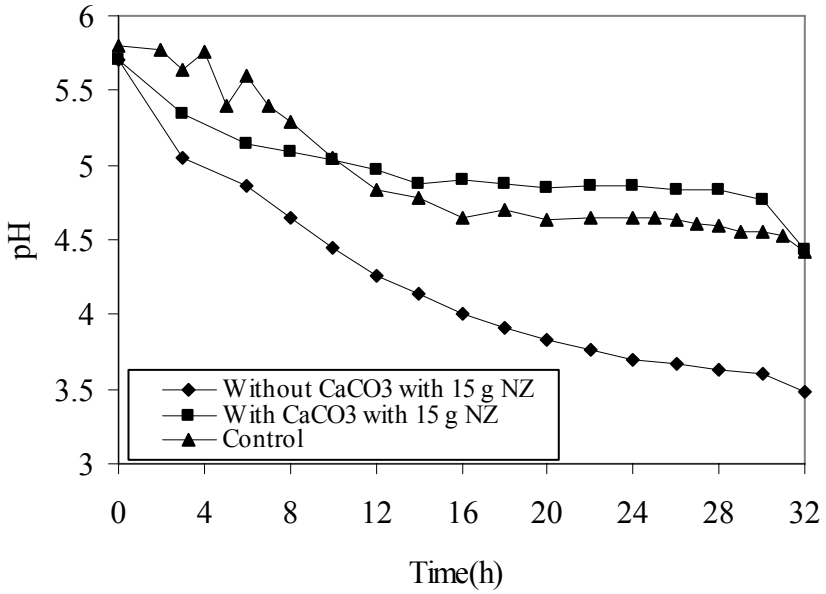

Figure 7.92 Lactic acid fermentation pH with and without $CaCO_3$ under the effect of natural zeolite addition (15g-NZ) (Temperature: 37°C, agitation speed: 150 rpm).

At the end of 32 h for the fermentation period, lactose in control fermentation was consumed and the lactose and lactic acid concentrations reached a plateau. On the other hand, for natural zeolite added samples, low amount of lactose consumption was observed. Besides, produced lactic acid concentrations at the end of the fermentation for

natural zeolite added samples were similar independent of the varying zeolite amounts. The pH range was around 3.5 whereas it was needed to be around pH 5 ±0.5.

The pH buffering effect of natural zeolites were not significant for regulating lactic acid fermentation pH. The usage of the Na-exchanged natural zeolites in fermentation media was investigated. By increasing the Na^+ ion content on natural zeolites, the fermentation study was performed. In Figure 7.100, the results of lactose concentration variations were belonging to the experiment performed with a control set that was containing $CaCO_3$. The other three sets were; one of them containing natural zeolite(NZ) but not containing $CaCO_3$, one of them was containing Na-exchanged natural zeolite but not $CaCO_3$ and the latter was neither containing natural zeolite nor $CaCO_3$. It was observed that if there was not $CaCO_3$ addition, lactose utilization and lactic acid production did not proceed as in Figure 7.93 and Figure 7.94. No matter the usage of natural zeolite containing higher Na amount, the amount was insufficient to proceed. Because Na- exchange in natural zeolites is balanced with the cation composition of itself before exchange, and the exchange capacity is low for fermentation requirement even the exchange process was performed at its maximum capacity level.

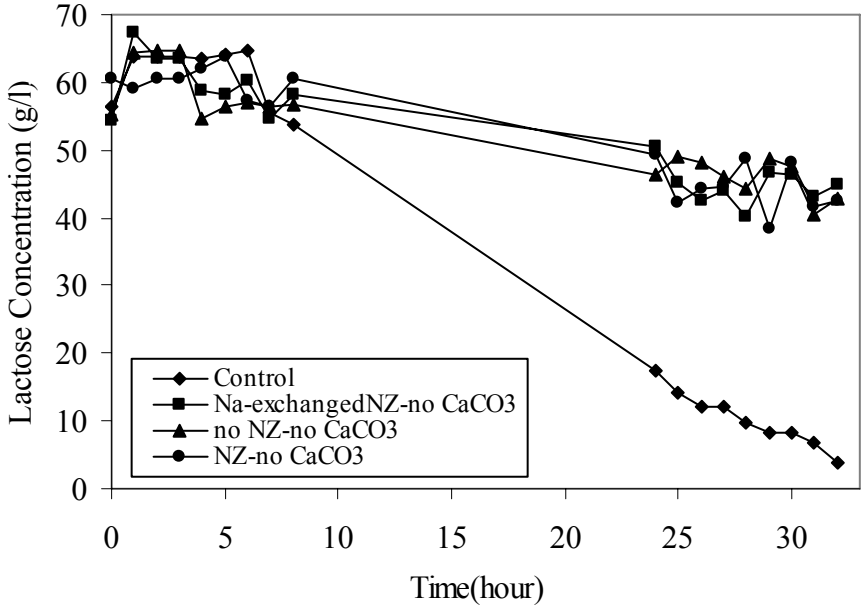

Figure 7.93. Lactose concentration profile of Na-exchanged natural zeolite and natural zeolite without $CaCO_3$ during ferrmentation (Temperature: 37^{o}C, agitation speed: 150 rpm).

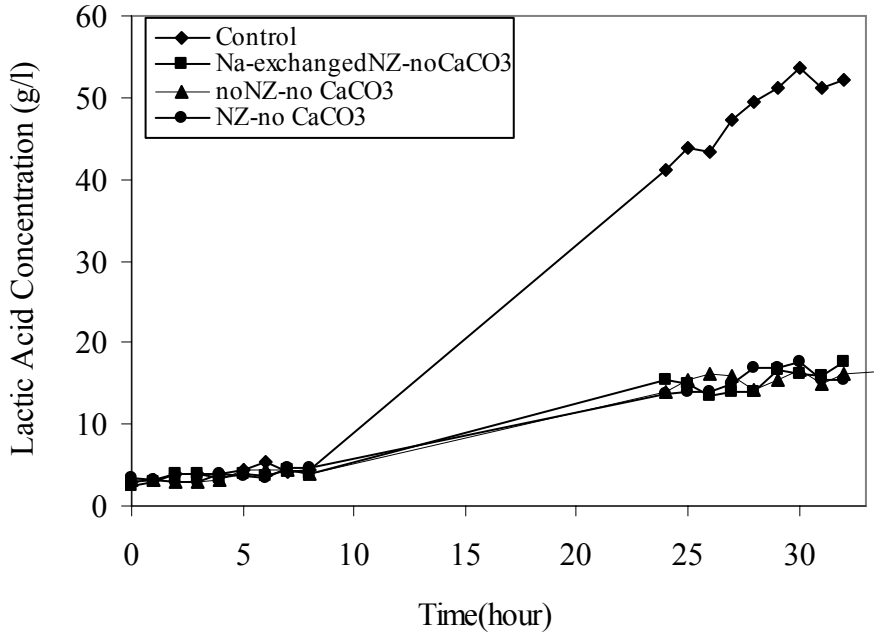

Figure 7.94. Lactic acid production in the presence of Na-exchanged natural zeolite and natural zeolite without $CaCO_3$ during fermentation (Temperature: 37^{o}C, agitation speed: 150 rpm).

7.5.2 Effect of Natural Zeolites as Elemental Source in Fermentation

Utilization of mineral salts such as KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, $FeSO_4.7H_2O$ and $MnSO_4.4H_2O$ were found to be required at different levels for the microorganism to grow and to produce the desired product. The study was carried on, to see the effect of natural zeolites in fermentation process without the addition of mineral salts due to the rich elemental composition of natural zeolites and their release properties. The experimental results are given in Figure 7.95 and 7.96. In those figures the compositions are given as; the first one containing all mineral salts (Control). The second one without mineral salts, is denoted as closed square (C-mineral). From the natural zeolite perspective, in order to compare with the second one the third one was containing 5g of natural zeolite but not any additional mineral salts, is depicted as closed triangle (C-min+zeo5). For the fourth data set, addition of all mineral salts except manganase (Mn) was studied (C-Mn). For the last one 5 g natural zeolite was inside fermentation media with all mineral salts except Mn (C-Mn+zeo5). The results showed that there is release of cations especially potassium and manganase by natural zeolite and the effect of Mn is quite significant for fermentation.

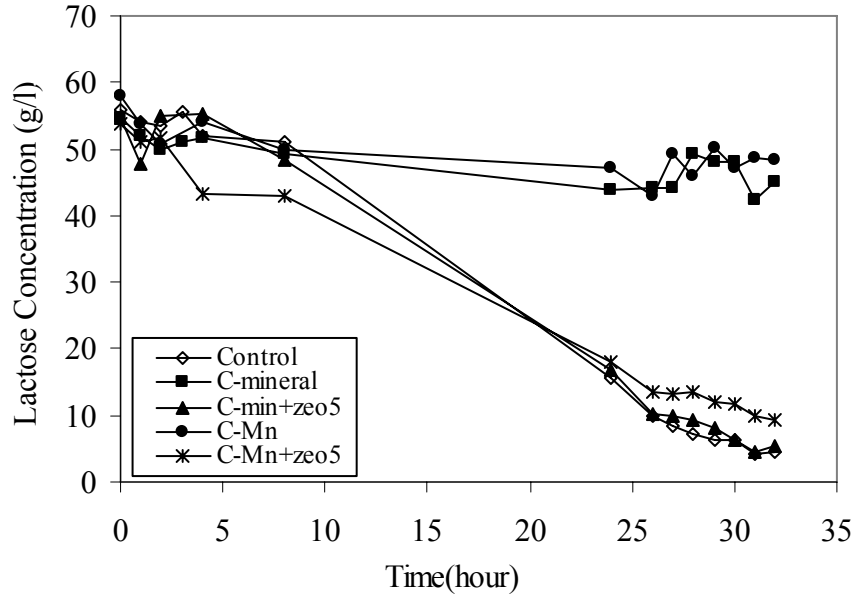

Figure 7.95. The Lactose concentration change during fermentation (Temperature: 37°C, agitation speed: 150 rpm).

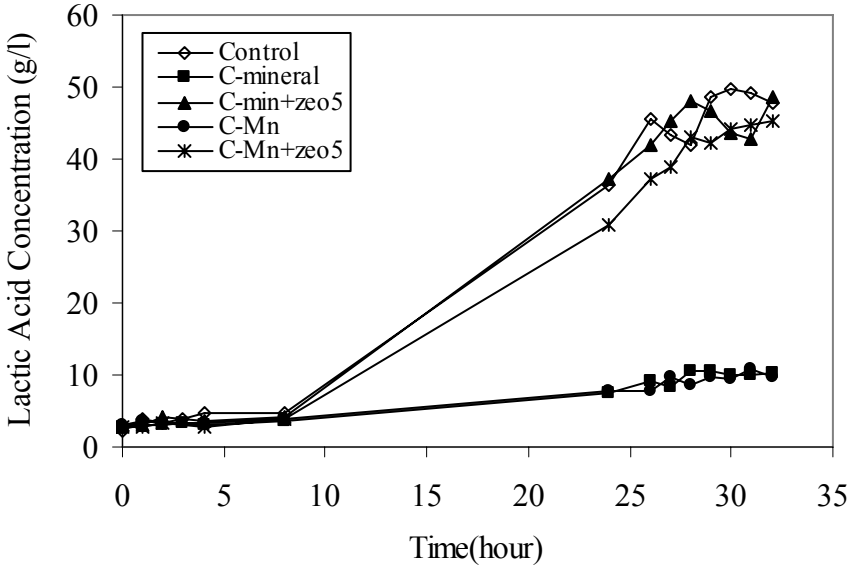

Figure 7.96. Lactic Acid concentration profile during fermentation (Temperature: 37°C, agitation speed: 150 rpm).

It was observed that 5 g natural zeolite could release needed Mn supplement amount for control set. The values of the control fermentation were similar with the zeolite added fermentation. On the other hand, when there was not Mn addition and there was not natural zeolite in fermentation media, microorganism could not consume lactose; and produce lactic acid. Mn was the most important element for the process

(Figure 7.95 and Figure 7.96). 5g of natural zeolites(NZ) were enough to be used as Mn source.

In Figure 7.97 the requirement to Mn by the microorganism during whole process was established. The results verified that natural zeolite can be a good Mn source for the microorganism to produce lactic acid. The pH profile of the fermentation study was observed and the results are plotted in Figure 7.98.

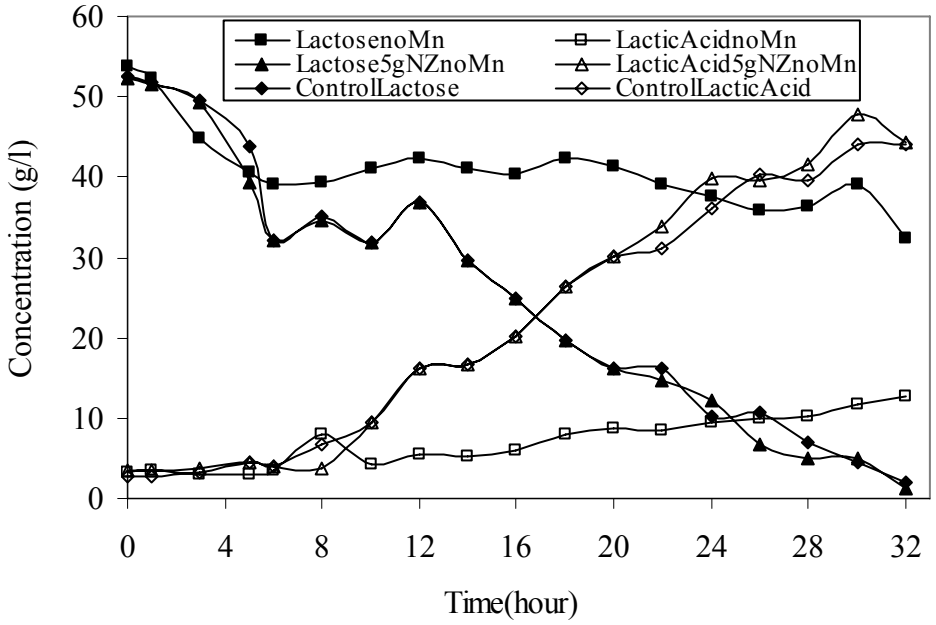

Figure 7.97. The effect of zeolite addition on Lactic acid fermentation without Mn (Temperature: 37^{o}C, agitation speed: 150 rpm).

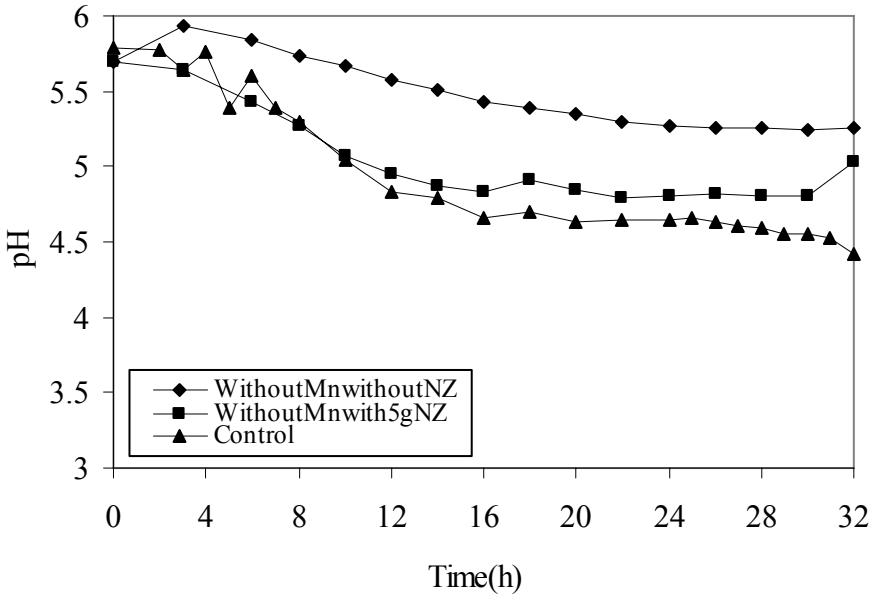

Figure 7.98. The Effect of Zeolite Addition on the pH of Lactic Acid Fermentation without Mn (Temperature: 37^{o}C, agitation speed: 150 rpm).

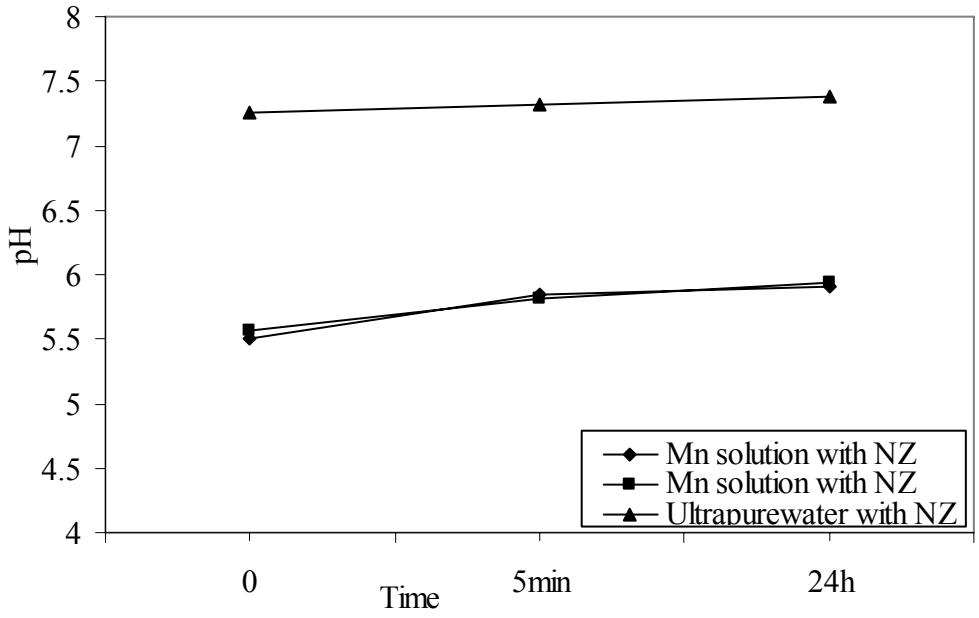

Figure 7.99. The pH profile for the Mn exchange process of natural zeolites

In order to see the effect of natural zeolites having higher Mn content, during fermentation study, natural zeolites were exchanged with manganase solution resulting an increase on its Mn content. The pH of the solution was measured at the beginning and at the end of the process (Figure 7.99). The pH of the natural zeolite in ultrapure water was also measured to make a comparison. The Mn content of natural zeolite is

0.23 mg/g natural zeolite and after the exchange process it was measured as 4.88 mg/g natural zeolite.

Fermentation with natural zeolites and Mn-exchanged zeolites were performed. The lactose and lactic acid concentrations during the process were monitored. The results are plotted in Figure 7.100. The pH profile for the fermentation process were recorded by monitoring under aseptic conditions. As it is seen in Figure 7.101 the pH of the media with Mn exchanged zeolites were quite smaller than the control fermentation. As it is observed in Figure 7.101, the pH of the solution, was lower with respect to natural zeolite. At the end of the fermentation process where the lactose was consumed by the microorganism, the pH of each flasks were identical. The explanation for that behavior is; in 5 g natural zeolite, Mn content was sufficient and even the increase in concentration does not make much difference.

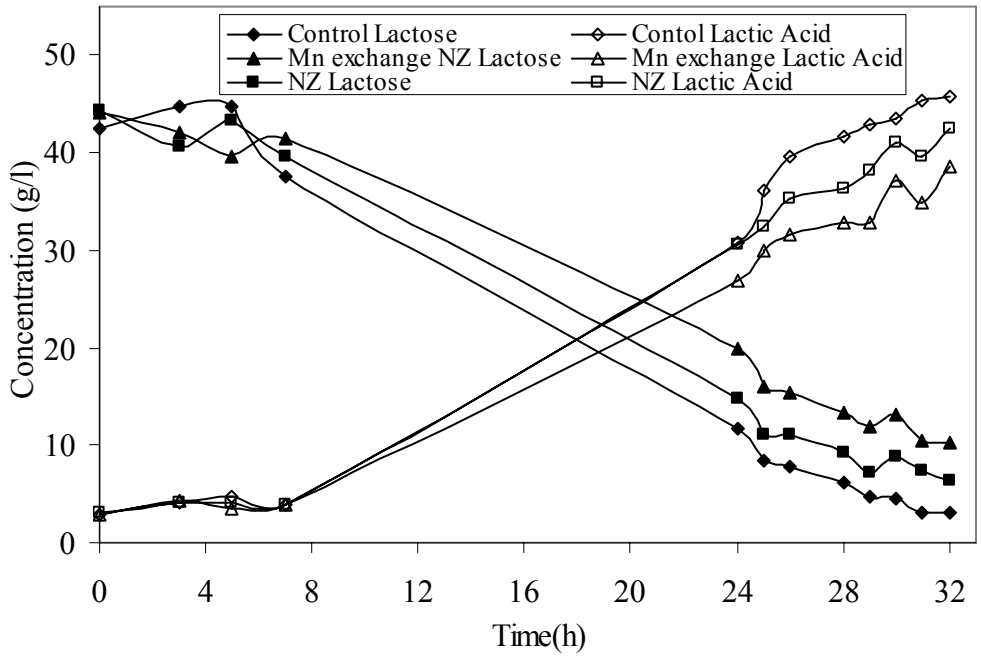

Figure 7.100. Lactose and Lactic Acid concentrations for the fermentation process performed with Mn exchanged natural zeolites and natural zeolites(NZ) (Temperature: 37^{o}C, agitation speed: 150 rpm).

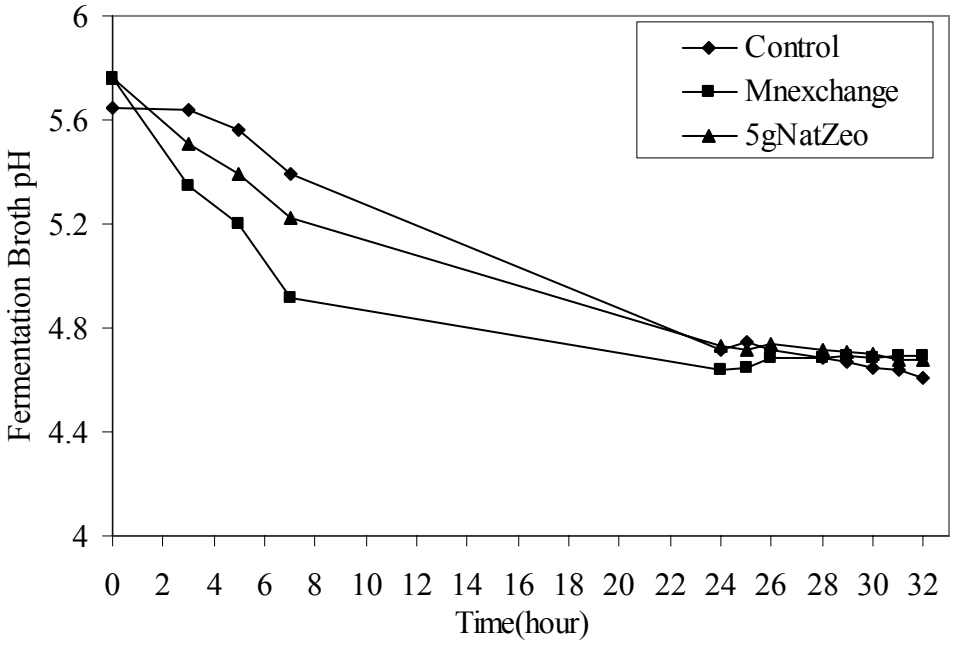

Figure 7.101. The pH profile for the fermentation process performed with Mn exchanged natural zeolites and natural zeolites (Temperature: 37^{o}C, agitation speed: 150 rpm).

7.5.3 Behavior of Natural Zeolites in Lactic Acid Medium

The adsorption of lactic acid in fermentation media and in aqueous solution by natural zeolites, dealuminated zeolites synthetic zeolite ZSM-5 were investigated at 37^{o}C, 150 rpm (Figure 7.104, Figure 7.105, Figure 7.106, Figure 7.107, Figure 7.108, Figure 7.109.) In addition, the pH of the zeolite in aqueous lactic acid solution was monitored for 26 hours (Figure 7.102, Figure 7.103, Figure 7.110).

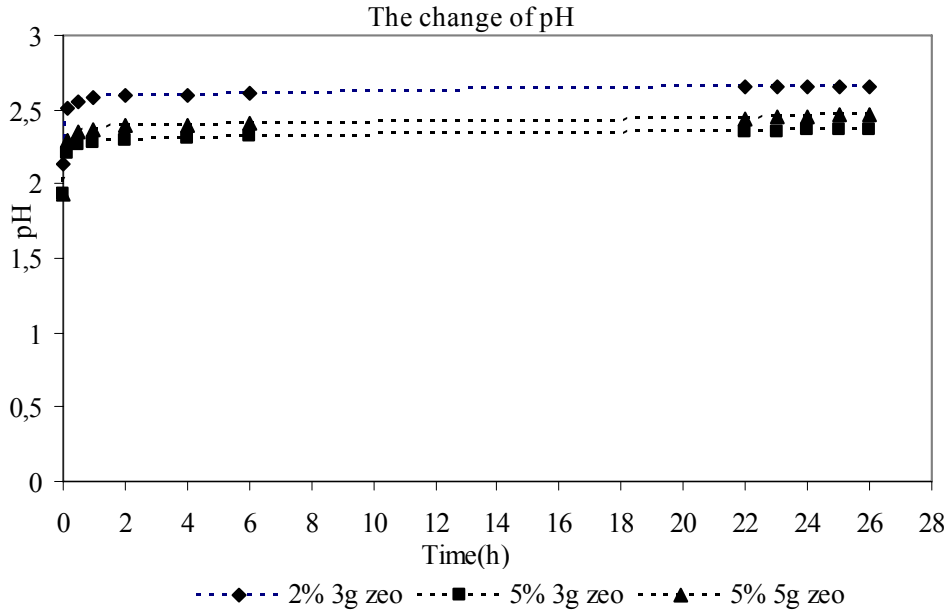

Figure 7.102 The change of pH in natural zeolite-aqueous lactic acid solution (Temperature: 37°C, agitation speed: 150 rpm).

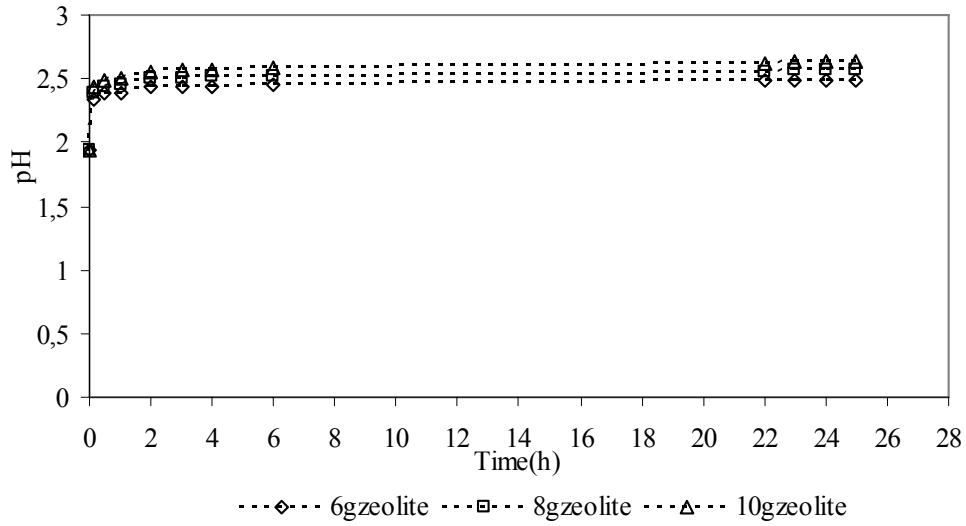

Figure 7.103 The Change of pH in Zeolite-Lactic Acid Solution (Temperature: 37°C, agitation speed: 150 rpm).

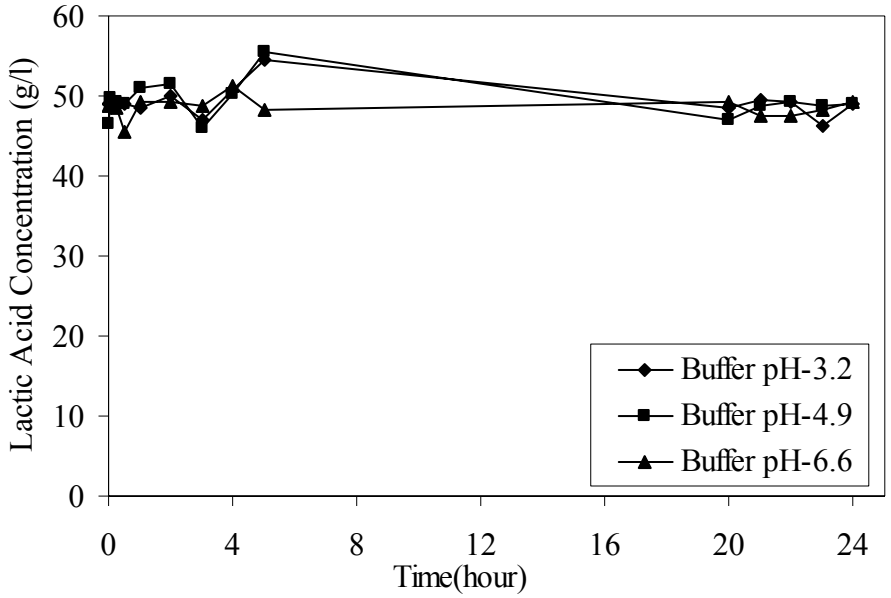

Figure 7.104. Lactic acid concentration profile in the presence of natural zeolites (Temperature: 37^{o}C, agitation speed: 150 rpm).

Aljundi et al., studied the recovery of lactic acid from fermentation broth and aqueous solutions by using Silicalite molecular sieves. The adsorbent was denoted as having low affinity to water (Aljundi et al., 2005) The acid treated samples were investigated for the adsorption of lactic acid solution. Acid treatment results the removal of Al ions in natural zeolite structure. Thus the Si/Al ratio of structure increases and the material gains a more hydrophobic character.

Figure 7.105 shows that there was not adsorption of lactic acid onto acid modified natural zeolites. The experiment was repeated with modified zeolites having higher Si/Al ratio. The results showed that there was not adsorption of lactic acid.

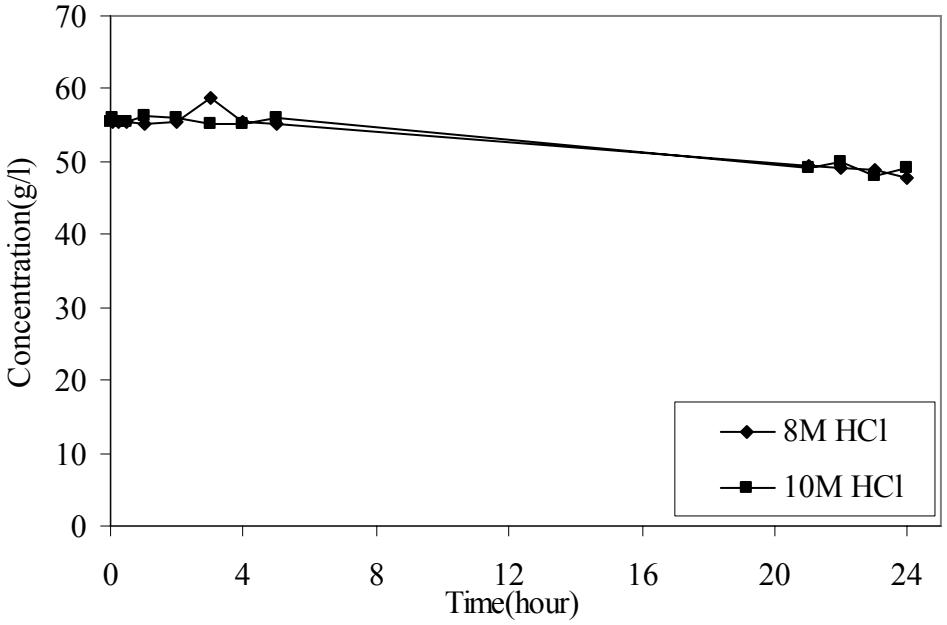

Figure 7.105. Lactic acid concentration variation with dealuminated natural zeolite (Temperature: 37°C, agitation speed: 150 rpm).

As the natural zeolites show hydrophilic character, an experimental study was performed with a hydrophobic adorbent which is synthetic zeolite ZSM-5 in order to make a comparison.

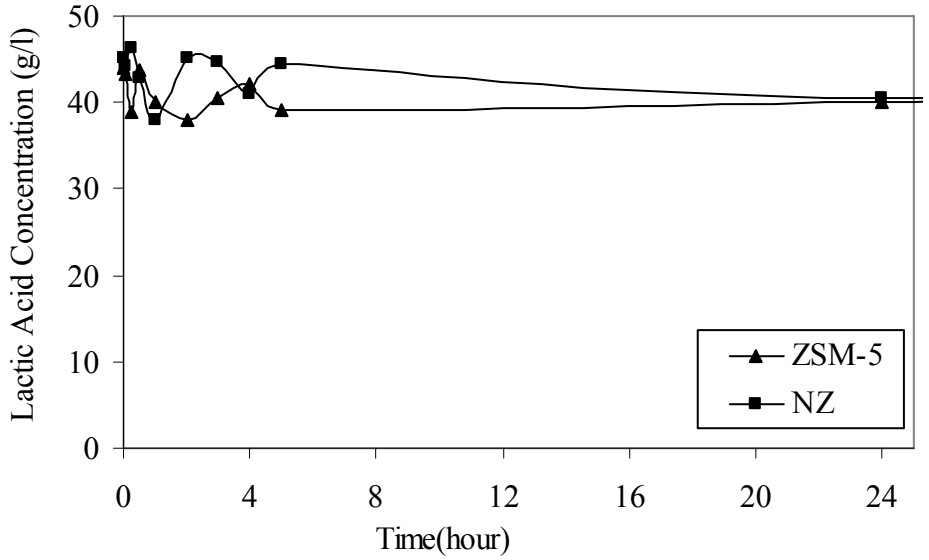

Figure 7.106. Adsorption kinetics study of aqueous lactic acid solution with ZSM-5(synthetic zeolite), and natural zeolite(NZ) (Temperature: 37°C, agitation speed: 150 rpm).

The concentration profile of aqueous lactic acid solution during the treatment with natural zeolites (NZ) and synthetic zeolite (ZSM-5) were given in Figure 7.106. The results were quite similar both for the natural zeolites and the synthetic zeolite ZSM-5.

The experiment was performed with the supernatant of fermentation broth for the adsorption study (Figure 7.107). The studies performed with aqueous lactic acid solution and cell free fermentation broth were compared. There was not any decrease in concentrations.

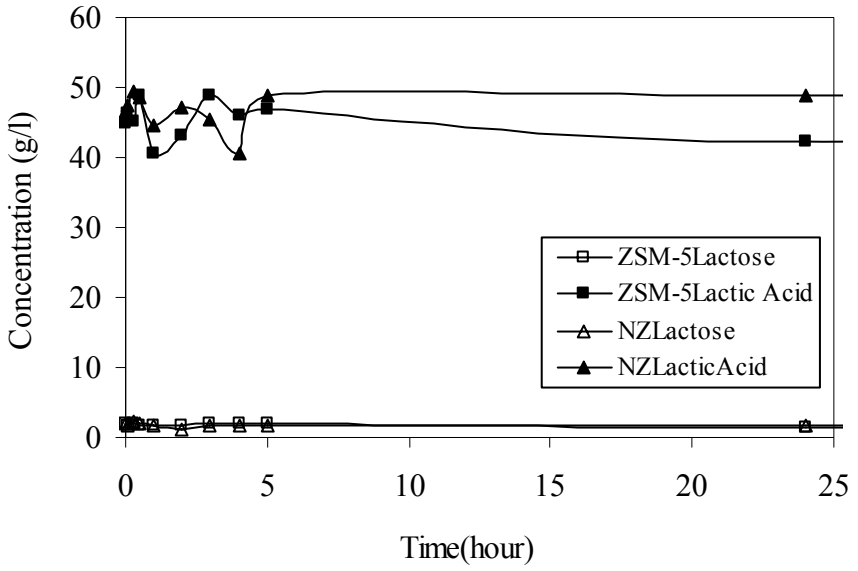

Figure 7.107. Adsorption kinetics study of cell free fermentation broth with ZSM-5(synthetic zeolite), and natural zeolite(NZ) (Temperature: 37^{o}C, agitation speed: 150 rpm).

The results of treatments with aqueous lactic acid solution and cell free fermentation broth were similar. In that case, the behavior of natural zeolite(NZ) and synthetic zeolite(ZSM-5) during fermentation was investigated (Figure 7.108)

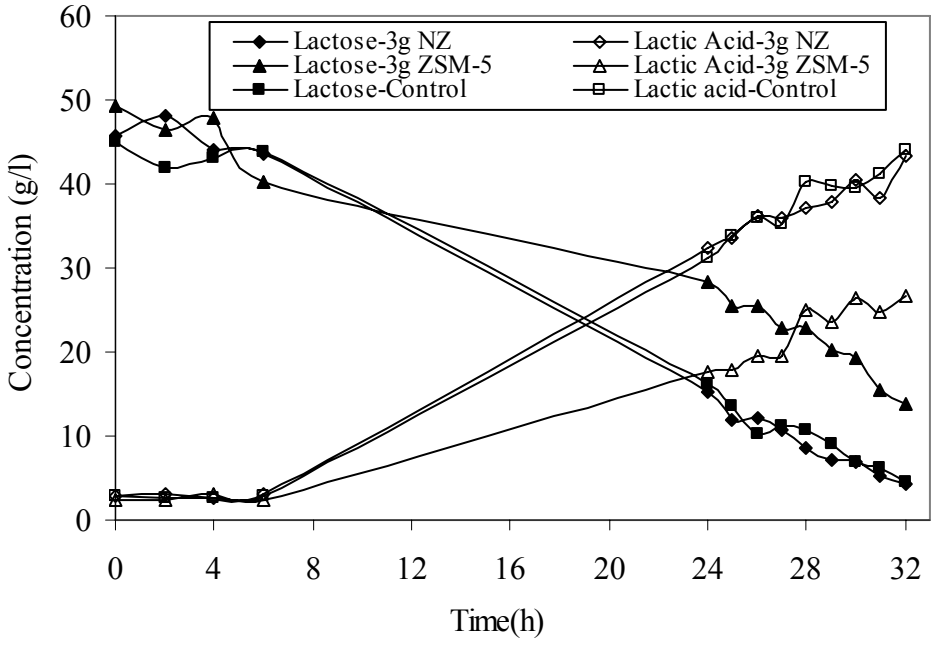

Figure 7.108. Fermentation process with natural zeolite (NZ) and synthetic zeolite (ZSM-5) (Temperature: 37^{o}C, agitation speed: 150 rpm).

It was seen that fermentation with natural zeolite and control fermentation were proceeding similarly whereas the addition of synthetic zeolite extended the process time. At the end of 32 hours the lactic acid concentration of synthetic zeolite addition was almost half of the control fermentation. The pH of the each batch was measured and presented in Figure 7.109. The values were very close with each other. The pH of the synthetic zeolite sample was slightly higher than the control set.

Sythetic zeolite ZSM-5 has a Si/Al ratio of 90. As the Si/Al ratio increase the material gains more hydrophobic character. In addition to that when the hydrophobicity increases, the adsorbent shows a tendency towards the chemical in aqueous solutions instead of water. However, according to the experimental results in aqueous solutions and fermentation studies, adsorption with synthetic zeolite ZSM-5 which is more hydrophobic than natural zeolites was not succesfull.

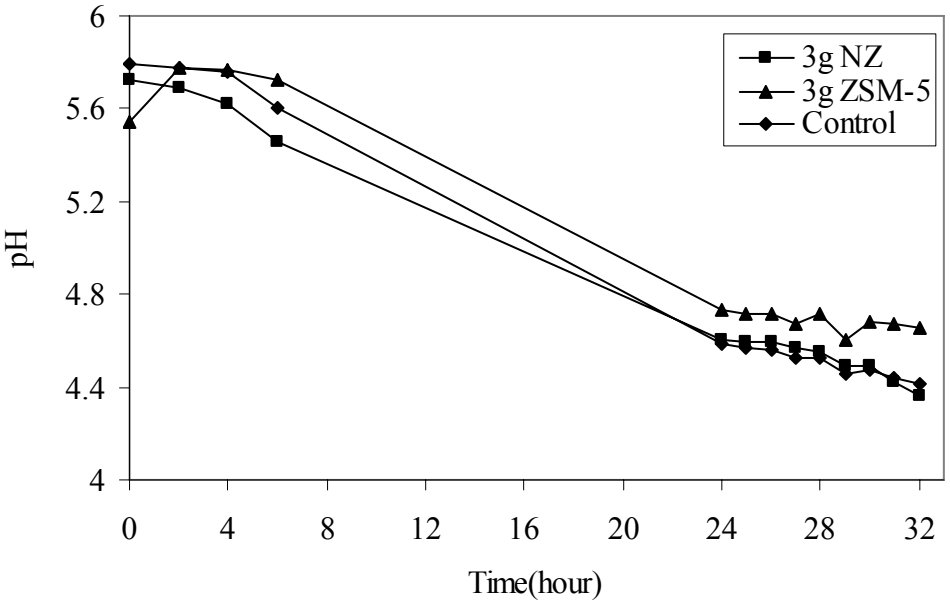

Figure 7.109. The pH profile of fermentation process with natural zeolite(NZ) and synthetic zeolite(ZSM-5) (Temperature: 37^{o}C, agitation speed: 150 rpm).

CHAPTER 8

CONCLUSION

In this study, an alternative solution for the discharge of whey which is the main by-product of dairy industry, by using natural zeolites as adsorbent material and mineral source was proposed. Whey is made of mainly high amount of lactose, valuable whey proteins and cations. The process was made up of two steps. The first part was the adsorption of whey proteins by natural zeolites, and subsequently transferring the residual supernatant for fermentation so as to convert lactose into lactic acid.

The deposits of natural zeolites from Gördes region having particle size between 75-150 μm was used as adsorbent in this study. Characterization techniques were applied to determine the structural characteristics of clinoptilolite rich local mineral.

The whey powder that was being used as carbon source for fermentation studies contains valuable whey proteins, namely β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La), Bovine Serum Albumin (BSA), Immunoglobulins, Glycomacropeptide and Lactoferrin. β-Lg, α-La and BSA were constituting 80 % of the whey proteins. The adsorption of whey proteins onto natural zeolite was studied. Adsorption equilibrium and adsorption kinetics studies were performed for BSA aqueous solution, whey powder solution and whey. Several parameters have been investigated. The effect of particle size and the effect of agitation speed had significant effect both on the time to reach equilibrium and equilibrium adsorbed amount. Besides, the results showed that providing a positive charge by adjusting the pH of the solution below the isoelectric point of proteins, presents fast adsorption. The maximum adsorption between opposite charges is the result of Coulombic forces. In addition to that as the experimental time period was extended, sharp decrease in figures of protein concentration was observed. The behavior was valid for all pH values and blank runs. When the blank runs were compared, the solution prepared in ultrapure water showed the maximum stability. In conclusion, the conformational changes of proteins with respect to the incubation time should be considered well in order to explain the mechanism. On the other hand, the effect of temperature was small. The maximum adsorption capacities for Langmuir isotherm were calculated as 35.59 (mg protein/g NZ) for 15^{o}C and 38.76 (mg protein/g NZ) for

25^{o}C. The adsorption kinetics study was performed to determine the rate controlling mechanism during adsorption. The results showed that the system was controlled more than one mechanism. External film diffusion, solid diffusion and surface reaction were the rate determining steps. Because of the small pores compared to the dimensions of protein molecules, pore diffusion was neglected. The whey protein adsorption ability of modified zeolites was investigated. Interestingly, increase in the hydrophobic character decreased the equilibration time but decreased the equilibrium adsorbed amount as well. The presence of Al^{+3} ions were ascribed to act an important role during protein adsorption. The whey powder solution after protein adsorption step was sent to fermentation without additional elements oppositely in conventional technique. The results clearly showed that fermentation was completed similarly as the control set. The proposition of the thesis was realized by those results. Additionally, desorption of proteins were also studied. NaCl and water were not good eluents for desorption step, where as the buffer solution having the highest pH value which was 8.9 gave the highest desorbed amount. Desorption was rapid which was completed within 1 hour. The effect of washing on whey proteins desorption was investigated. Comparison between the samples eluted after washing step and without washing step was made. The difference in desorbed protein concentrations between the two sets was about two fold.

The buffering effect of natural zeolite is common in studies throughout the literature. The pH profile of natural zeolites in ultrapure water presents us a comment about the tendency of natural zeolites to behave as a proton acceptor. In this case the H^{+} ions present in the aqueous solution.

Furthermore, the modification of natural zeolites in order to increase the Si/Al ratio which would increase the hydrophobicity was performed with 1 M HCl, 5 M HCl, 8 M HCl and 10 M HCl. Although, the Si/Al ratio was increased to 18.52 by (10 M HCl) acid treated samples while it was 5.26 for natural zeolites, the X-ray diffraction patterns of these dealuminated samples showed significant change of the intensities and places of the characteristic clinoptilolite peaks.

In addition to the aim of the thesis, the adsorption of lactic acid from fermentation broth and aqueous solutions by natural zeolites was investigated. The kinetics of lactic acid adsorption with natural zeolites in a batch system was studied during fermentation, in aqueous lactic acid solutions and in cell free fermetation broth at the end of the fermentation process. The production of lactic acid from whey was carried out at 37 ^{o}C, pH 5±0.5 using *Lactobacillus casei* NRRL B-441. At the end of

fermentation about 50 g/l lactic acid was produced within 32 hours. L(+) lactic acid was present inside the broth. Unfortunately we did not observe the affinity of adsorption by natural zeolites towards lactic acid. However, the rich cation composition of natural zeolites served as a mineral source especially from the manganese perspective. The presence of natural zeolites during fermentation could be a good alternative as a mineral source for the microorganism. During lactic acid production, 4 % (w/v) of natural zeolites were sufficient to supply the requirement of the previously determined fermentation conditions which was 50 ppm. Moreover, the candidacy of natural zeolites as a pH regulator during lactic acid fermentation was investigated. The conventional process was being performed with $CaCO_3$ to provide media pH 5±0.5. On the other hand, when natural zeolites were added as a replacement of $CaCO_3$, the conversion of lactose to lactic acid was diminished significantly. The pH value of media was decreased to 3.4 which was causing an inhibition to proceed the lactic acid fermentation. Natural zeolites are not suitable as a pH regulator agent during lactic acid fermentation.

The adsorption of lactic acid by modified natural zeolites was studied with 50 g/l initial lactic acid concentration. The results showed us that there was no change in lactic acid concentration even the Si/Al ratio was increased. The Si/Al ratio is not the only limitation point for lactic acid adsorption as it was clearly observed in the results of synthetic zeolite ZSM-5 where the Si/Al ratio was 90.

The element composition in whey powder and natural zeolite were also tested by ICP, and it is observed that there is exchange of ions between the solution and natural zeolite. The change of element composition of whey in the presence and absence of natural zeolites provides a strong evidence both for adsorption and desorption of the major and minor elements.

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APPENDICES

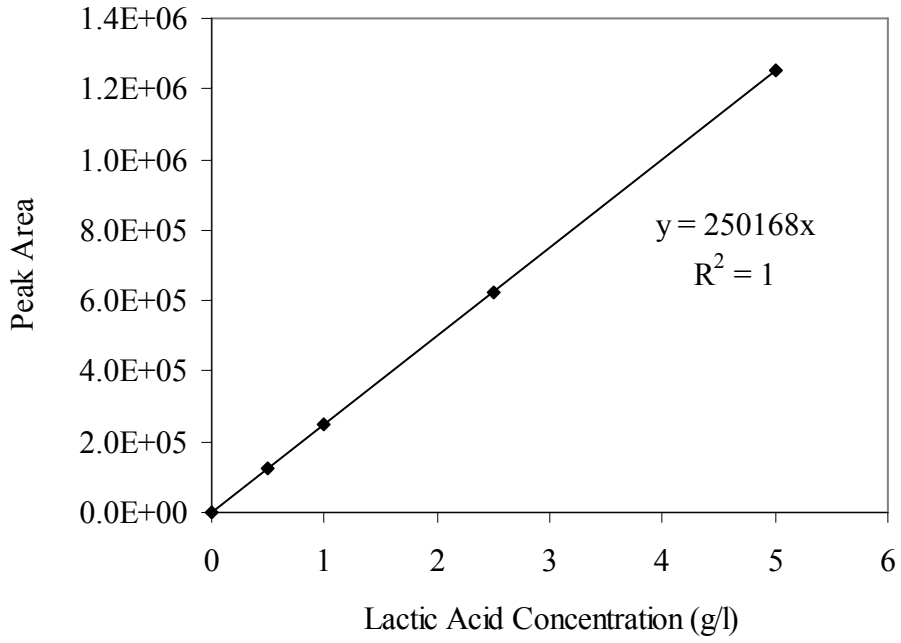
APPENDIX A. BASIC PROPERTIES OF CHEMICALS

Table A1. Chemicals Used During Study.

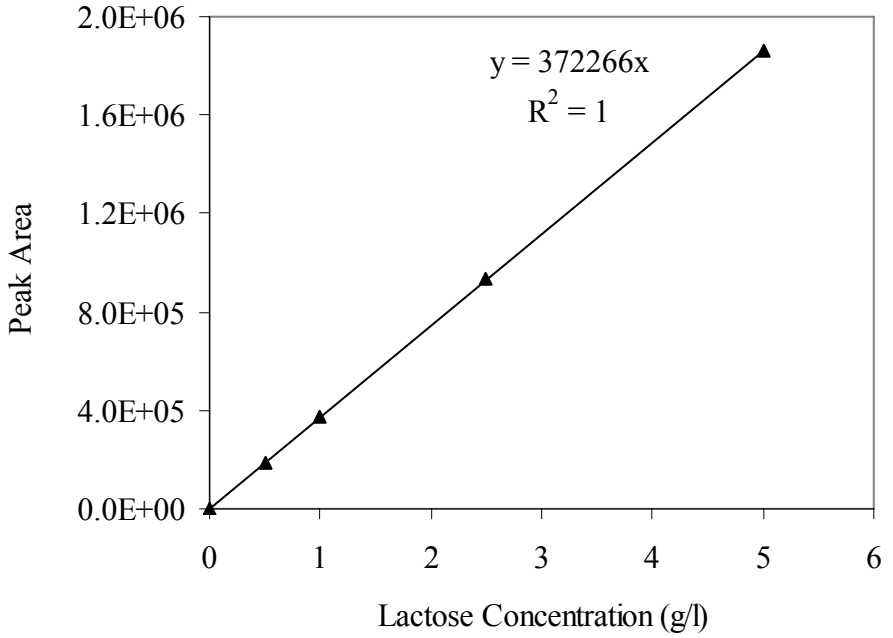
Chemical	**Features**	**Source**
L(+)-Lactic acid, 30 %, d=1.21 kg/cm^3	FW: 90.08 g/mole,	Sigma
α-Lactose monohydrate	FW: 360.3 g/mole	Sigma
Calcium carbonate, >99%	FW: 100.09 g/mole	Fluka
Sodium Hydroxide, >99 %	FW: 40.00 g/mole	Sigma
Sulfuric acid, 95-98 % ,d=1.84 kg/cm^3	FW: 98.08 g/mole	Merck
Litmus Milk	pH:5-6	Difco
Magnesium sulfate anhydrous,>99 %	FW: 169.02 g/mole	Sigma
Hydrocloric acid, 37 %	FW: 36.5 g/mole	Merck
Manganese sulfate monohydrate	FW: 138 g/mole	Merck
Potassium phosphate monobasic, >99.8 %	FW: 136.09 g/mole	Sigma
Potassium phosphate dibasic, >99 %	FW: 174.18 g/mole	Sigma
Yeast extract 99.5 %		Oxoid
Zeolite ZSM-5	Si/Al= 90	SüdChem
Sodium carbonate anhydrous, 99 %	FW:105.99 g/mole	Riedel-de Häen
Folin Ciocalteus phenol reagent,	2.0 N	Sigma
Cupper sulfate pentahydrate	FW: 250 g/mole	Merck
Sodium phosphate monobasic dihydrate,>98%	FW:156.01 g/mole	Riedel-de Häen
Potassium sodium tartrate tetrahydrate, >99%	FW: 282.23 g/mole	Fluka
ICP Standard Solutions	Multielement, Si	Merck
Albumin fraction V	from bovine serum	Merck
β-Lactoglobulin	from bovine milk	Sigma
α-Lactalbumin, approx.85 %	from bovine milk	Sigma

APPENDIX B. CALIBRATION CURVES

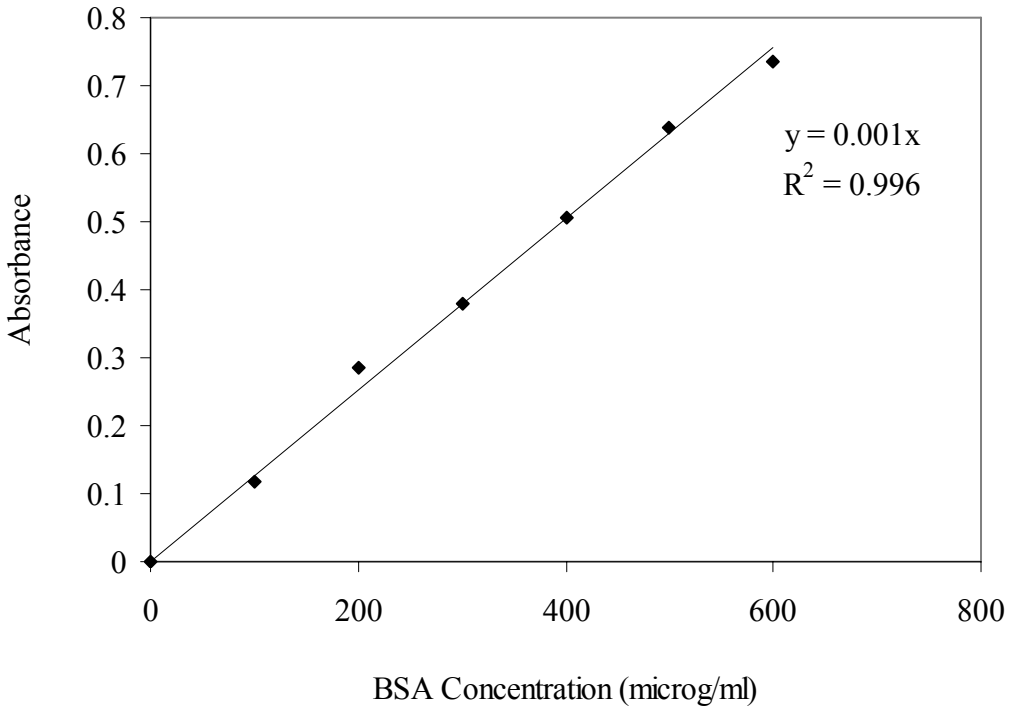
B.1. Calibration Curve For Lactic Acid during HPLC Analysis



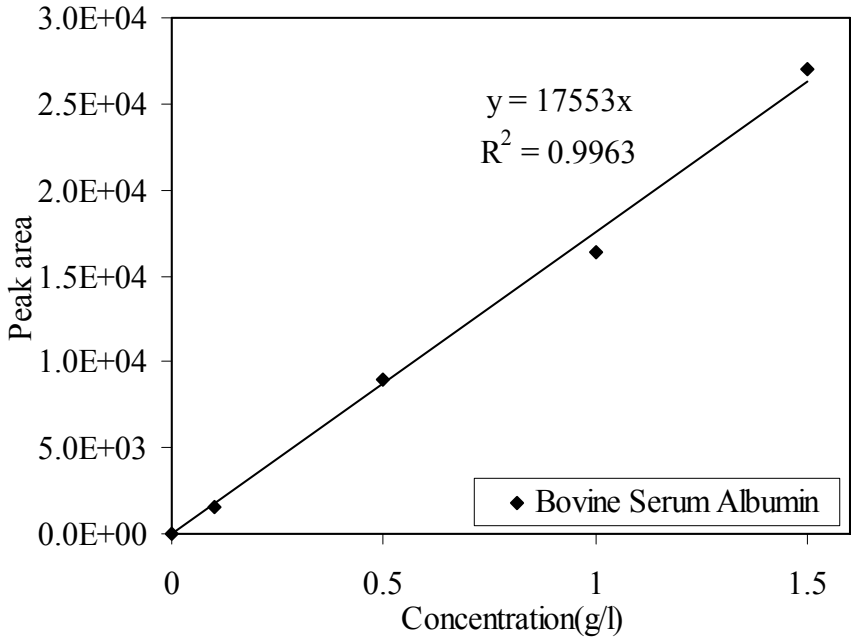
B.2. Calibration Curve for Lactose during HPLC Analysis



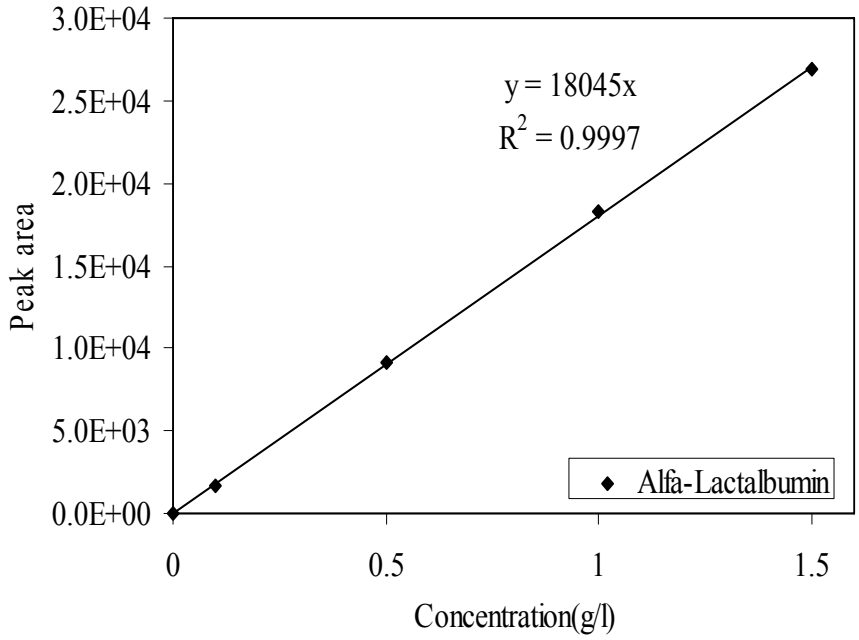
B.3. Calibration Curve for Bovine Serum Albumin during UV-Spectrophotometer Analysis



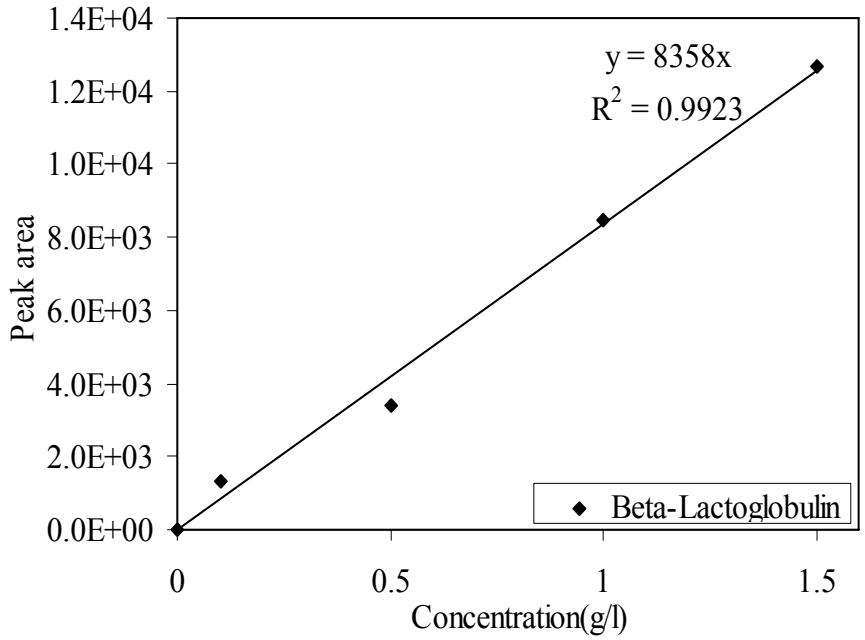
B.4. Calibration Curve for Bovine Serum Albumin during HPLC Analysis



B.5. Calibration Curve for α-Lactalbumin during HPLC Analysis



B.6. Calibration Curve for β-Lactoglobulin during HPLC Analysis



APPENDIX C. LOWRY PROTEIN ANALYSIS

Reagents:

Reagent 1 (R1): 1 % copper sulfate

Reagent 2 (R2): 1 % potassium sodium tartarate

Reagent 3 (R3): 2 % sodium carbonate in 0.1 N NaOH

Dye: Folin-ciocalteau (1:1 dilution with water)

Reagent R: 1 % R1 + 1 % R2→+ 98 % R3

Blank: 0.2ml water/buffer + 2.1 ml R + 0.2 ml dye

Procedure:

The samples were diluted according to the linear detection range. 0.2 ml samples were pute inside glass tubes. Next 2.1 ml reagent R was added inside tubes. Tubes were vortexed with Yellowline TTS 2 for 3 seconds at a speed of 2200. There should be a waiting period for 10 minutes. Subsequently, 0.2 ml dye was added into the tubes. The mixture was readily vortexed for 7 seconds and the tubes were put inside dark place for the 1 hour waiying period. The liquid mixtures were put inside spectrophotomter cells and the absorbance values were measured at 750 nm. It is advised to perform a scanning step in order to get the operation wavelength at which the sample gives maximum absorbance value.

APPENDIX D. HPLC OPERATION SPECIFICATIONS

Table D1. The properties of the column and analysis conditions for HPLCmeasurements

Property	**Specifications**	**Specifications**
Type of Analysis	Lactose Lactic acid	Bovine serum albumin (BSA), β-Lactoglobulin, α-Lactalbumin
Retention Time	Lactose : 7.6 min Lactic acid : 12.4 min	BSA and its dimer: 7.6 and 9.97 β-Lactoglobulin: 11.19 min α-Lactalbumin:12.05 min
Column	Aminex HPX- 87H ion exclusion Column (Biorad Laboratories)	Agilent Zorbax GF-250 Bio Series
Column Length	300 mm	250 mm
Column Diameter	7.8 mm	4.6 mm
Particle Size	9 μm	4-4.5 μm
Guard cartridge	Micro-Guard cation –H cartridge (30x4.6)	9.4 mm Idx15 mm
Mobile Phase	5 mM H_2SO_4	130 mM NaCl/20mM KCl/50mM Na_2 HPO_4
Flow rate	0.6 ml/ min	1.0 ml/min
Temperature	45 ^{0}C	25 oC
Detector	Refractive index	Diode array detector- 210 nm
Elution Type	Isocratic Elution	Isocratic Elution

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