ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IMMOBILIZATION OF β-GALACTOSIDASE ONTO CHITOSAN NANOFIBERS

M.Sc. THESIS

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Thesis Advisor: Assoc. Prof. Dr. Filiz ALTAY

JUNE / 2016

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

KİTOSAN NANOLİFLERİ ÜZERİNE β-GALAKTOSİDAZ İMMOBİLİZASYONU

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FOREWORD

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TABLE OF CONTENTS

Page

FOREWORD ..... **vii**
TABLE OF CONTENTS ..... **ix**
ABBREVIATIONS ..... **xi**
LIST OF TABLES ..... **xiii**
LIST OF FIGURES ..... **xv**
SUMMARY ..... **xvii**
ÖZET ..... **xix**
1. INTRODUCTION **1**
1.1 Purpose of the Thesis 2
1.2 Literature Review 3
1.2.1 Nanotechnological applications and immobilization 3
1.2.1.1 Nanotechnology and immobilization 3
1.2.1.2 Nanofibers as immobilization supports 5
1.2.1.3 Production of nanofibers- electrospinning 6
1.2.1.4 Chitosan nanofibers 8
1.2.1.5 Safety issues and regulations 10
1.2.2 Immobilization 11
1.2.2.1 Immobilization of enzymes 11
1.2.2.2 Immobilization of β-galactosidase 14
1.2.2.3 Immobilization on carriers 15
1.2.2.4 Performance of immobilized enzymes 22
2. MATERIAL AND METHOD **25**
2.1 Materials 25
2.2 Methods 25
2.2.1 Production of uniaxial chitosan nanofibers 25
2.2.2 Surface activation 26
2.2.3 Immobilization of β-galactosidase 27
2.2.4 Measurement of enzyme activity and enzyme loading efficiency 28
2.2.5 Measurement of thermal stability 30
2.2.6 Measurement of reusability 31
3. RESULTS AND DISCUSSION **33**
3.1 Electrospinning 33
3.2 Surface Activation and Immobilization of β-Galactosidase 34
3.3 Thermal Stability and Reusability 37
4. CONCLUSIONS **43**
REFERENCES **45**
CURRICULUM VITAE **53**

ABBREVIATIONS

CLEA **:** Crosslinked enzyme aggregates
DCM **:** Dicloromethane
GA **:** Glutaraldehyde
ONPG **:** o-Nitrophenyl ß- D – Galactopyranoside
PVA **:** Polyvinyl alcohol
TFA **:** Trifluoroacetic acid
U **:** Enzyme unit

LIST OF TABLES

Page

Table 1.1 : Advantages and disadvantages of immobilization on nanoparticles. **5**
Table 1.2 : Stability, reusability and immobilization efficiencies of immobilized β –Galactosidase. **24**
Table 3.1 : Efficiency of immobilization and treatment conditions..... **36**
Table 3.2 : One-way ANOVA test results of immobilized and free enzyme..... **38**
Table 3.3 : Results of independent samples test for reusability assay. **42**

LIST OF FIGURES

Page

Figure 1.1 : Electrospinning jet- Whipping instability. **7**
Figure 1.2 : Uniaxial and coaxial nanofibers. **7**
Figure 1.3 : Number of publications between 2000 and 2013. ..... **13**
Figure 1.4: Symbolic representation of different binding methods. **21**
Figure 2.1 : Chitosan nanofibers on the collector plate. **26**
Figure 3.1 : Nanofiber membrane from chitosan and PVA dissolved in acetic acid. **33**
Figure 3.2 : Chitosan nanofiber treated with GA. **34**
Figure 3.3 : Calibration curve-1. ..... **35**
Figure 3.4 : Calibration curve-2. ..... **35**
Figure 3.5 : Changes in amount of enzyme at varying temperatures. **37**
Figure 3.6 : Activity of the immobilized and free enzyme at varying temperatures. **37**
Figure 3.7 : Relative activity of the immobilized and free enzyme at varying temperatures. **38**
Figure 3.8 : Recycled chitosan nanofibers. **40**
Figure 3.9 : Reusability of the first nanofiber set. **40**
Figure 3.10 : Reusability of the second nanofiber set. **41**

IMMOBILIZATION OF β-GALACTOSIDASE ONTO CHITOSAN NANOFIBERS

SUMMARY

Enzymes are biocatalysts which offer a wide variety of specific functions. Large amounts of enzymes are used in native forms at industrial scale. Use of free enzyme means one-time application with no chance of recovery and reusability. Additionally free enzymes show low stability in harsh processing environments. They are sensitive to changing processing conditions such as pH and temperature change. Immobilized enzymes serves some opportunities for recovery and multiple use of enzymes providing higher stability and selectivity. Enzyme immobilization is defined as the attachment of a free enzyme on a carrier (supporting) surface which restricts or prevents the mobility of the enzyme. Immobilization technique was started to be used in 1916. The studies showed that immobilization facilitates higher stability in challenging processing conditions. In the following years, the performance of immobilized enzyme was studied to improve it. It was discovered that performance of the immobilized enzyme is highly dependent on the surface characteristics and binding methods. Afterwards, novel carrier materials and methods were studied. Inorganic or organic, hydrophilic or hydrophobic, porous or nonporous features of the carrier has altered the interaction of the enzyme with the surface. It affected the activity and the stability of the immobilized enzyme. With development of nanofibers it was found that nanomaterials has a promising potential as carrier surfaces due to their high surface area. The change of some biological and chemical characters in nanoscale compared to the macro or micro scales, pointed out that nanomaterials nano-materials can be utilized in broad range of applications. Nanotechnology involves production of materials, devices or a system in the range of 1 to 100 nm. Nanosheets, nanoparticles, nanotubes, nanofibers are examples of nanostructures. Among these, nanofibers has attracted the most attention due to their high surface to volume ratio, simple and cost-effective production, easily modifiable surface properties. The most preferred method is electrospinning for production of nanofibers. Electrospinning system consists of a high voltage power supply, pump, syringe and a collector plate. The polymer is dissolved in a suitable solvent and located on the pump in a syringe. The power supply gives a charge to the system which creates an electrical field between the capillary of the syringe and the collector plate. With the pump the solution is fed with a definite rate to the electrical field and collected on the collector plate.

In this study nanofibers were fabricated using electrospinning, which is a simple and cost effective technique. The polymer was preferred as chitosan which is a non-toxic, biocompatible, biofunctional material. Since the type of solvent changes the solubility of polymer hence structure of the nanofiber, acetic acid, trifluoroacetic acid (TFA) and dicloromethane (DCM) were studied. The best solubility was observed for the solution of TFA and DCM. A solution of TFA and DCM was prepared in the ratio of 70:30. Chitosan was dissolved in TFA-DCM solution at 3% concentration. Chitosan nanofibers were fabricated at 25 kV with feed rate of 0.4 ml/h. Distance of the plate from needle was changed between 10 and 15 cm. β-galactosidase was preferred for

immobilization, since it is one of the mostly used enzymes in food industry. It is used for degradation of lactose into glucose and galactose. Unlike chitosan, chitosan nanofibers are water soluble. Because of that, chitosan nanofibers reqiure to be functionalized to become insoluble and active by a coupling agent. Glutaraldehyde (GA) was used as functionalizing agent at concentration of 4%. Chitosan nanofibers were treated in GA and with GA vapor. After functionalization, chitosan nanofibers were incubated in enzyme solutions with different enzyme concentrations. The enzyme immobilization efficiency, reusability and thermal stability were measured by simply measuring the activity of enzyme solutions by use of a spectrophotometer. Assays were conducted by comparing the activities of free and immobilized β-galactosidase. The activity was determined by use of a substrate o-Nitrophenyl ß- D – Galactopyranoside (ONPG) which gives a colorful product after hydrolzed by β-galactosidase.

A successful enzyme loading efficiency was obtained up to 69±20%. The immobilized and the free β-galactosidase showed highest activity at 50 °C. The activities at 50 °C were considered as 100% activity to observe the percental change in activity clearly. The activities of the immobilized and free enzyme decreased when the temperature was increased up to 90°C. At 70°C the activity of the free enzyme decreased sharply to 31±0.01% as the activity of the immobilized enzyme was lowered to 73.5±0.1%. After 10 cycles of use of immobilized β-Galactosidase at 40°C, 68±26% of the enzyme activity was retained. These results was found to be promising when compared with the data from literature. The reusability of chitosan nanofibers can be improved by production of magnetic chitosan nanofibers in further studies.

It should be taken into consideration that nanomaterials have different chemical and physical characteristics compared to their macro and micro size. The results of intake of nanofibers into the body is not known for certain. For use of nanofibers in food applications, toxicological studies and regulations are required.

KİTOSAN NANOLİFLERİ ÜZERİNE β-GALAKTOSİDAZ İMMOBİLİZASYONU

ÖZET

Enzimler, gıda, eczacılık, kozmetik gibi bir çok endüstriyel alanda kullanılan proteinlerdir. Spesifik reaksiyonlara girebilmeleri ve prosesteki etkinlikleri nedeniyle geniş kullanım alanlarına sahiptirler. Ancak günümüzdeki enzim kullanımının büyük kısmı enzimlerin serbest şekilde proses ortamına katılmasıyla gerçekleşmektedir. Bu da yüksek miktarda enzim kullanımı anlamına gelmektedir. Serbest halde kullanılan enzimleri prosesten geri kazanmak zordur. Bu nedenle çoğu proseste geri kazanılmadan yalnızca bir kez kullanılmaktadırlar. Ayrıca değişken proses şartlarına, yüksek veya düşük pH, yüksek sıcaklık gibi zorlu şartlara dayanıksızdırlar. Bu da, enzimlerin kullanım alanını daraltmaktadır. Enzimlerin saflaştırılması ve üretiminin zorluğu düşünüldüğünde bu durumun büyük bir maddi kayba yol açtığı sonucuna varılmaktadır. Enzimlerin geri kazanımı için kullanılan mevcut bazı yöntemler yüksek enerji tüketiminin yanında uygulama zorluğu ve zaman kaybı açısından tercih edilmezler. Bu amaçla enzimlerin serbest olarak değil; immobilize, yani bir yüzeye tutunmuş halde kullanılması denenmiştir. İmmobilizasyon ile enzimin stabilitesinin, yarı ömrünün önemli ölçüde arttırıldığı görülmüştür. Bunun ardından immobilize edilmiş enzimin aktivite yönünden de performansını arttırmak için çalışmalar yapılmıştır. Bu çalışmalarda, aktivitenin ve hassasiyetin önemli ölçüde enzimin bağlandığı yüzeye ve bağlanma metoduna bağlı olduğu görülmüştür. Çeşitli inorganik veya organik, hidrofilik veya hidrofobik, porlu veya porsuz yüzeyler denenerek farklı maddelerin aktiviteyi nasıl etkilediği araştırılmıştır.

Nanoteknolojinin de gelişmesiyle nano boyuttaki malzemelerin immobilizasyon yüzeyi olarak kullanılabileceği anlaşılmıştır. Nano malzemeler makro ve mikro boyutlarına göre farklı fiziksel ve kimyasal özellikler taşırlar. Bu özellikler, enzim performansını belli yönlerden geliştirdiğinden, nano yapılar enzim immobilizasyonu için tercih edilebilir olmuşlardır. Nanolifler, nano malzemeler arasında yüzey alanı genişliği, üretiminin kolaylığı, ucuzluğu ve yüzey özellikleri bakımından en dikkat çekici yapılardan biridir. Nanoliflerin çapı 1-100 nm aralığındadır. Elektroeğirme (elektrodöndürme) metoduyla kolaylıkla üretilebilirler. Elektroeğirme basit ve ucuz bir yöntemdir. Elektroeğirme metodu bir güç kaynağı, bir pompa ve toplayıcı plakadan oluşur. Kullanılacak polimerin çözeltisi bir şırınga içerisinde pompaya yerleştirilir. İğnenin ucu güç kaynağına bağlanır. Güç kaynağının çalıştırılmasıyla sisteme yük verilir ve bir elektriksel alan oluşur. Pompa belli bir hız ile elektriksel alana çözeltiyi besler. Elektrik alanın etkisiyle polimer metal toplayıcı plakada toplanır.

İmmobilizasyon yüzeyi olarak porlu cam ve silika gibi inorganik maddeler; polisakkaritler ve sentetik polimerler seçilebilir. Sentetik polimerlerin fonksiyonel özellikleri, ucuzluğu ve kolay ulaşılabilirliği; yüzey olarak kullanım açısından avantajlıdır. Bunun yanında sentetik polimerle nanolif üretimi, polisakkaritlere göre daha kolaydır. Biyopolimerlerin (polisakkaritler ve proteinler) elektroeğirme yöntemiyle nanolif haline getirilmesi kompleks kimyasal yapılarından dolayı zordur. Bu nedenle biyopolimerlerle sentetik polimerler karıştırılarak nanolif üretiminde

kullanılabilir. Bu çalışmada polimer olarak kitosan seçilmiştir. Kitosan biyobozunur, biyofonksiyonel, toksik olmayan ve antibakteriyel özellik gösteren bir maddedir. Proteinlerle etkileşiminin iyi olmasının yanında, mekanik dayanıklılığı yüksek bir polimerdir. Organik polimerle nanolif üretiminin zorluğu nedeniyle kitosanın uygun bir çözeltide iyice çözünmesi ve nanolif üretiminin ardından aktive edilmesi gerekir. Aktivasyon ile kitosan nanolifi üzerindeki enzimlerin bağlanabileceği aktif kısımların çoğaltılması amaçlanır. Aktivasyon işlemi ile kitosanın fonksiyonel grupları eşleşir ve stabil bir yapı elde edilir. Böylelikle suda çözünebilir olan kitosan nanolifinin sulu çözeltide çözünebilirliği engellenir.

Çalışmada immobilize edilmek üzere gıda endüstrisinde laktoz parçalamada sıkça kullanıldığı bilinen β-galactosidaz seçilmiştir. Laktoz intoleransı olan insanların dünya nüfusunun yaklaşık %70'ini oluşturduğu bilinmektedir. Laktoz intoleransı, laktaz enziminin vücuttaki eksikliğini ifade eder. Bu kişilerde laktoz alımı, kramplar, kusma, ishal gibi sağlık problemlerine yol açar. β-galactosidaz enziminin dışarıdan alımı ile laktoz glukoz ve galaktoza parçalanır ve bu etkiler azaltılabilir veya giderilebilir. β-galactosidaz süt endüstrisinde ürün kalitesini ve proses verimini arttırmak ve süt ürünlerinin sindirilebilirliğini arttırmak için kullanılır. İmmobilizasyon ile bu enzimin ısıl stabilitesinin arttırılabileceği ve tekrar kullanılabilirliğinin sağlanabileceği bilinmektedir.

Bu tezin amacı kitosan nanolifleri üzerine immobilize edilmiş β-galaktosidaz'ın ısıl stabilitesini ve tekrar kullanılabilirliğini araştırarak etkin bir enzim tutuklama yüzeyi oluşturmaktır. Çalışmada çözelti olarak trifluoroasetik asit (TFA) ve diklorometan (DCM) ve ayrıca asetik asit kullanılmıştır. TFA uçucu bir asit olduğundan çalışılması zor ve tehlikeli olabilmektedir. Bunun için öncelikle asetik asit ile kitosan çözeltisi hazırlanmış ve nanolif üretimi denenmiştir. Kitosanın asetik asit içerisinde çözünmesiyle nanolif elde edilememiştir. Kitosana polivinil alkol (PVA) katılarak nanolif üretimi gerçekleştirilmiştir. Ancak PVA'nın uzaklaştırılması ayrıca bir işlem gerektirdiğinden ve oluşan nanolifin çoğu PVA olduğundan verimli sonuç alınamamıştır. Bunlar üzerine kitosan TFA ve DCM içerisinde çözünmüştür. 95 saatlik nanolif üretimi sonucunda, 1.35 g kitosan nanolifi elde edilmiştir. Enzim bağlama metodu olarak kovalent bağlama seçilmiştir. Bunun sebebi kovalent bağlamanın yüzey ile enzim arasında en stabil bağı oluşturan yöntem olmasıdır. Kovalet bağlama ile enzimin taşıyıcı yüzeyden ayrılması minimize edilir. İmmobilize enzimin ortamdaki diğer moleküllerle etkileşime geçmesi engellenir. Kovalent bağlama için kimyasal aktive edici ajanlar kullanılması gerekir. Bu ajanlar ile enzimin bağlanma verimi ve yüzey üzerindeki mobilitesi arttırılır. Bu çalışmada kitosan nanoliflerini aktive etmek için glutaraldehit (GA) kullanılmıştır. Üretilen nanolifler ayrı ayrı GA buharı ve GA'nın %4'lük çözeltisi ile aktive edilmiştir. Çalışmadaki ölçümler enzim aktivitesinin spektrofotometrik olarak belirlenmesi ile gerçekleştirilmiştir. Substrat olarak β-Galactosidase ile reaksiyonu sonucunda sarı renkli bir bileşik veren o-Nitrofenil ß- D – Galactopiranosit (ONPG) seçilmiştir. Belirli konsantrasyonlarda enzim ve substrat reaksiyonu sonrasında oluşan sarı renkli çözeltinin, spektrofotometrede absorbansı ölçülmüştür. Absorbansların bulunmasının ardından kullanılan eşitliklerle enzim aktivitesi ölçülmüştür. GA ile aktif hale getirilmiş kitosan nanolifler farklı konsantrasyonlardaki enzim çözeltilerinde inkübe edilerek enzim yüklemesi yapılmıştır. Buna göre 0,25 mg enzim/ml konsantrasyondaki enzim çözeltisinde inkübe edilen nanoliflerin 69±20% oranına kadar enzim yüklenebildiği görülmüştür. 0,025 mg enzim/ ml konsantrasyonlu enzim çözeltisinde bekletilmiş nanoliflerde ise immobilizasyon verimi % 59±20' ye kadar çıkarılabilmiştir. Bu sonuç,

artan enzim konsantrasyonunun, enzimin yüzeye bağlanma şansını arttırarak immobilizasyon verimini arttırabileceğini göstermiştir. İmmobilizasyon verimini arttırabilmek için nanolifler GA buharıyla muamele edilmiş, sonrasında ise GA çözeltisinde bekletilmiştir. Her iki işlemin ardından da enzim yükleme verimi ölçülmüştür. Nanoliflerin GA buharı ile muamele edilip, sonrasında %4'lük GA içinde bekletilmesiyle; immobilizasyon verimliliği sadece GA buharı ile muamele edilmiş nanoliflere göre %46±20 arttırılmıştır.

Enzim yüklenmiş kitosan nanoliflerine termal stabilite ve tekrar kullanılabilirlik testleri yapılmıştır. Termal stabilite için 30°C, 50°C, 70°C ve 90°C'lerde immobilize ve serbest enzim için aktivite ölçümleri yapılmıştır. Ölçümler 3 tekrarlı gerçekleştirilmiştir. En yüksek aktivite hem serbest enzim hem de immobilize enzim için 50°C'de ölçülmüştür. 50°C'deki bu aktivite %100 aktivite olarak kabul edilip farklı sıcaklıklardaki aktivite değişimi yüzde olarak ifade edilmiştir. 50°C üzerindeki sıcaklıklarda aktivite hem serbest enzim hem de immobilize enzim için azalmıştır. Ancak 70°C'de serbest enzimin aktivitesi 50°C'deki aktivitesinin%31±0,01 'ine kadar düşerken immobilize enzimin aktivitesi %73,5±0,1'e kadar düşmüştür. Bu sonuçlar immobilizasyonun termal stabiliteyi arttırdığını göstermiştir. Tekrar kullanılabilirlik testinde 0,1 mg enzim içeren 5 mg'lık iki farklı nanolif seti farklı şartlarda 10'ar defa kullanılmıştır. Her iki sette de tekrar olarak 5 farklı nanolif kullanılmıştır. Birinci nanolif seti her kullanımda 40'ar dakika bekletilirken, ikinci nanolif seti 15'er dakika bekletilmiştir. Her kullanım sonrasında nanolif üzerine immobilize edilmiş β-galaktosidazın aktivitesi ölçülmüştür. 10 kullanım sonunda, her kullanımda 40 dakika işleme maruz bırakılan nanolifler başlangıç aktivitesinin %29±7'sini korurken ikinci nanolif seti %68±13'ünü koruyabilmiştir. Buradan uzayan proses süresinin enzim aktivitesini ciddi ölçüde azalttığı sonucuna varılmıştır. İkinci nanolif setinin 10 kullanım sonucunda aktivitesinin %68±13'ünü koruması, literatürdeki değerlerle karşılaştırıldığında kitosan nanoliflerinin tekrar kullanılabilirliği açısından umut verici bir sonuçtur.

Bu çalışmadan elde edilen sonuçlarla, literatür verileriyle karşılaştırıldığında, kitosan nanoliflerinin endüstriyel uygulamalarda enzim tutuklaması için uygun materyaller olduğu çıkarılmıştır. Ancak kitosanın toksik olmaması, biyobozunur olması ve gıda uygulamalarında kullanılabilir olması dışında, kitosanın TFA ve DCM gibi sağlık açısından tehlikeli çözücülerde çözünerek üretilmiş olması gıdada uygulama açısından risk oluşturmaktadır. Nanolifi aktive etmek için kullanılan ajanlardan biri olan GA toksik bir madde olduğundan kalıntısı sağlık açısından tehdit oluşturabilir. Üretilen nanolif üzerinde çözelti kalıntısı olabileceğinden ve bu kalıntı proses sırasında ürüne karışabileceğinden gerekli toksikolojik çalışmalar yapılmalıdır. Bunun dışında nano boyutta maddelerin kimyasal ve fiziksel özelliklerinin değiştiği bilinmektedir. Bu nedenle nano-malzemelerin vücuda alındığında ne gibi sonuçlara yol açacağı, vücuttaki moleküllerle nasıl etkileşime gireceği tam olarak bilinmemektedir. Bunlar dışında, kitosan nanoliflerinin tekrar kullanılabilirliği enzim immobilizasyon yüzeyinin geri kazanımınn önemini göstermiştir. Bunu göz önünde bulundurarak gelecek çalışmalarda kitosan nanolifinin geri kazanımı araştırılmalıdır. Bunun için üretim sırasında nanolife manyetik özellik kazandırılarak proses sonrası geri kazanım sağlanabilir. Kitosan nanolifleri enzim endüstrisinde kullanım açısından gelecek vadetmektedir. Ancak gıda endüstrisinde kullanılması için öncelikle gerekli düzenlemelerin ve mevzuatın oluşturulması gerekmektedir.

1. INTRODUCTION

Functional bioactive materials such as antioxidants, probiotics and bioactive peptides are counted as health-supporting agents in food industry. Their ability to decrease long-term risks of growing diseases and their positive physiological effects make them important ingredients in functional foods. However, functional ingredients have an instable character, which requires a protection (Zhao et al., 2011). Encapsulation and immobilization provide a mild "living space" for functional molecules. Thus, these molecules can be protected from harsh effects of temperature, oxygen, light, pH, enzymes or other nutrients (Zhao et al., 2011). Use of enzymes in industry is highly important since they can show activity under mild conditions and they can work for very specific reactions with limited by-product formation (Chen et al., 2014). It is known that enzymatic applications take place in many industrial area including food processing and medicine. According to the report of BCC Research, the value of global market of industrial enzyme has raised from nearly $4.5 billion to nearly $4.8 billion between the years of 2012 and 2013. The expected value of the market is around $7.1 billion by 2018. The estimated compound annual growth rate (CAGR) is 8.2% from 2013 to 2018 (Anon. 3, 2014). This wide use of enzymes creates some economic concerns due to high consumption of soluble (free) enzyme (Buchholz et al., 2012). Enzymes are considered to have a fragile and unstable character and only work in aqueous medium. For most of the enzymatic reactions, these properties are undesirable. In order to overcome some of these drawbacks, immobilization has been found to be a cost-efficient technique (Buchholz et al., 2012; Singh et al., 2013). Enzyme immobilization is defined as the attachment of a free enzyme on a carrier (supporting) surface, which restricts or prevents the mobility of the enzyme (Khan & Alzohairy, 2010). Immobilized enzyme industry has a huge potential and a production amount in the range of several million down to a few hundred tons per year (Buchholz et al., 2012). In order to utilize the immobilized enzyme to the maximum benefit, it is important to choose the right carrier, right reactants, right binding method and suitable treatment conditions. All of these factors have some effect on stability or

catalytic activity of the enzyme. Carrier surface is one of the most effective factors since it is directly in interaction with the enzyme. There are many types of organic or inorganic supporting materials. The developing nanotechnology, nanomaterials started to be used as carriers due to their unique physicochemical properties. Nanomaterials offer ideal characteristics such as surface area, mass transfer resistance and effective enzyme loading to optimize enzyme activity and efficiency (Ahmad & Sardar, 2015). Nanoparticles and nanofibrous materials can be utilized as good supports for enzyme immobilization in terms of large surface area leading to high enzyme loading capacity and high volumetric enzyme activity (Xu et al., 2013). Especially, nanofibrous supports offer many advantages of their high porosity and interconnectivity compared to nanostructured supports like mesoporous silica and nanoparticles (Wang et al., 2009). Surface structure of nanofibers, materials used for nanofabrication and binding method applied for immobilization are factors, which affect enzyme activity and stability. Insoluble (immobilized) enzymes are advantageous in terms of separation and reuse after reaction (separation by filtration, centrifugation etc.), application in continuous processes (in fixed bed, fluidized bed, stirred tank reactors with a filter system). Immobilized enzymes used in continuous process offer easier process control, automation, recovery and purification opportunity. Recovery and purification may be an essential step in case that expensive enzyme is used in order to make the industrial application economic (Buchholz et al., 2012). In addition, possible increase in thermal and pH stability of the enzyme is another reason of immobilization (Belhacene et al., 2015).

1.1 Purpose of the Thesis

The aim of this thesis is to obtain a firm and smooth chitosan nanofiber mat for enzyme immobilization, to investigate the suitability of the chitosan nanofiber as an immobilization surface for enzymes and the potential of chitosan nanofiber in industrial applications. The main objective is to obtain a chitosan nanofiber, which can be used in food applications. In this study, the β-galactosidase enzyme was preferred since it is widely used in food industry for production of non-lactose containing foods. β-galactosidase was immobilized on chitosan nanofiber mats. In order to understand if chitosan nanofibers are promising for food applications; the activity of the immobilized β-galactosidase in various conditions were investigated. Some

parameters involving thermal stability, immobilization efficiency and reusability were determined.

1.2 Literature Review

1.2.1 Nanotechnological applications and immobilization of enzymes

1.2.1.1. Nanotechnology and immobilization

Nanotechnology is a relatively new technology in food industry. It attracts great attention due to its prevalent application areas such as material science, medicine and electronics. Nanobiotechnology, which is a branch of nanotechnology includes biochemical and biological elements, production of materials devices or a system in the range of 1 to 100 nm. Fabricated nanostructures can be used with the biological materials. Unfortunately, nanotechnological applications in food cannot be easily commercialized due to concerns about health and environmental risks. Commercialized products have been produced mainly in countries which are not the member of EU. US, China and Japan have been pioneers of nanotechnological applications in food. It has been suggested that the food nanotechnology market would grow rapidly by improving food innovation (Momin et al., 2013; Food Safety Authority of Ireland, 2008).

Because of the change in physical, chemical and biological properties compared to their macro scale counterparts, nano-materials can be utilized in broad range of applications. Nano-sized materials are considered as good supports for enzymes, since they improve the performance of enzyme in a manner. Ratio of surface area to mass is relatively greater in some nanostructures such as nanoparticles and nanofibers. For this reason, nanomaterials are expected to have higher biological activity than its macro- or micro-sized counterparts. Unsurprisingly; –macro, -micro and –nanosized chitosan carriers were compared in a research in terms of enzyme activity, it was seen that the nano-system provided the highest enzyme activity (Sulaiman et al., 2014, Zhao et al., 2011).

Nanotechnology is expected to enhance stability and texture of food, create "intelligent" food contact materials, packages and provide controlled release or immobilization of functional compounds. Some of current application areas in food

industry are sensors (flavor/colour enhancement, texture modification), targeted delivery of bioactive compounds, stabilization of active ingredients, packaging materials and antimicrobials to improve food safety. (Momin et al., 2013; Food Safety Authority of Ireland, 2008).

Some potential application fields in food are given below (Ravichandran, 2010);

- Organic and inorganic nanoadditives
- Nanosensors for food quality control (and smart packaging)
- Nanocoating or nanofilms for kitchenware and foodstuff
- Antimicrobial and hygiene coatings (for detection of pathogens)
- Self-sanitizing surfaces with high antimicrobial characteristics
- Nanosized freshness indicators
- Nanoemulsions for fat reduction

There are also some current examples of application in food industry. For example, addition of nanoparticles of carotenoids to fruit drinks provides enhanced bioaavailibility. As an application of controlled delivery, nanosized micellar structures containing canola oil were used for delivery of a range of molecules such as vitamins, minerals or phytochemicals. Nanocages or nanoclusters included in nanoceutical products, are benefited as delivery systems. Mineral supplements in nano-range are used in Chinese nanotea which is declared to increase selenium uptake. "Nanodrop"s are used for delivery of encapsulated materials such as vitamins (Ravichandran, 2010). Nanostructures can be also used as support materials in enzyme immobilization. Nanoporous silica, nanotubes, nanoparticles, nanofibers, nanocomposite, and nanosheets are nanostructured materials which are used as carriers for immobilization. Nano materials are promising in terms of developing novel technologies in enzyme immobilization field. (Sulaiman et al., 2014).

Besides the positive effects, some nano sized materials create some restrictions in terms of enzymatic activity. The mass transfer of substrate between enzyme-carrier system and the medium should be high for an efficient processing. As an example, nanoporous silica encloses the enzyme molecule in its pores. It restricts the mass diffusion of substrate and product which reduces enzymatic production. Non-porous nano materials such as nanotubes or nanoparticles provide higher mass transfer, however they are difficult to recycle and reuse. Nanocomposites can be harmful to

reactive functional groups of enzymes. On the other hand, nanofibers have promising properties which solve these problems, as they can be produced with smooth surface structure and can be easily recycled and reused (Sulaiman et al., 2014). The advantages and disadvantages of the use of nanoparticles as enzyme carriers, are given on the Table 1.1.

Table 1.1. Advantages and disadvantages of immobilization on nanoparticles (Ahmad & Sardar, 2015).

Advantages	Disadvantages
Minimization of diffusional problems	Cost of fabricational process
Effective enzyme loading	Large scale application is difficult
High surface area	Separation is difficult (except magnetic nanoparticles)
High mechanical strength	

High surface area, high mechanical strength, effective enzyme loading and less diffusional problems are advantages of use of nanoparticles. However the production of nanoparticles is complicated and expensive for industrial production. Recycling is difficult when no magnetic nanoparticle is used. Thus, it makes the large scale application hard (Ahmad & Sardar, 2015).

1.2.1.2 Nanofibers as immobilization supports

The most remarkable feature of nanofibers is their extremely high surface area-to-volume ratio. The size of nanofiber is determined measuring its diameter. The diameter of a nanofiber may change from less than 40 nm to 2 μm. The high surface area of nanofibers make surface properties more important than bulk properties. The functionality of nanofibers can be altered by changing surface characteristics. As an example, thin porous nanofibers showed increased accessibility and low diffusion resistance to reactive materials. It was stated that finer nanofibers generally have relatively higher tensile strength and higher Young's modulus when compared to nanofiber with larger diameters. Additionaly, the beads on the nanofiber would decrease mechanical strength (Kriegel et al., 2008).

Whereas critical surface area and diffusional limitations inhibits the efficient application of immobilization, use of electrospun water-soluble nanofibers as enzyme

supports can overcome these limitations. Nanofibers exhibit increasing surface area to volume ratio, improved storage stability, improved catalytic efficiency and reducing diffusion limitations. Fabricated nanofibers may offer a surface area of about 100-1000 m^2 /g. While immobilization in or on nanofibers increases enzyme stability, it also increases the durability on the broadened working pH and temperature (Wong et al., 2014). As reported by Wong et al. (2014), immobilization onto nanofibers requires additional surface functionalization steps or post processing cross-linking for better activity as also mentioned previously. Studies have focused on surface modification after electrospinning and extra addition of enzymes by functionalized end groups and zero-length cross linkers. As an example, in a study poly (An-co-MMA) nanofibers were treated with polyethylenimine before immobilization of β-galactosidase. Additionally, polyacrylonitrile nanofibers can be used for binding lipase by amidination or functionalization of nitrile groups. Lipase is attached to amino side chains (Wong et al., 2014).

1.2.1.3 Production of nanofibers- electrospinning

Nanofibers can be produced by sol-gel method, chemical deposition method, drawing, template synthesis, phase separation, self- assembly, thermal oxidation or electrospinning. Nanofibers and nanofiber mats can be easily produced by electrospinning, which is known for its versatility, fashionability and flexibility. Industrial applications of electrospinning started in 1990. It is the most accepted method in literature for production of nanofibers. Since it is a simple and cost effective method, electrospinning can be considered as a suitable technique for industrial applications. (Austero et al., 2012; Kriegel et al., 2008; Zhao et al., 2011; Fallahiarezoudar et al., 2014a).

Electrospinning system consists of a high voltage power supply, pump, syringe and a collector plate. The polymer is dissolved in a solvent before electrospinning. Afterwards, the polymer is fed into a syringe. The feeding rate of the pump is adjusted and the syringe is located on the pump. Power supply injects charge of a certain polarity to the spinneret as the polymer is fed through the spinneret. A strong electrical field is created. A drop is formed on the top of the capillary. The electrical field induces a force against surface tension and interfacial forces of the solution. When the surface tension is overcome, the solution tends to accelerate towards the target electrode

(collector). The hemispherical shape of the solution on the top of the syringe turnes into a conical shape which is named as Taylor cone. When the polymer jet is accelerated toward the metal plate of opposite polarity, solvent is evaporated and nanofiber is obtained on the grounded collector. The collector provides that an electric field is created between capillary tip and the target by completing the circuit (Bhardwaj & Kundu, 2010; Fallahiarezoudar et al., 2014b; Kriegel et al., 2008).

As the electrical field is applied between needle tip and the collector, polymer jet comes out from the tip following a straight way. As it approaches to the collector it begins to follow a spiral path at some point. This point of alternation is called as the whipping instability point (Figure 1.1). Taylor cone and whipping instability are the two remarkable parameters in order to produce nanofibers with good morphology. Three types of instability are observed in electrospinning; (a) Rayleigh, (b) bending, and (c) whipping. Rayleigh and bending instabilities are axisymmetric and whipping is nonaxisymmetric instability. Rayleigh and bending instability occurs when the electrical field is high and viscosity of the polymer is lower than the optimum (Fallahiarezoudar et al., 2014a).

Figure 1.1. Electrospinning jet-Whipping Instability (Schultz, 2008).

Nanofibers can be fabricated in uniaxial and coaxial forms. The structures of uniaxial and coaxial nanofibers are illustrated on Figure 1.2.

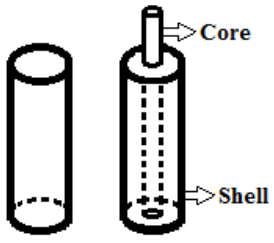

Figure 1.2 Uniaxial (on the left) and coaxial nanofibers (on the right).

In fabrication of uniaxial nanofibers one pump/syringe is used as previously explained. The coaxial electrospinning is applied with a dual-nozzle spinneret, which has inner capillary inside and larger outer capillary. Two immiscible liquid are fed through these capillaries for core/shell structure. Immiscibility of two liquids is critical to fabricate fine core/shell nanofibers. When inner and outer solutions are highly immiscible, Taylor cone shows instability (Fallahiarezoudar et al., 2014a; Sung et al., 2012).

The diameter of the electrospun nanofiber is affected by some parameters such as molecular weight, concentration, surface tension, viscosity, conductivity of the polymer, temperature, pressure, humidity and electrospinning conditions. Control parameters such as applied voltage, flow rate and distance between nozzle and the collector can be adjusted during operation. Viscosity is one of the most important factor, since it effects entanglement of polymer in the solution. Voltage designates electrostatic interaction forces, which creates an ejection of polymer jet. By changing these parameters, nanofiber diameter, porosity of the mat and morphology can be adjusted and ultra-fine quality nanofibers with porous surface can be obtained. (Austero et al., 2012; Zhao et al., 2011; El-Aassar et al., 2013).

1.2.1.4 Chitosan nanofibers

Chitosan is a biocompatible, non-toxicand an antibacterial material. It is known with its harmlessness to environment. Free reactive amino and hydroxyl groups on chitosan surface enable good amount of enzyme immobilization. Its biofunctionality, biocompatibility and metal chelating characteristics are also well known. It also establishes good interactions with proteins and shows good mechanical strength. Considering all these factors, chitosan is a suitable surface for immobilization in food industry (Hosseinipour et al., 2015; Kriegel et al., 2008).

As stated by Zhao et al., chitosan is a partially deacetylated polymer of N-acetyl glucosamine. It can be gained by alkaline deacetylation of chitin. It can be gained from waste of fishing industry Chitosan molecule includes a β-(1,4)-linked-D-glucosamine residue with the amine groups. The amine groups can be randomly acetylated. These amine groups and –OH groups make chitosan an easily utilizable molecule in many applications (Ye et al., 2005; Zhao et al., 2011). Properties of chitosan changes according to its molecular weight, the degree of deacetylation, the distribution of acetylation sites, solution pH and ionic strength. Only the deacetlyated amino groups

may gain or lose protons. Because of that, the charge density depends on the degree of deacetylation (Kriegel et al., 2008).

Since it is difficult to produce pure chitosan nanofibers, different techniques and solvents were studied to overcome this issue. Huang et al. (2007), studied blending chitosan (3 wt% aqueous acetic acid solution) with 9 wt % PVA dissolved in water. The ratio of chitosan to PVA solution was 7:3. They obtained nanofibers with mean diameters of 150-300 nm. The nanofibers were treated with NaOH. At the end porous chitosan nanofibers with low amount of PVA was obtained . Chitosan (3 wt %) solutions in 90 wt % aqueous acetic acid were used for fabrication of nanofiber. Nanofibers with diameters between 70 ± 45 nm were produced. Chitosan with varying molecular weight (low, medium and high) formed beads and thin nanofibers in solution of acetic acid with concentration of 30%. Increasing concentration of acetic acid up to 90wt% lowers surface tension and increases the net charge of polymers which provides continuous nanofiber formation. In addition to acetic acid, trifluoroacetic acid (TFA) and dichloromethane (DCM) are used for dissolving chitosan. It has been stated that pure chitosan nanofibers with good structure were produced in TFA and DCM (Kriegel et al., 2008).

Chitosan becomes a polyelectrolyte in acidic solutions. During application of high electrical field, the repulsive forces between ionic groups of the polymer increase. It inhibits continuous fabrication and causes beads on nanofibers. TFA is given as the most suitable solvent of chitosan, since the amino groups of chitosan form salts with TFA. These salts provokes destroying the interactions between chitosan molecules. Increasing chitosan concentration in TFA changes the morphology of nanofibers collected on the plate. It is converted from spherical beads to interconnected fibrous network. When DCM is added to TFA, homogeneity of nanofibers is enhanced without interconnected fibrous network. (Sun & Li, 2010; Zhao et al., 2011).

By producing nanofibers and nanofiber mats from chitosan, chemical functionality of chitosan is improved. Thus, it can be used for food processing, biomedical applications, food packaging, filtration membranes and tissue engineering scaffolds. In order to obtain a mechanically and chemically stable chitosan, it is needed to be stabilized before application. Crosslinkers are utilized for stabilizing polymers. Stabilizing occurs through bonding and coupling of functional groups of chitosan. It means the retention of functional groups. Thus, dissolution of the polymer in aqueous

medium is inhibited. Glutaraldehyde (GA), genipin, diisocynates and epoxides are used as crosslinking agents. (Austero et al., 2012).

1.2.1.5 Safety issues and regulations

It is known that nanoparticles of 100 nm or less have the ability of entering the body through inhalation. Exposure to nanoparticles may also occur through skin. Water, food and air are potential primary sources of nanoparticles. This makes the nano-sized structures suspicious in terms of food safety. Also it is unclear how they interact with other components in food and how they are treated in body and removed from digestive system. Thus, toxicological studies and risk assessment about nano-sized structures in food should be conducted (Anon. 6, 2008).

In the United States, the US Food and Drug Administration (FDA) demands manufacturers to demonstrate that the product does not contain a risk for health. However there is no regulation for nanotechnological applications in food. It was stated by FDA that no formal definition of "nanotechnology", nanomaterial"," nanoscale" was adopted. The overall attitude of FDA about a nano technological application is based on questioning "(1) whether a material or end product is engineered to have at least one external dimension, or an internal or surface structure, in the nanoscale range (approximately 1 nm to 100 nm) and (2) whether a material or end product is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometer (1,000 nm)." In addition, it was clarified by Ravichandran (2010) that the FDA make regulations for products instead of technologies. According to the definition of European Commission from the Commission Recommendation 2011/696/EU, a "'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm". In 2012, nanotechnology was considered as a "key enabling technology". It was stated in a regulatory review that not all nanomaterials have toxic effects. The statement that 'size reduction means more reactivity and toxicity' cannot be proved. Toxicity did not reported as related to the size but rather the type of the material. According to the Regulation No 1333/2008 on Food Additives, if there is a

significant change in the production methods of a food additive which is already included in Community list or if there is a change in particle size through nanotechnology, this food additive is considered as a new additive. Community lists are accepted by the European Parliament and the Council. Community lists include food additives approved for use in foods, food enzymes, flavourings and their conditions of use. Before a new product can be used in the food market, a new entry in the Community lists is required (Anon. 4., 2015.,; Anon. 5., 2014; Anon. 7., 2008; Ravichandran, 2010).

1.2.2. Immobilization

1.2.2.1. Immobilization of enzymes

Enzymes are widely used as biocatalysts due to their high level of catalytic efficiency, substrate specificity, region specificity and stereo-specificity. They also speed up reactions. Thus, enzymes can be used as biocatalysts instead of conventional chemical catalysts. Since enzymes reduce the activation energy of the reaction, they can be utilized in environmental-friendly purposes. However, there are some challenges in application of enzymes. Free enzymes can be easily affected by pH, temperature or other environmental conditions. Native enzymes are not able to show long-term stability in processing environment. They cannot be easily recovered and reused. Some methods of recycling of free enzymes are applied such as capillary gel electrophoresis. However, it requires high-energy consumption and it is hard and time consuming to apply in industrial processing. The high cost and instable character necessitate immobilization. (Cao et al., 2012; Sulaiman et al., 2014; Ye et al., 2005). Immobilization of an enzyme has definite benefits in terms of reuse, longer half-lives and less degradation. The rigidity of the protein increases by covalent binding on a surface, which prevents dissociation related inactivation. The arrested enzyme can be used repeatedly without time consuming and costly purification. Thus, efficient recycling and control of the process leads to decrease in processing costs. Immobilization provides easy handling of the enzyme. Reaction compounds are not contaminated by enzyme, which is desired in food and pharmaceutical industries. The performance of a free enzyme can be enhanced in terms of pH tolerance, heat stability, functional stability, activity in organic solvents. (Ahmad & Sardar, 2015; Cao, 2006; Zhao et al., 2011). In addition to the advantages of immobilization, enzyme

inactivation or denaturation are other consequences of immobilization, which causes decrease of activity. However, the level of activity loss can be minimized by taking into consideration, carrier type, immobilization technique, immobilization conditions. Achieving a good activity retention is dependent on not changing the chemical characteristics or reactive groups in the enzyme's binding site (Sulaiman et al., 2014).

Immobilized enzyme was started to be used in 1916. Developing phase of immobilization technology has occured in 1970s. Following 1990s, a rational design phase has started. In 1916, it was discovered that activity of invertase enzyme is not hindered when it is absorbed on a solid matrix. This was the first step of the current immobilization technology. In these early years, inorganic carriers such as glass, alumina or hydrophobic compound-coated glass were used as surface. At that time some methods of immobilization such as covalent binding and physical adsorption were reported. However, these carriers did not give satisfying results in terms of activity retention of enzymes. This effect was probably reasoned by highly hydrophobic characteristic of the carriers used in these years or unsuitable active functionality of activation agents. In the 1960s the extent of bio-immobilization was developed with the use of more hydrophilic insoluble carriers such as cross-linked dextran, agarose, and cellulose beads. Also new activation techniques were studied for example cyanogen bromide and triazine for polysaccharide, isothiocyanate for coupling amino groups, and Woodward reagents for activation of carboxyl groups. Additionally, the range of enzymes to be immobilized is extended. In the further years, the awareness about supports has increased. New carriers of different physical or chemical characteristics, different hydrophilicity or hydrophobicity, different size or shape (i.e. beads, sheet, film, membrane) were studied. By the end of 1970s, it was known which method (entrapment, encapsulation, covalent attachment, adsorption or combination of these methods) and carrier (organic or inorganic, natural or synthetic, porous or non-porous, film, beads, foam, capsules or disks) and to use for immobilization of a certain enzyme. However in these years, the immobilized enzymes usually did not show considerable activity and stability in organic solvents compared to conventional aqueous media. Since 1990s, the focus of enzyme immobilization studies has been production of more robust immobilized enzymes, which can perform well in harsh conditions. (Ahmad & Sardar, 2015, Cao, 2006). Many studies have been

conduct with this objective. Figure 1.3 shows the number of publications between the years of 2000 and 2013 (Sulaiman et al., 2014).

The continuous increase in studies points out that there is always a need for novel methods and enzyme technology is open to new applications such as development of nanostructured materials as supports.

Some of the application areas of immobilized enzymes are given as reusable heterogeneous biocatalysts, selective adsorbents for purification of proteins and enzymes, controlled released protein drugs, stable and reusable analytical applications and solid phase protein chemistry (Cao, 2006). Biosensors are another field of application, which are used for detection of biological compounds. Biological

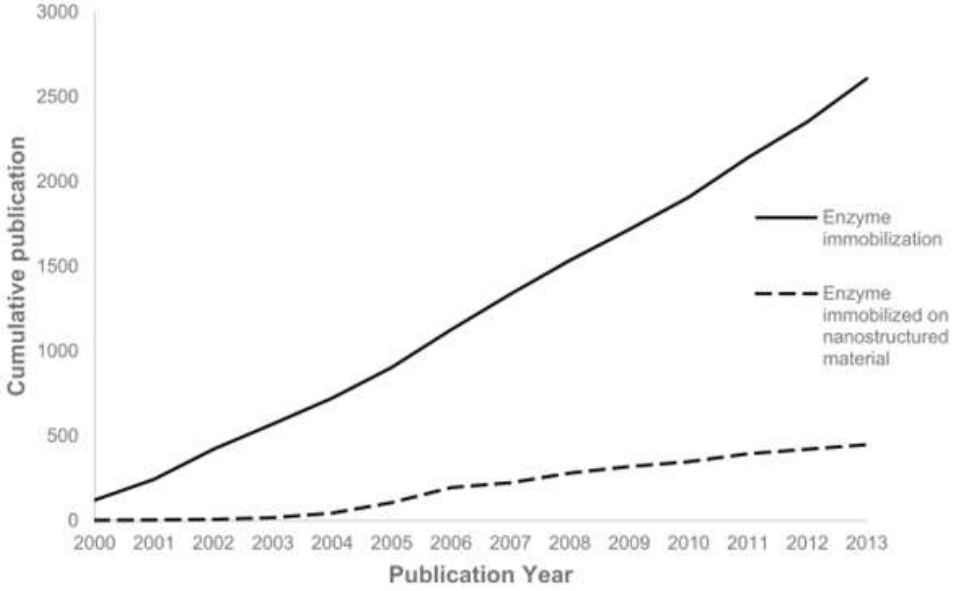

Figure 1.3 Number of publications between the years of 2000 and 2013 (Sulaiman et al., 2014).

detection molecules involve antibodies, enzymes and oligonucleotids. A good biosensor must work sensitively even if at low concentrations of the analyte. It must be able to distinguish the strange molecules that are bound on its surface. In addition to advantages of immobilized enzymes mentioned previously, low cost and relatively compact size of biosensors, make them important actors in detection of multifarious components. Biosensors can be used for medical purposes, environmental monitoring, microbiological and toxin detection in water and food (Khan & Alzohairy, 2010). There are two requirements, which an immobilized enzyme must fulfill. These include non-catalytic and catalytic functions. Non-catalytic functions comprises the ability of easy separation of the enzyme from processing environment, reusability. Catalytic

function involves the ability of turning the substrate into the targeted compound within the expected time and space. Catalytic parameters are activity, selectivity and stability (in harsh environment). Additionally, non- catalytic parameters are geometry, mechanical and chemical stability (Cao, 2006).

Laccase, pectinase, β-Galactosidase, trypsin, protease, pectin lyase are some examples of enzymes used in immobilized form in food industry. Immobilization supports that have been used in food industry include bone powder, anion exchange resin, silica gel, cellulose, agarose glutaraldehyde support, alginate beads, polyacrylic acid nanotubes, calcium alginate beads, duolite-A-568 and chitin (or chitosan) (Khan & Alzohairy, 2010).

1.2.2.2 Immobilization of β-galactosidase

β-galactosidase is utilized for production of non-lactose containing foods. It hydrolyses lactose, which causes problem in lactose-intolerant people, which are approximately 70% of the world population (as cited in Belhacene et al., 2015; as cited in Benavente et al., 2015). Lactose intolerance refers to deficiency of lactase or hypolactasia. Individuals who have lactose intolerance are not capable of digesting lactose due to deficiency of β-Galactosidase in their digestive system. Consuming considerable amount of lactose causes abdominal bloating, cramps, flatulence, diarrhea, nausea and vomiting in lactose-intolerant individuals. (Facin et al., 2015). It also stimulates formation of galactooligosaccharides, which have prebiotic functions. Inhibition of lactose crystallization and increase of sweetness during production of ice creams, condensed milk are other aims of use of β-Galactosidase (Benavente et al., 2015). Hydrolysis of lactose into glucose and galactose improves product quality and process efficiency in the dairy industry. Solubility and digestibility of dairy products are increased when compared to the lactose-containing foods (El-Aassar et al., 2013).

Commercial β-Galactosidase can be obtained from strains of *Kluyveromyces lactis*, *Bacillus circulans*, *Aspergillus Niger*, *Eschericia coli*, etc.. High prices of this enzyme make use of immobilized form of the enzyme more advantageous. By immobilization, it is possible to use the enzyme several times (as cited in Belhacene et al., 2015). In addition, low specific activity, low thermostability or high prevention of this enzyme by other reaction by-products are the some restrictions. In order to optimize the use of β-Galactosidase inhibition effect of by-products should be lowered to achieve full

elimination of lactose in a short time. Accordingly, applicability under a wide range of pH for the hydrolysis of lactose from acid whey, optimized transglycosylation process and enhanced stability by immobilization are needed (as cited in Benavente et al., 2015).

Oral intake of β-Galactosidase is possible by achieving controlled release by immobilization. The enzyme can be immobilized in a polymer matrix. It can also be encapsulated into tablets. However, the problem with tablets is fast release of the enzyme into the digestive system. β-Galactosidase can be chemically adsorbed by on an active porous polymeric matrix which enables controlled release. In this system release rate of the enzyme can be monitored (as cited in Facin et al., 2015).

1.2.2.3. Immobilization on carriers

The carrier material in immobilization attracts notice since it is directly in contact with enzyme. Interaction between support and enzyme may change the activity and the stability of the enzyme (Ye et al., 2005). Immobilization to a carrier can be applied by physical adsorption, ionic binding and covalent binding. Insoluble porous carriers are used as a standard. Adsorption of internal surface of the carrier, binding to ion exchangers, ultimately crosslinking to prevent desorption of enzyme during processing and covalent binding are used both in industrial and laboratory scale (Cao, 2006).

Types of carriers

Enzyme immobilization is widely applied on insoluble porous carriers. In order not to restrict diffusion of enzyme into the porous area pore should be sufficiently large and accessible. Diameters of technical enzymes are in the range of 4-8 nm. Accordingly pore diameter should be larger than 20 nm. Since excessed diameter of pores would decrease available internal surface, an adsorption isotherm showing the capacity of carrier for enzyme adsorption should be measured under optimal conditions (Buchholz et al., 2012). Particles should be favorably in regular shape and have a narrow particle size distribution for an optimal flow in a fixed bed reactor (Buchholz et al., 2012). The residual stationary charges on the pore surfaces affect the rate of immobilization of negatively charged enzymes. Residual charges on pore surfaces are mainly negative. It influences selectivity of negatively charged enzymes in interactions with charged substrates (Buchholz et al., 2012). Mostly used carriers are classified according to their

origins as inorganic, organic from natural sources and organic synthetic materials (Buchholz et al., 2012).

Inorganic carriers

Inorganic carriers have high pressure stability. Porous glass and silica are examples of inorganic carriers widely used in industry. (Buchholz et al., 2012). Magnetic iron oxide is also one of the inorganic supports. These materials show good mechanical stability, rigidity and regeneration character. However, their biocompatibilities are low and they are expensive (Ye et al., 2005). A study of adsorption of lipase onto a silica carrier showed high efficiency of adsorption (Buchholz et al., 2012). Functionalizing of carriers can be actualized by increasing the density of functional groups. SiO_2 carriers can be functionalized due to 3 amino groups bound to spacers (surface groups). It can be applied by treating with aminopropyltriethoxysilane (APTS). In order to have a stabile carrier in aqueous solution a complete functionalization is needed (Buchholz et al., 2012). Celite is a carrier, which is known for its good adsorption and stabilization capacity. Bentonite offers also a good adsorptive surface for enzymes such as penicillin amidase without inactivation. Crosslinking with glutaraldehyde hinders desorption while the carrier is entrapped in alginate for creating biocatalysts in proper size (Buchholz et al., 2012).

Polysaccharides

The source of the carrier surface is an important aspect. So that organic materials from natural sources show good compatibility with proteins with weak interactions and no inactivation effect. Polysaccharides have typical wide network structure and hydrophilic properties. Wide network structure infers a rather low mechanical and pressure stability. Biopolymers are widely used especially in food industry other related fields due to their nontoxic, edible and digestible, biocompatible and biodegradable, renewable and sustainable features (Buchholz et al., 2012; Kriegel et al., 2008) .

There are some limitations in fabrication of electrospun nanofibers from organic polymers. First, biopolymers may require complicated and expensive purification steps before electrospinning. They show less solubility in most of the organic solvents due to their high degree of crystallinity or high polarity (e.g.. chitosan, alginate). Additionally most of the biopolymers form strong hydrogen bonds which results with

high viscosity and gel formation. Nanofibers from biopolymers have low mechanical strength and they are generally sensitive to processing treatments. Electrospinning of biopolymers (polysaccharides and proteins) can be difficult because of their complex chemical structure and distribution of molecular weight. These problems may be partially overcome by blending organic polymers with synthetic polymers, which lead to entanglement of the polyelectrolyte macromolecules. The blending with synthetic polymers facilitates required linkage for electrospinning and increases biocompatibility of the nanofiber as improving mechanical strength. Chitosan, alginates, cellulose and cellulose derivatives, dextran and agarose are organic polymers used in nanofiber production (Buchholz et al., 2012; Kriegel et al., 2008; Zhao et al., 2011).

Synthetic polymers

Most of the fabrication of nanofibers is conducted by use or synthetic polymers due to low cost high availability and availability of well-defined molecular and functional characteristics. Besides, biopolymers such as polysaccharides have distributed molecular weights or have complex chemical structures (Kriegel et al., 2008). It is claimed that synthetic, water- insoluble nanofibers has showed higher mechanical and structural strength upon contact with aqueous environments compared to the nanofibers fabricated from biopolymers. Also, synthetic polymers have suitable chemical structure for production of uniform nanofibers. Except these, synthetic polymers such as poly ε-caprolacton (PCL), polyglycolic acid (PGA), polylac- tic acid (PLA), polyethyleneoxide (PEO), and polyvinylacetate, polyvinylalcohol (PVA) are utilized for fabrication of electrospun nanofibers (Fallahiarezoudar et al., 2014a; Kriegel et al., 2008).

Organic synthetic polymers show high chemical stability as they can easily be adjusted for a good compatibility with proteins. Polymeric ion exchange materials are widely used for their effectiveness. Ion exchange is the equivalent exchange of ions between two or more species including at least one ion exchanger phase (Zagorodni, 2007). Polypropylene, polyacrylonitrile and nylon are some examples of synthetic polymers. Due to their good mechanical stability, reactive functional groups and simple preparation in various geometrical shape, they can be used for enzyme immobilization (Ye et. al., 2005).

In order to produce a perfect support for enzyme immobilization, the biocompatibility of synthetic polymers or inorganic supports should be increased by generating a biofriendly surface on the support. Interactions between the enzyme and the support causes non-biospecificity, which reduces enzyme activity. Natural macromolecules such as chitosan, can be directly tethered onto synthetic polymers. Thus, a biomimetic layer for enzyme immobilization can be obtained (Ye et al., 2005).

Ion exchange materials are composed of three dimensional polymeric networks and physically trapped large molecules of organic electrolytes. Polymeric ion exchange materials include functional groups or functional sites in the structure differently from non-functional polymers (Zagorodni, 2007). Ion exchange materials (containing anion or cation exchangers) have been found to be economic and to have good enzyme loading capacity. Because of that, these polymers are widely used mainly due to cost effectiveness and simplicity of preparation. Polystyrene derivatives, polyacrylic ester derivatives are examples of commercial ion-exchange resins (Buchholz et al., 2012).

Methods of binding on a carrier

Adsorption

Physical or ionic adsorption of enzymes is a very old and simple technique. As a first step of immobilization, proteins are adsorbed on the surface. Immobilization is occurred by mixing the enzymes with an appropriate adsorbent under optimal conditions of pH and ionic strength. After adsorption the carriers are rinsed in order to remove poorly bound and unbound enzymes (Ahmad & Sardar, 2015). The rate of adsorption increases with increasing enzyme concentration. On the purpose of improving enzyme efficiency, the immobilization can be done quickly. By this application maximal enzyme loading is reduced and diffusion depth inside the carrier is shortened (Buchholz et al., 2012). Use of hydrophobic interfaces inhibits agglomeration of adsorbed enzymes. The adsorbent should be chosen carefully to minimize the leakage of the used enzyme. The preferred carrier surface should not lead chemical modification or harm to enzyme (Ahmad & Sardar, 2015). Molecular details for surface modification and enzyme loading by immobilization can be observed by several methods including AFM (atomic force microscopy), transmission Fourier transform infrared spectroscopy (FTIR) and x-ray photoelectron spectroscopy (XPS). Ion-exchange carriers with high efficiency are available in a broad range with

reasonable prices. In order to select the right carrier for enzyme immobilization optimal ratio of enzyme and carrier amounts, pH, buffer and temperature should be investigated. Stabilization of an adsorbed enzyme can be carried out by cross-linking with glutaraldehyde (Buchholz et al., 2012). The main disadvantage of physical adsorption is easy cleavage of the enzyme from the surface by the effect of changes in temperature, substrate and ionic concentrations (Ahmad & Sardar, 2015).

Covalent binding

Covalent binding is stated as the most stable binding method between enzyme and the support. It minimizes the dissociation of the enzyme from the carrier. Via covalent immobilization, an unlimited contact between the enzyme molecule and the substrate occurs. The stability is reached since an immobilized enzyme is not able to interact with any additional molecules. On the contrary, the free enzyme tends to aggregate and interact via a hydrophobic interface. Greater stability leads to higher resistance to temperature, decomposition, pH and organic solvents (Sulaiman et al., 2014).

Covalent binding is relatively difficult to apply especially for nanosized support materials since it requires a chemical coupling agents. The use of chemical activating agents should be conducted carefully due to their toxic and reactive characteristics. These characteristic may cause modification or alteration of the active site of the enzyme which reduces enzyme activity. As chemical coupling agents, ligands and spacer are mostly preferred as they can enhance binding efficiency, provide a good mobility and lower steric hindrance significantly (Sulaiman et al., 2014).

The increasing density of functional groups on carriers also increases binding the overall protein. In a study, for the functionalization of porous silica or glass, the carrier is treated with aminopropyltriethoxysilane in order to introduce amino groups. Right after, glutaraldehyde (GA) is used for activation of carrier. Subsequently enzyme binding takes place. After immobilization, the unreacted functional groups should be inactivated. This method is suitable for both laboratory and industrial scale due to its reasonable cost, effectiveness and simplicity. Surface activation is required to achieve a good yield of immobilization. However, it must be considered that GA solution is composed of oligomers, which may also react (Buchholz et al., 2012). It is reported that chitosan from trifluoroacetic acid (TFA) can be successfully crosslinked by one-step application of GA. GA is a homobifunctional crosslinker. It can interact with

chitosan via a Schiff base and/or through Michel-type adducts with terminal. These interactions cause imine functionality and formation of carbonyl groups respectively. It is also reported that GA improves conformational flexibility of enzymes bound to the surface. By surface activation, it is desired to obtain a high immobilization yield and high enzyme activity. Applying low concentration of GA results with low yield of immobilization (Auster et al., 2012; Pal & Khanum, 2011). As Schiffman and Schauer stated, chitosan nanofibers are soluble due to its high Young's modulus (tensile elastic modulus). Crosslinking results with decreasing of Young's modulus, hence dissolubility (2007). Since it is hard to immobilize enzymes on a soluble nanofiber mat, crosslinking was definitely needed. GA can be applied both in vapor form and liquid form. Ahmed Ali et al, applied the GA vapor in a desiccator (nd.). Considering this, 25% GA was filled in a dish and placed in a desiccator. Onto the ceramic plate of the desiccator, chitosan nanofibers were placed. It was allowed to be incubate for 48 hours at room temperature.

Surface activation can also be achieved by direct interaction of chitosan with varying GA concentrations. As Pan et al. (2009) stated, immobilization efficiency can be enhanced by increasing GA concentration up to 8%. Similarly, relative activity of the enzyme increases with increasing GA concentration and reaches maximum at 4% of GA. Above 4% concentration of GA relative activity decreases sharply. This effect is explained by the conformational changes of enzyme due to extensive interaction with GA. It should be also taken into consideration that GA is a compound which may cause damage in food and human health (Belhacene et al., 2015). Immobilization is recommended to be conducted in two steps including activation of the carrier as first and subsequent enzyme immobilization. In several studies the reaction is carried out in the range of minutes (at 25°C) or a few hours (at 4 °C) (Buchholz et al., 2012). Multipoint covalent immobilization provides stabilization and rigidification of enzymes against agents like heat, organic solvents, pH (Buchholz et al., 2012).

Entrapment

By entrapment, the flexibility and movement of the enzyme is restricted in a solid matrix such as porous gels or fibers. Entrapment may applied both by physical caging or covalent binding. Covalent binding (or crosslinking) is applied as a second step to strengthen the beads and avoid leakage. As a disadvantage, large molecules cannot reach the catalytic sites of an entrapped enzyme. It limits the catalytic activity in the

presence of large substrates. Various synthetic polymers such as polyvinylalcohol hydrogel, polyacrylamide and also natural polymers like agar, agarose, gelatin, alginate and carrageenan can be used as carriers of entrapment (Ahmad & Sardar, 2015; Cao, 2006).

Crosslinking

Crosslinking is defined as binding of biocatalysts to each other by bi- or multifunctional reagents or ligands. By crosslinking, very high molecular weight insoluble enzyme aggregates are obtained. Cross linked enzyme aggregates (CLEAs) have been developed in order to improve enzymatic activity. However it is not exactly an immobilization method since enzyme aggregates are not attached to any surface. Crosslinking is a relatively easy process (Ahmad & Sardar, 2015). Physical aggregates of protein molecules are precipitated from aqueous medium by addition of ammonium sulfate or polyethylene glycol. Subsequently crosslinking is carried out. As a result, most of the activity remains (up to 100%). This method combines two steps including purification and immobilization into a single unit. The enzyme used does not have to be highly pure. In a study, seven commercially used lipases were investigated in CLEAs form. They showed activities up to 12 times those of free forms (Buchholz et al., 2012). Part et al. (2013) reported that CLEA applications result in increased thermal and environmental stability and effective inhibition of enzyme denaturation by multipoint attachment. It also extends the storage time and enhances reusability. On the contrary, covalent-type bonding between enzyme molecules results in conformational changes which restricts the activity (Ahmad & Sardar, 2015).

On the Figure 1.4, a symbolic representation of different binding methods can be seen.

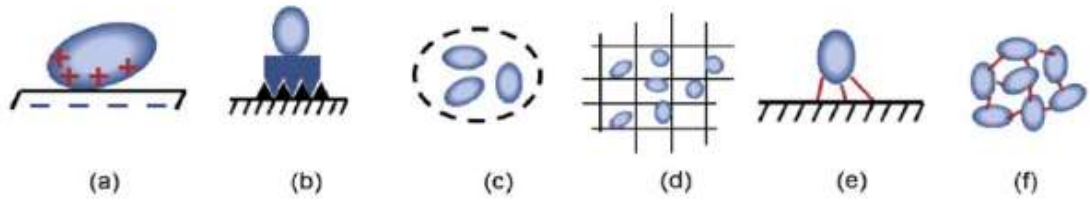

Figure 1.4. Symbolic representation of different binding methods (ionic physical adsorption (a), high-affinity physical adsorption (b), encapsulation (c),entrapment (d), covalent binding on an insoluble support (e), cross-linked enzymes (f)) (Zhang et al., 2011).

1.2.2.4 Performance of immobilized enzymes

The need for immobilization has occurred due to high cost of non-reusable soluble enzymes. However, immobilization has some shortcomings in addition to wide range of benefits. Although immobilization offers a possibility to reduce cost by reuse of enzyme, cost of carriers and immobilization method have been limitations against it. Use of immobilized enzymes in continuous processing leaded some mass transfer limitations (Buchholz et al., 2012). Despite the low residence time, that provides high volumetric activity, some problems were observed in multienzyme systems (Buchholz et al., 2012). Some other impacts of immobilization on performance of enzyme are given in the further sections.

Activity

While immobilized enzyme systems provide simple separation and recovery, they can change the sensitivity, selectivity of enzymes resulting in activity loss. The forces that lead interaction between surface-active groups of both of the matrix and of the protein. In addition, conformational changes also causes inactivation (Buchholz et al., 2012). Enzyme activity is given as enzyme unit (U). One enzyme unit represents the amount of enzyme that catalyzes 1 μmol of substrate per minute (Martinez Villaluenga et al., 2008). The catalytic effect of an enzyme may change according to varying environmental conditions.

It has been reported that many enzymes immobilized by different immobilization methods have higher activity than the free enzymes (Ahmad & Sardar, 2015). The yield of immobilization is affected by the method applied for immobilization, concentration, pH, temperature, the type of carrier and reaction time (Buchholz et al., 2012). The features of the external protein surface and the functional groups of enzymes have a great impact on binding to carrier surface. Hydrophilic and hydrophobic characteristics are highly effective on adsorption treatment. Ionic groups of enzymes and their interaction are related with the amino acids with overall surface charge. The charge of amino acids and their density are specifically dependent on the pH. According to the pH, overall charge is determined. Accessible functional groups on protein surface play a role in covalent binding. In practice, some of the amino groups such as lysine, arginine, and the carboxyl groups of aspartic and glutamic acid are utilized for covalent binding (Buchholz et al., 2012). For industrial and laboratory applications of immobilized enzymes, porous insoluble carriers are widely used

(Buchholz et al., 2012). Pores provide a larger surface area for enzyme loading. Pores should not be smaller than 20 nm and larger than 160 nm in diameter. Below 20 nm, pores do not serve a suitable surface area for binding (Buchholz et al., 2012).

As a summary, ionic, hydrophobic-hydrophilic, hydrogen bonding have an impact on interactions between carrier surface and enzyme as well as enzyme stability. When the interactions are too strong, undesirable and irreversible adsorption may occur which results in loss of enzyme activity or conformational changes of the tertiary structure of the protein. These changes were observed for multiple interactions on rigid carriers (Buchholz et al., 2012). It is inevitable that activity of immobilized enzyme increases compared with free enzyme. The decrease in activity may be caused by multipoint attachment of enzyme to surface, which restricts flexibility of enzyme. Thus, enzyme protein cannot easily adapt suitable conformation for catalysis. A second reason can be non-biospecific interactions between support and enzyme. It disrupts the conformation of enzyme and changes the variation of microenvironment. For redox enzymes, electron transfer between enzyme and carrier may be restricted by surface. It is another reason for activity decrease. It is also believed that use of coupling agents such as GA could cause structural change of enzymes which refers to a decrease of activity (as cited in Cao et al., 2012). Considering these factors, activity and stability of immobilized enzymes can be enhanced by tailoring the surface chemistry of nanofibers (Wang et al., 2009).In order to avoid inactivation, adsorption of cheap inactive proteins can be applied as a protection (Buchholz et al., 2012).

Stability and reusability

As already mentioned, stabilizing effect of immobilization can be obtained by applying multipoint covalent binding (Buchholz et al., 2012). In this study, enzyme stability is accepted as sustainability of the catalytic activity of the enzyme. The stability of an immobilized enzyme is dependent on many parameters, such as the nature of interaction with the surface, binding position and number of bonds, the micro-environment in which the enzyme is used, the chemical and physical characteristic of the carrier, spacer properties (charged or neutral, hydrophilic or hydrophobic, size, length), flexibility of conformational change and the environmental conditions in which the enzyme was immobilized. By the effect of time, temperature, experimental and storage conditions; the stability may change positively or negatively (Ahmad & Sardar, 2015). Reusability of an enzyme means use of an enzyme repeatedly in a

process. After each use, in most cases enzyme activity decreases. A high residual activity after a certain number of cycle of use means that the immobilization surface is suitable for reuse of the enzyme. Thermal stability and reusability of β-Galactosidase, which is immobilized on surfaces with different characteristics, is given on Table 1.2 from the literature.

Table 1.2. Stability, reusability and immobilization efficiencies of immobilized β – galactosidase ([1]: not applicable).

SUPPORT SURFACE	IMMIBILIZATION EFFICIENCY	THERMAL STABILITY	REUSABILITY	REFERENCE
magnetic chitosan nanoparticles	78% at pH 6	80% activity loss (from 30 to 80°C)	92% activity after 15 cycles	Pan et al., 2009
Chitosan	na[1]	95% activity loss (from 45 to 70 °C)	na	Klein et al., 2013
carrageenan coated with chitosan (hydrogel)	50% (opt. pH 5-5,5)	up to 100% activity loss (from 55 to 70 °C)	97% activity after 9 cycles	Elnashar & Yassin, 2009
silver nanoparticles	93%	25% activity loss (from 50 to 80)	88% after 6 cycles	Ansari et al., 2012
nanofibers of poly (AN-co-MMA) copolymer	na	50% activity loss (from 45 to 70)	na	El-Aassar et al.,2013
insoluble carrier Eupergit C	95%	50% activity loss (from 50 to 60)	na	Nakkharat et al., 2006
Chitosan	na	na	80% after 4 cycles	Gaur et al., 2006
Silica gel	na	na	50% after 10 cycles	Song et al., 2010
Aggregated silica nanoparticles	na	50% activity loss (from 50 to70)	94,2% after 9 cycles	Wu et al., 2013
silver nanoparticles	na	20% activity loss (from 50 to 70)	80% after 6 cycles	Agnps et al., 2015
Activated agar disks	na	60% activity loss (from 50 to 65)	90% after 15 cycles	Wahba et al., 2015
PVA lenses	89%	na	95% after 7 cycles	Jovanovic-Malinovska et al., 2012

2. MATERIALS AND METHODS

2.1.Materials

Low molecular weight chitosan and polyvinyl alcohol (PVA) were used as materials of nanofibers. As solvent, acetic acid, trifluoroacetic acid (TFA) and dichloromethane (DCM) was used to dissolve chitosan. A 25% glutaraldehyde (GA) solution was purchased for activation of the nanofiber surface. β-Galactosidase was used as the enzyme. As a substrate, o-Nitrophenyl ß- D – Galactopyranoside (ONPG) was studied. Sodium carbonate (Na_2CO_3) solution was used in enzyme activity assay to stop the reaction of ONPG and β-Galactosidase. Enzymatic activity was determined by using a UV visible spectrophotometer.

2.2.Methods

2.2.1. Production of uniaxial chitosan nanofibers

First step was finding the right solvent for chitosan. Firstly, a solution of 90% (v/v) acetic acid was prepared to produce chitosan nanofibers. Chitosan was added in ratio of 3% (w/v). Low molecular weight chitosan was used, since use of high molecular weight chitosan causes increase in nanofiber diameter (Zhao et al., 2011). Molecular weight of the chitosan was between 50-190 kDa. The solution was filled into a syringe. The syringe was placed on the pump and the feeding rate was adjusted to 0.3 ml/hour. A voltage of 25 kV was applied. The distance of the collector plate from the needle was 7 cm.

In order to increase the electrospinning character of chitosan, PVA was added to chitosan-acetic acid solution (Huang et al., 2007). PVA was dissolved in pure water in ratio of 9% (w/v). Chitosan solution was prepared in ratio of 3% (w/v) in 90% acetic acid. PVA and chitosan solutions were blended in ratio of 70:30. The concentrations were selected according to the method of Huang et al. (2007). A feeding rate of 0.45 ml/h was applied at 25 kV. The distance of the collector plate was 10 cm.

Within the scope of this study, chitosan nanofibers were produced using electrospinning method. A solution of TFA and DCM was prepared in the ratio of 70:30 (As cited by Sun and Li, 2011). Chitosan was dissolved in TFA-DCM solution at 3% concentration (As cited by Zhao et al., 2011). Chitosan nanofibers were fabricated at 25 kV with feed rate of 0.4 ml/h. Distance of the plate from needle was changed between 10 and 15 cm. Nanofibers were collected on an aluminum foil and tore off after operation. A membrane-like structure was observed on the aluminum foil (Figure 2.1).

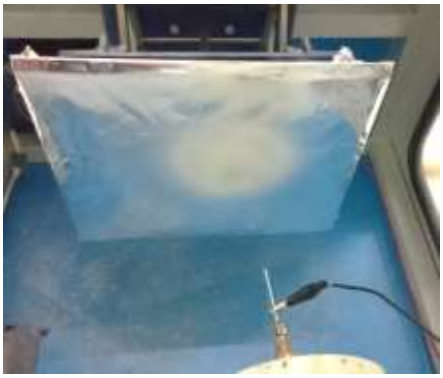

Figure 2.1. Chitosan nanofibers on the collector plate

2.2.2. Surface activation

Chitosan nanofibers were weighed as 5 mg pieces as in the study of Park et al. (2013). Nanofibers were treated with GA in two different ways. Firstly, all chitosan nanofibers were exposed to the vapor of 25 % GA for 48 hours in a desiccator (Ahmed Ali et al., nd.). Firstly, 10 ml of 25% GA was placed into the desiccator in a petri dish. The chitosan nanofibers were put on the perforated plate which belongs to the desiccator. The cap of the desiccator was closed carefully. After 48 hours, the desiccator was opened and the nanofibers were washed with deionized water in order to remove excess GA from the nanofiber surface, since the residuals can interact with active sites of the enzyme. Enzyme immobilization efficiency was measured. Since enzyme loading efficiency was found too low when compared to the efficiency values on the Table 1.2, it was decided to apply the GA directly to the nanofibers which were previously treated with GA vapor. A 4% solution of GA was prepared in deionized water. This concentration of GA was selected according to the method of Pan et al. (2009). The purpose was to achieve the maximum immobilization efficiency by applying the most suitable GA concentration. The nanofibers were immersed in 1 ml of 4% GA solution and were treated in an orbital shaker at 28 °C, 150 rpm for 3 hours.

Shaking rate and the time was adjusted according to the study of Pal and Khanum (2011). Nanofiber mats were collected and washed 3 times with deionized water in order to remove excess GA from the nanofiber surface.

2.2.3. Immobilization of β-galactosidase

β-galactosidase from *Aspergillus oryzae* (Enzyme Comission number: 3.2.1.23) was selected to immobilize on chitosan nanofiber. It was containing ≥8 units/ mg solid. 1 unit of the enzyme is defined as the amount that hydrolyzes 1μmole of ONPG or lactose per minute. After functionalization with GA, aqueous solutions of β-Galactosidase were prepared in two different concentrations such as 0.025 mg/ml and 0.25 mg/ml. The concentrations were selected according to the method of Sigma, which is clarified in the section 2.2.4.

5 mg pieces of nanofiber functionalized only with GA vapor were taken into tubes and submerged in the 3 ml of enzyme solution (0.025 mg/ml) and shaken gently in an orbital shaker at 40 °C for 1 hour. After that, the nanofibers in the enzyme solution were kept at 4 °C for the next 16 hours. At the end of 16 hours the nanofibers were taken out and rinsed with deionized water to remove excess enzyme from the nanofiber surface and stop the immobilization. This treatment will be mentioned as the"1st treatment" in the next sections. The method of El-Aassar et al (2013), was taken as a reference at this immobilization step.

5 mg pieces of nanofiber which were activated with both GA vapor and 4% GA solution, were put into the tubes. 3 ml of enzyme solution (0.025 mg/ml) was added to each tube. The tubes were shaken in an orbital shaker for 20 hours (150 rpm, 30°C). After 20 hours, nanofibers were rinsed with deionized water. This treatment will be mentioned as the "2nd treatment" in further sections

Since increasing enzyme concentration also increases the immobilization efficiency, an enzyme solution with the concentration of 0.25 mg/ ml was prepared. 3 ml of enzyme solution and 5 mg of the nanofiber were taken into tubes and the tubes were put in an orbital shaker. The orbital shaker was adjusted to 150 rpm at 30 °C. The nanofibers were shaken gently for 20 hours. After 20 hours, nanofibers were rinsed with deionized water. This treatment will be mentioned as the "3rd treatment".

2.2.4. Measurement of enzyme activity and enzyme loading efficiency

Enzyme loading efficiency was determined by measuring the activity of the enzyme solution at the beginning and after the immobilization. At the end of the immobilization, chitosan nanofibers were taken out. A certain amount of the enzyme in the solution was immobilized by the nanofiber. The enzyme solution remained in the tube is stored for determination of the immobilization efficiency. The enzymatic activity of the remaining solution was compared with the activity of the enzyme solution used at the beginning. Measurements were conducted in a spectrophotometer at 405 nm, using o-Nitrophenyl ß- D – Galactopyranoside (ONPG) as a substrate. The method of Sigma, which is named as "Enzymatic Assay of β-Galactosidase" (EC 3.2.1.23), was used as a base at this step (Anon. 1., 1994). The principle of the assay is based on the spectrophotometric stop rate determination of the enzymatic reaction. ONPG is a synthetic compound, which is cleaved in the presence of β-Galactosidase into o-Nitrophenol and β-D-Galactose. The absorbance of o-Nitrophenol solution is read spectrophotometrically. A 2 mM pH 6.0 solution of ONPG is prepared with buffer solution. The enzymatic reaction should be stopped before testing on spectrophotometer. A 1000 mM sodium carbonate solution (Na_2CO_3) is prepared to stop the reaction. Enzyme solutions are prepared in cold deionized water. The procedure includes preparation of a test solution and blank solution. Firstly, 500 µl of ONPG solution, 300 µl of deionized water were added into two different tubes named as test and blank tube for each replicate. The tubes are mixed by inversion. 200 µl of enzyme solution is added to test tube and mixed by inversion. The tubes are incubated for 10 minutes. 4 ml of Na_2CO_3 solution is added to each tube to complete the volume to 5 ml. Lastly 200 µl of the same enzyme solution is added to the blank tube and the absorbance is read for each tube at 405 nm. According to the method of Sigma, the enzyme solution, which will be used, should contain 0.02-0.04 unit/ml of ß-Galactosidase in cold deionized water. Because of that, concentrations of enzyme solutions were determined predicting the final enzyme concentration after immobilization. The enzyme concentration of the enzyme solution should be between 0.02 and 0.04 unit/ml after immobilization. The immobilization efficiency was assumed 90% at most by taking into consideration the values on the Table 1.2. After immobilization, 10% of the enzyme is expected to be left in the solution. This amount of enzyme should be measurable spectrophotometrically. Thus, this remaining 10% of

the total enzyme content should involve at least 0.02 and 0.04 unit/ml according to the method of Sigma. The final concentration of the enzyme enzyme solution was assumed to be 0.02 unit/ ml. Since the enzyme contains more than 8 units per mg solid, ≥ 0.02 unit enzyme corresponds to 0.0025 mg solid enzyme. In order to leave 0.0025 mg solid enzyme ml after immobilization, the enzyme solution should include 0.025 mg solid enzyme/ml at the beginning, since the immobilization efficency was expected to be 90% at most.

The assays were each conducted in triplicate. Three enzyme solutions were prepared and three replicates were used for each enzyme solution. The exact amount of enzyme in the solution was determined according to the equation, which was obtained from the calibration curve. The enzymatic activity was calculated according to the equation 2.1 and 2.2 (Anon. 1., 1994). The relative enzymatic activity was determined using the equation 2.3.

$$\frac{Units}{ml\ enzyme} = \frac{\left(Abs\ (Test) - Abs\ (Blank)\right) \times 5 \times Df}{10 \times 4.6 \times 0.2} \tag{2.1}$$

$$\frac{Units}{mg\ solid} = \frac{\frac{units}{ml\ enzyme}}{\frac{mg\ solid}{ml\ enzyme}} \tag{2.2}$$

5: Total volume (in milliliters) of assay

Df: Dilution factor

10: Time of assay (in minutes) as per the Unit Definition

4.6: Millimolar extinction coefficient of o-Nitrophenol at 405 nm

0.2: Volume (in milliliters) of enzyme used

$$Relative\ Activity = \frac{A_T}{A_{Tmax}} \times 100 \tag{2.3}$$

A_T: Enzymatic activity at the temperature of T

A_{Tmax}: Maximum enzymatic activity of the enzyme at a certain temperature degree

The efficiency of immobilization was calculated using the equation 2.4.

$$Ei = \frac{Ab - Af}{Ab} \times 100 \quad (2.4)$$

Ei: Efficiency of immobilization

A_b: Activity of the enzyme solution at the beginning

A_f: Activity of the enzyme solution at the end

2.2.5. Measurement of thermal stability

Thermal stability of β-Galactosidase was tested for both immobilized and free form at 30°C, 50°C, 70°C and 90°C. The temperature values were selected according to the method of Pan et al. (2009). Enzymatic assay was conducted according to the procedure of Sigma (EC 3.2.1.23) which was explained above. After the efficiency of immobilization was determined according the equation 2.4, the amount of enzyme which was arrested on the nanofiber was identified. The similar amount of solid enzyme was used to prepare 3 enzyme solutions as replicates. These enzyme solutions was used to measure the activity of the free enzyme at different temperatures. 500 µl ONP, 300 µl deinozed water and 200 µl of the free enzyme solution was used do determine the activity at different temperatures. The tubes were firstly incubated in a 30°C water bath for 15 minutes. When incubation has ended, Na_2CO_3 was added to stop the reaction. The absorbances of the reaction tubes and blanks were measured at 500 nm. This treatment was repeated at 50°C, 70°C and 90°C. In order to test the thermal stability of the immobilized enzyme, 5 mg pieces of chitosan nanofibers are put into the 3 different tubes. 500 µl ONP solution and 500 µl deionized water was added on the nanofibers. The tubes were incubated at 30°C water bath for 15 minutes. At the end of 15 minutes the tubes were taken out and 4 ml of Na_2CO_3 solution was added to each tube. After the reaction was stopped, the absorbance was measured with spectrophotometer at 500 nm. It was repeated at 50°C, 70°C and 90°C. Measurements at each temperature value were conducted in triplicate. The activity of β-Galactosidase was calculated by the Equation 2.1.

2.2.6. Measurement of reusability

At this step, the reusability of immobilized β-Galactosidase was tested. The reusability was assayed by testing the activity of the enzyme after each use. The activity of the enzyme after the first use is determined as 100% of activity. The enzymatic assay was conducted using the procedure given in the Section 2.2.4. 5 mg pieces of chitosan nanofibers were taken into tubes. 500 µl ONPG solution and 500 µl deionized water were added to the tubes. 5 nanofiber pieces were used as repetition. Reusability was tested 2 times with different testing conditions. The first set of nanofibers was incubated for 40 minutes in 40 °C water bath for each use. The second set of nanofibers was incubated for 15 minutes at 40°C for each use. Nanofiber mats were used 10 times. After each use, nanofibers were rinsed with deionized water in order to remove remaining o-Nitrophenol. Excess water was removed as much as possible from the nanofiber surface.

3. RESULTS AND DISCUSSION

3.1. Electrospinning

As acetic acid was used as the only solvent, at the end of a 15 hour operation no reasonable amount of nanofibers was observed on the collector plate. Since it is hard to obtain an electrospun nanofiber membrane from pure chitosan, it is important to use a good solvent (Huang et al., 2007). PVA is known for its interference with chitosan through hydrogen bonding at molecular level. It provides a good electrospinning in aqueous medium. After electrospinning, nanofiber is treated in a NaOH solution, in order to remove PVA (Huang et al., 2007). By considering this, PVA was used as an additive. After a couple of hours of operation with PVA, a visible nanofiber membrane as observed on the aluminum foil. The photo of the aluminum foil is given in Figure 3.1.

Figure 3.1 Nanofiber on an aluminum foil from chitosan and PVA dissolved in acetic acid.

At the end of a 21 hour application, 0.24 g chitosan-PVA nanofiber was produced. Taking into account that a considerable amount of this nanofiber is PVA, this operation did not seem efficient enough to obtain chitosan nanofiber. In addition, removal of PVA means an extra treatment.

Finally, the blend of TFA and DCM was used as a solvent. At the end of 95 hours of operation, 1.35 g of chitosan nanofiber was obtained which was found to be enough for enzymatic assay. TFA is known as a good dissolvent of chitosan. However, it is a

highly volatile caustic acid which makes it unsafe to study with. Considering this, firstly acetic acid was used as a solvent since it is a safe alternative compared to TFA. However TFA was found to be more advantegous for production of chitosan nanofibers.

3.2. Surface Activation and Immobilization of β-Galactosidase

At this step the chitosan nanofibers were activated in order to obtain a good immobilization efficiency. After that, the enzyme was immobilized and the effect of different immobilization and activation conditions were compared by measuring enzyme activity for each case. Surface activation was conducted by use of GA. As a visual result, the color of nanofibers were turned to straw yellow from white. Figure 3.2 shows a chitosan nanofiber which was exposed to GA vapor.

Figure 3.2 Chitosan nanofiber treated with GA.

As a second application, the nanofibers which were treated with GA vapor, were immersed in 4% GA solution for 3 hours. As a result of GA applications, nanofibers have gained a brittle structure. Activated nanofibers were incubated in enzyme solutions for immobilization of β-galactosidase. Two different concentrations of enzyme (0.025 and 0.25 mg ml) were applied as explained in the section 2.2.3. Enzyme activities of enzyme solutions were measured before immersing the nanofiber in the enzyme solutions. After completion of immobilization, the activity of the enzyme

solutions were measured again. . The amount of enzyme in mg/ml was calculated using the calibration curve-1 and -2 which are given in Figure 3.3 and Figure 3.4.

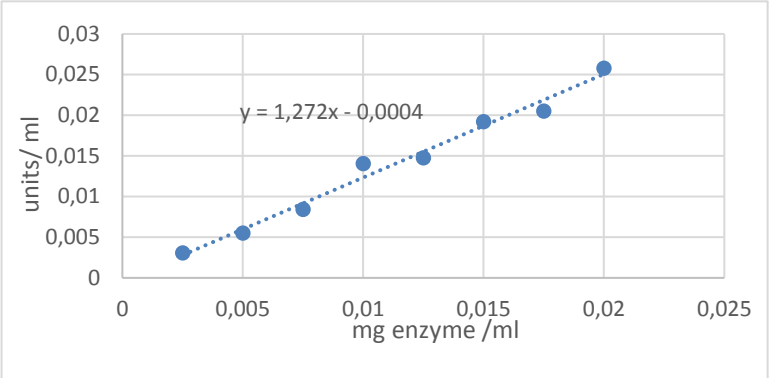

Figure 3.3 Calibration curve-1 (for the enzyme solution with concentration of 0,025 mg/ml).

Calibration assays were conducted in triplicate.

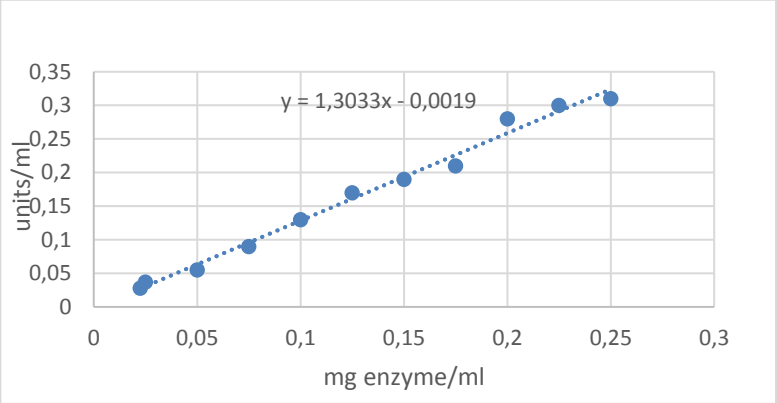

Figure 3.4 Calibration curve-2 (for the enzyme solution with concentration of 0,25 mg/ml).

Enzyme loading efficiency of chitosan nanofibers from 1st, 2nd and 3rd treatments are summarized in Table 3.1. According to the table, enzyme loading efficiency for 1st treatment was found as 13±0.2% which is too low considering the data from literature. After obtaining this result, another method of immobilization was applied as 2nd and 3rd treatment. For the enzyme, solution with concentration of 0.025 mg/ml immobilization efficiency was calculated as 59±20%, which seemed considerable when compared to applications given on the Table 1.2. When concentration of enzyme solution was enhanced up to 0.25 mg/ml, immobilization yield also increased up to 69±20%. It means 69±20% of the enzyme in the enzyme solution was immobilized on the chitosan nanofiber.

Table 3.1. Efficiency of immobilization and treatment conditions ([1]: Immobilization at 40°C for 1 hour, incubated at 4°C for 16 hours, pH 6; [2]: Immobilization at 30°C for 20 hours, pH 6).

	1st treatment	**2nd treatment**	**3rd treatment**
GA activation	Exposed to GA (25%) vapor[1]	Exposed to GA vapor and immersed in GA solution (4%)[2]	Exposed to GA vapor and immersed in GA solution (4%)[2]
Concentration of enzyme solution	0.025 mg/ml	0.025 mg/ml	0.25 mg/ml
Immobilization efficiency	13±0.2%	59±20%	69±20%

As stated by Mariotti et al. (2008), increasing concentration of enzyme solution up to 20 mg/ml enhances the immobilized enzyme activity. The increase in activity of the immobilized β-galactosidase in this study has verified this information. The same result was obtained in the study of Pan et al. (2009). The reason was explained as the increase of the chance that the attachment with reactive GA. However, this phenomenon is not valid for higher concentrations. After some point of enzyme concentration, activity remains constant in the study of Mariotti et al. (2008). Pan et al. (2009) declared that the activity was at its maximum at a concentration of 0.5 mg/ml. Higher concentrations resulted in sharp decrease of activity. This effect was believed to be a result of enzyme aggregation at higher concentrations of enzyme solution. Immobilization of 69±20% of the enzyme on a 5 mg of nanofiber was interpreted as an acceptable degree of immobilization compared to the values given on the Table 1.2. Pan et al. has achieved 78% immobilization efficiency of β-galactosidase using magnetic chitosan nanofibers (2009). Ansari et al., reported an immobilization efficiency of 95% on silver nanoparticles (2012). Considering these results, immobilization efficiency on the chitosan nanofibers can be improved by changing process conditions. The changes in enzyme content of enzyme solutions before and after immobilization are given in Figure 3.5 for 1st, 2nd and 3rd treatments. The assays were conducted in 3 repeats with 3 replicates for each repeat. Thus 9 samples were examined in total for each test. Standard deviations are also shown on the Figure 3.5.

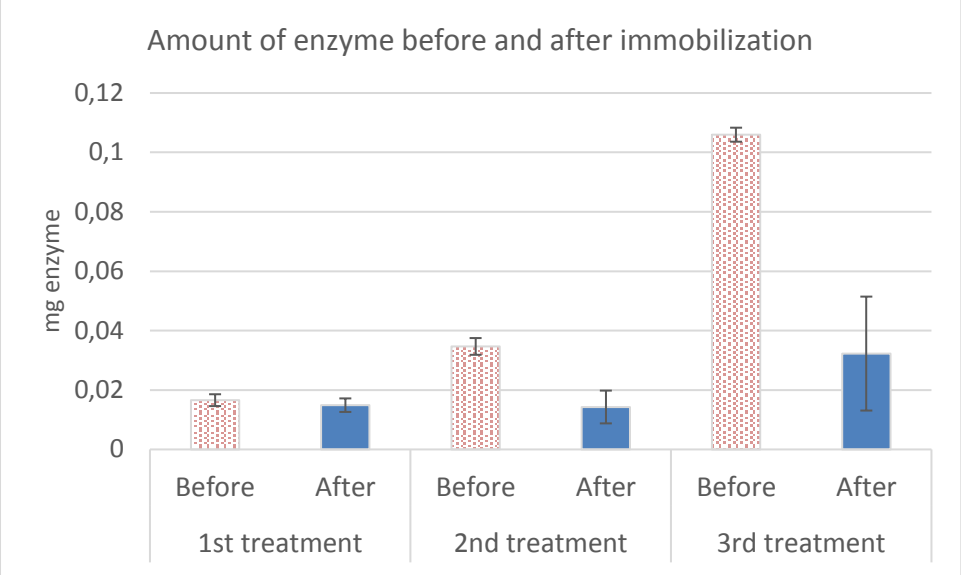

Figure 3.5 Changes in amount of enzyme in three different enzyme solutions (1st treatment: GA vapor treatment and enzyme solution with 0.025 mg/ml concentration, 2nd treatment:GA vapor and GA solution treatment and enzyme solution with 0.025 mg/ml concentration, 3rd treatment: GA vapor and GA solution treatment and enzyme solution with 0.25 mg/ml concentration).

3.3 Thermal Stability and Reusability

Thermal stability of immobilized and free β-Galactosidase was tested at 30 °C, 50°C, 70°C and 90°C. The activities are given with standard deviations on the Figure 3.6. It was observed that, at 50 °C enzymatic activities of both immobilized and free form were at their highest level. The activities of the immobilized and free enzyme decreased when the temperature was increased. At 70°C the activity decreased sharply for free β-galactosidase, as the decrease in the activity of the immobilized enzyme was slight.

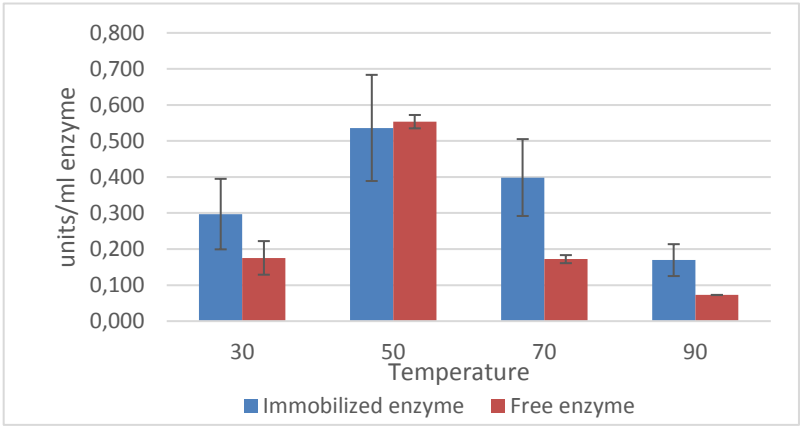

Figure 3.6 Activity of the immobilized and free enzyme at varying temperatures (15-minutes incubation at each temperature, pH 6, replicates:3).

The activity of immobilized β-Galactosidase was more than 2-fold higher than the activity of the free form at 70˚C. At 90°C the difference in activities of free and immobilized enzyme has decreased. However, the activity of the immobilized enzyme was still higher than the free enzyme. The activity of the immobilized and free enzyme were compared using one-way ANOVA. According to the test, there was no significant activity difference between immobilized and free enzyme (α=0.05). The statistical results were given on the Table 3.2.

Table 3.2 One-way ANOVA test results of immobilized and free enzyme

ANOVA

Activity		Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)	,004	1	,004	3,066	,097
	Linear Term Contrast	,004	1	,004	3,066	,097
Within Groups		,021	18	,001		
Total		,025	19			

In order to observe the percental change of the activity, activities at 30, 70 and 90˚C were expressed relatively to the activity at 50˚C, since the highest activity was observed at 50˚C. The activities of the immobilized and free enzymes were accepted as 100% at 50˚C. At 70°C the activity of the free enzyme decreased sharply to 31±0.01% of its activity at 50°C as the activity of the immobilized enzyme was lowered to 73,5±0.1%. Changes in relative activities of immobilized and free enzyme are given in the Figure 3.7. When the temperature was increased up to 90°C, the immobilized enzyme lost approximately 70% of its activity as the free enzyme lost around 87 % of its relative activity.

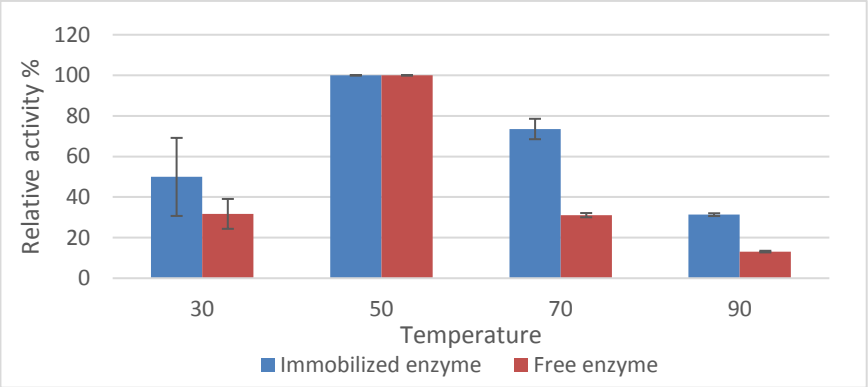

Figure 3.7 Relative activity of the free and immobilized enzyme at varying temperatures (15-minutes incubation at each temperature pH 6, replicates: 3).

The results of thermal stability testing were compared with examples in literature. Increasing stability of immobilized enzyme is believed to be related to improved stabilization by the multipoint covalent attachment (Pan et al., 2009). In the study of Pan et al. (2009), activity of β-Galactosidase was tested between 30-80 °C. Unlike my study, highest activity was observed at 30°C. The activity of free β-galactosidase decreased rapidly when compared to the immobilized form. The final relative activity of immobilized enzyme was higher than the free form. They claimed that the immobilized enzyme could work at high temperatures and challenging environmental conditions. On the Figure 3.7, it can be observed that relative activity of immobilized enzyme is obviously higher than the free enzyme 70°C. Some studies has shown similar results in literature. In the study of Klein et al. (2013), the immobilized enzyme was more active in a wider range of temperature. This result was attributed to the effect of immobilization. The investigation of Ansari et al. (2012) also points out that the relative activity of covalently immobilized β-Galactosidase is significantly stabilized than the native enzyme at high temperatures. The activity decrease at high temperatures was explained as denaturation of enzyme molecules, which resulted in the rupturing of polypeptide chain and degradation of polymer matrix. In their study, free and immobilized enzyme showed the maximum activity at 50°C. At 80°C, the decline in relative activity of immobilized enzyme and free enzyme was approximately 25% and 70% relatively. At 90˚C, the residual activities of immobilized and free enzyme were approximately 30% and 15% relatively. In the study of El-Aassar et al. (2013), the decline of relative activity was found approximately 50% and 70% relatively for immobilized and free enzyme (between 45°C and 70°C). Elnashar and Yassin (2009) investigated the effect of temperature on β-Galactosidase, which was immobilized on chitosan-coated carregenan. The activity assay between 30-70°C, showed that the immobilized enzyme had its highest activity at 45-55°C. In this range, the immobilized enzyme was stable. The free enzyme reached its maximum activity at 50°C. However, the activity of the free enzyme decreased sharply after this point. In the study of Elnashar and Yassin (2009), the use of the immobilized enzyme seemed advantageous at the temperature range of 45-55°C. At higher temperature values, relative activity of immobilized enzyme decreases significantly even beyond the free form. In my study, immobilized β-Galactosidase was clearly more durable at 70˚C. This result showed similarity with the results given from literature.

The reusability test gave distinctive results for two different nanofiber sets. The structure of the nanofiber was firm but not fragile for the most of the nanofibers. In the first set of nanofibers, some of the nanofibers were relatively soft and tended to disperse during regaining. The Figure 3.8 shows some of the recycled nanofibers.

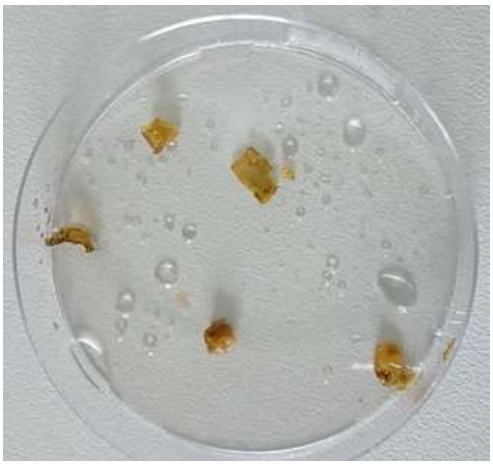

Figure 3.8 Recycled chitosan nanofibers.

Two different testing conditions were applied. The first set of nanofibers which were incubated for 40 minutes for each cycle, has retained lower activity at the end of the 10 cycles compared to the nanofibers which were incubated for 15 minutes for each cycle. After 10 cycles of use, first nanofiber set preserved 28±7% of its initial activity. However, second nanofiber set kept 68±13% of its initial activity after 10 use.

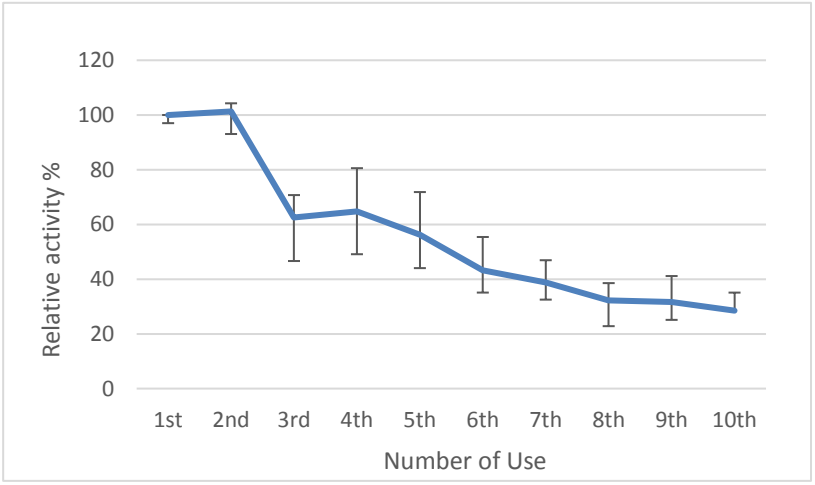

Figure 3.9 Reusability of the first nanofiber set (40 minutes incubation for each cycle at 40 °C in condition of pH 6, replicates: 5).

The reason of this difference between the two sets of nanofibers can be explained from the point of processing time. The first nanofiber set was incubated for 400 minutes at 40°C in total. The incubation time of the second nanofiber set was 150 minutes for 10 cycles of use. It can be interpreted that the exposure to heat for long time can decrease

the enzymatic activity. It was already mentioned that after each use nanofibers were rinsed with deionized water. During washings, it was observed that the first nanofiber had the tendency to rupture easily. The big difference in activity may be caused by the loss of nanofiber mat during washings. The tendency to rupture may be due to long exposure to heat or nonhomogeneous structure of chitosan nanofiber mat. Ansari et al. (2012) investigated the effect of processing time on activity of β-Galactosidase during exposure of heat. They incubated the immobilized enzyme at 60 °C for 120 minutes. The activity of β-Galactosidase which was immobilized on GA treated silver nanoparticles, decreased with time. The activity retention was about 70% at the end of the 120 minutes. Also in the study of Gaur et al. (2006), at the end of a 2 hour incubation at 60°C the residual activity was about 30% (β-Galactosidase immobilized on chitosan). It may clarify the distinctive decrease in stability of the first nanofiber in my study.

Figure 3.10 shows changes in relative activity of the enzyme on the nanofiber 2.

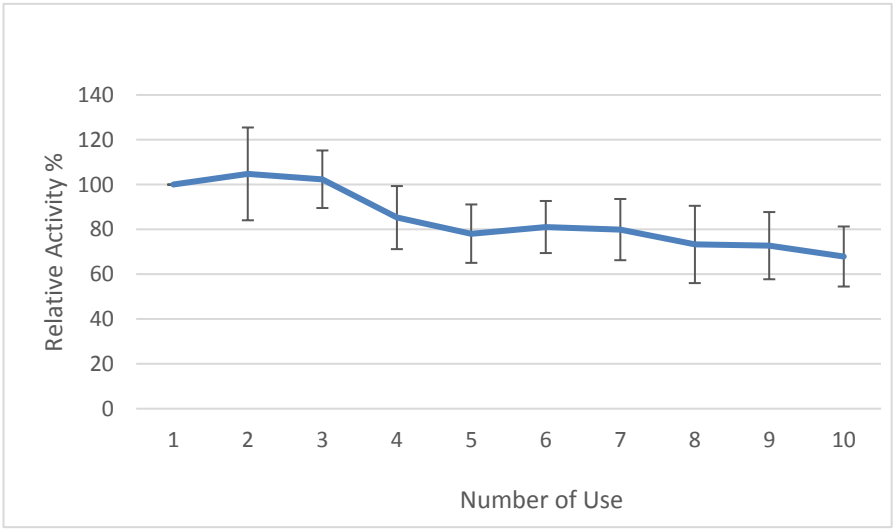

Figure 3.10 Reusability of the second nanofiber set (15 minutes incubation for each cycle at 40 °C in condition of pH 6, replicates: 5).

Independent samples t-test was applied to these two sets of nanofibers. The results are given on the Table 3.3. As a result of Levene's Test, the variances were not assumed equal. Since significance is higher than the 0.05; null hypothesis was accepted. There was found no significant difference between two sets in terms of mean of the activities (α=0.05).

Tablo 3.3 Results of independent samples test for reusability assay

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Activities	Equal variances assumed	8,505	,009	-1,751	18	,097	-,02678	,01530	-,05892	,00535
	Equal variances not assumed			-1,751	11,46	,107	-,02678	,01530	-,06028	,00672

4. CONCLUSION

With the scope of this study, the use of chitosan nanofibers for enzyme immobilization was investigated. The study gave promising results in terms of potential industrial applications. It was concluded that exposure to direct GA in addition to GA vapor, increases enzyme loading efficiency. Besides, increasing concentration of enzyme, also enhanced immobilization yield up to 69±20%. This degree of immobilization may be enhanced by applying different processing conditions taking into consideration the other studies. In thermal stability assays, the enzyme-chitosan nanofiber mat retained a good activity (31±0.4% of its activity at 50°C) even at 90°C when compared to the data from literature. The activity decrease with rising temperature was lowered by immobilization on to chitosan nanofibers. It points out that the β-galactosidase, which is immobilized on the chitosan nanofibers can be used at high temperatures. In reusability tests, it was observed that long exposure to heat (40˚C) causes an obvious decrease in relative activity. Hence, it can be concluded that enzyme immobilized on chitosan nanofibers are not suitable for long time-high temperature applications. Reusability assay of the chitosan nanofiber also gave acceptable results for processing time of 15 minutes for each use. After 10 cycles of use of immobilized β-Galactosidase, 68±13% of the initial enzyme activity was retained. During the reusability tests the chitosan nanofiber mats performed well with regard to recycling due to their rigid structure. These results indicate a promising lowering of processing costs and time in terms of enzyme purification and preparation.

It should be taken into consideration that, any presence of residuals on the nanofiber which come from the preparation step may create a significant risk for health. The highly volatile and toxic solvents used for production and activation of chitosan nanofibers may contaminate the processing bulk in industrial applications. It must be considered that GA is a toxic compound, effects of which changes according to the exposure type (Anon. 2, nd.). The residual GA on the nanofiber is unknown. Hence, it could lead some risks in industrial applications. In further studies, other activating agents such as genipin which is a natural crosslinker can be examined for

functionalization of chitosan nanofibers (Li et al., 2015). Additionally, the results of intake of nanomaterials into the body is not known for certain. The interaction of nanomaterials with other molecules in the body should be investigated. In order to use nanomaterials in food industry, required research and toxicological tests should be conducted and legislations should be made in the near future.

In conclusion, this study showed the stabilizing effect of immobilization on to chitosan nanofibers. Thermal stability of β-galactosidase was improved compared to most of the immobilization surfaces given in literature. The reusability of chitosan nanofibers requires further improvement considering reusability results on the Table 1.2. The reusability of chitosan nanofibers can be utilized by production of magnetic chitosan nanofibers in further studies.

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