Studies on Alkaline Protease Production from *Bacillus* sp.

By

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ABSTRACT

The aim of this study was optimization of the conditions for the production of alkaline protease from the *Bacillus* strains coded as L18, L21 and I18, which were isolated from our natural habitats. Additionally, the goal was also to optimize the production of alkaline protease from *Bacillus* sp. L21 in a new low-cost media formulation by employing design of experiments and response surface methodology. Lastly, the focus was given to the determination of the characteristic properties of the crude alkaline protease of *Bacillus* sp. L21.

The strains L21 and L18 were supplied by İYTE Department of Biology, whereas I18 was supplied by Ege University Department of Biology. These potential alkaline protease producer strains identified as *Bacillus* sp. were isolated from the by products of a leather factory (strains L21 and L18) and from the soil of the Ege University Campus (strain I18).

Specific growth rates calculated from the slope of the logarithmic phase were as $0.49h^{-1}$, $0.60\ h^{-1}$, $0.70\ h^{-1}$ for I18, L18 and L21 respectively.

When the effects of environmental conditions on alkaline protease production from strains L21, L18 and I18 were studied, the optimum temperatures were determined as 30°C for strain I18 and 37°C for the strains L18 and L21. Similarly the optimum agitation speeds were 100 rpm for I18 and 180 rpm for L18 and L21. The optimum inoculation ratio was determined as 5% (v/v) for all three strains. The optimum incubation time was determined as 96 hours for the strains I18 and L21 and 125 hours for L18. The optimum initial media pH was estimated as pH 10 for strain L18 and L21.

The constant medium constituents in the development of a low-cost medium study were peptone, KH_2PO_4, $Mg_2SO_4.7H_2O$, and Na_2CO_3. At the end of screening experiments, the factors considered include, soybean meal and corn steep liquor as nitrogen source, maltose as carbon source, $CaCl_2$ and Tween 80 as elements. The relative importance of these factors on alkaline protease production was investigated by using a resolution IV fractional factorial design with single replicate of treatment combinations of four factors (soybean meal, corn steep liquor, $CaCl_2$, Tween 80). Soybean meal, maltose, Tween 80 concentrations and initial pH were found to significantly influence the alkaline protease production.

For obtaining the mutual interaction between the variables and optimizing these variables, Box-Behnken design and central composite design (CCD) using response

surface methodology was employed. Among six interaction terms only those of soybean meal and maltose (X_1*X_2) and Tween80 and pH (X_3*X_4) were found to be significant. The neutral pH values of the medium and Tween 80 around its maximum level has a positive effect on proteolytic activity. Additionally, 4 sets of validation experiments were employed. The validation experiment, which state soybean meal, maltose, Tween 80 and pH as 2.5 g/L, 15 g/L, 0.35 g/L and 8.5, respectively, yielded an actual protease activity of 294.3 U/mL, where the model estimated a value of 338 U/mL. The Box-Behnken design and validation experiments were not sufficient to determine the true optimum values for the significant factors because of the saddle nature of the response surfaces. Therefore pH and Tween 80 were kept constant and a central composite design with two factors (soybean meal and maltose concentrations) was conducted. The adjusted R2 of the model was 93.3% with an insignificant lack of fit (p value=0.141). The CCD was able to determine the optimum value of soybean meal as 3.0 g/L, however it could be determined for maltose concentration after a set of additional experiments. Consequently, the optimum values of the four factors were determined as 8.0 for pH, 0.35 g/L for Tween 80, 3.0 g/L for soybean meal and 30-40 g/L for maltose concentration. The maximum activity under these conditions was 269.2 U/mL.

In the characterization part of this study, the crude alkaline protease of Bacillus sp. L21 displayed a pH optimum of 11.0 and retained about 73-78% of its original activity between pH 4.0 and 11.0 in the stability studies. The optimum temperature for protease activity was found to be 60ºC and retained about 90% of the original activity even at 80°C. By analyzing the thermal stability, the alkaline protease was found to be stable in a temperature range of 30°C to 50°C but lost about 30% of its activity at 60°C, after 30 min and 1 hour incubations both in the presence and absence of Ca2+. The protease from strain L21 showed high stability against both 5% and 15% (v/v) concentrations of H_2O_2, which is a bleaching agent, and retained approximately 82% and 94% of its activity, respectively, therefore, the present alkaline protease is thought to be a bleach-stable enzyme, so that it can be used in detergent formulations. There was no inhibitory effect observed from EDTA that further show that the enzyme is not a metalloprotease. However, PMSF (phenylmethylsulphonyl fluoride) strongly inhibited the protease activity, suggesting that the enzyme is a serine protease. In addition, $CaCl_2$ showed inhibitory effect on the enzyme, decreasing the activity to 90% of the control and this can be explained that the enzyme do not require the presence of Ca^{2+} ions to be active and stable.

ÖZ

Bu çalışmada doğal kaynaklarımızdan izole edilip, L18, L21 ve I18 olarak kodlandırılan alkalifilik *Bacillus* suşlarından alkali proteaz enzimi üretmek için gerekli olan optimum şartların belirlenmesi amaçlanmıştır. Çalışmanın bir diğer amacı ise, *Bacillus* sp. L21'den endüstriyel üretime uygun düşük maliyetli bir besi ortamı geliştirmek suretiyle alkali proteaz üretmek ve geliştirilen bu besi ortamının bileşenlerini deneylerin dizaynı ve yüzey yanıt yöntemi yardımıyla optimize etmektir. Son olarak, L21 suşundan elde edilen ham alkali proteazın bazı karakteristik özelliklerinin belirlenmesi hedeflenmiştir.

L21 ve L18 suşları İYTE Biyoloji Bölümü'nden, I18 suşu ise Ege Üniversitesi Biyoloji Bölümü'nden temin edilmiştir. *Bacillus* olarak tanımlanan ve potansiyel alkali proteaz üreticisi oldukları belirlenen bu suşlardan L18 ve L21 deri fabrikası yan ürünlerinden, I18 ise Ege Üniversitesi kampüs toprağından izole edilmiştir.

Her üç bakterinin logaritmik büyüme eğrileri oluşturularak spesifik üreme hızları sırasıyla I18 için 0.49, L18 için 0.60 ve L21 için 0.70 $saat^{-1}$ olarak hesaplanmıştır.

Çevresel koşulların alkali proteaz üretimi üzerine etkisini belirlemek için yapılan çalışmalarda L18 ve L21'in 37°C, I18 ise 30°C 'de en iyi ürediği ve bu sıcaklıklarda yüksek aktivitede enzim sentezleyebildikleri saptanmıştır. Aynı şekilde optimum çalkalama hızları L18 ve L21 için 180 rpm, I18 için ise 100 rpm olarak tespit edilmiştir. Her üç bakteri suşu için optimum inokulasyon oranı % 5 (v/v) olarak belirlenmiştir. En uygun fermentasyon süresi I18 ve L21 için 96 saat iken L18 için 125 saattir. Ayrıca L18 ve L21 suşları için optimum başlangıç pH değerleri 10.0 olarak belirlenmiştir.

L21 suşundan alkali proteaz üretmek amacıyla endüstriyel üretime uygun düşük maliyetli bir besi ortamı geliştirme çalışmaları sırasında pepton, KH_2PO_4, $Mg_2SO_4.7H_2O$, ve Na_2CO_3 besiyerinin sabit bileşenlerini oluşturmaktadır. Tarama faaliyetleri sonucunda karbon kaynağı olarak maltoz, azot kaynağı olarak mısır şurubu ve soya küspesi, elementler olarak ise $CaCl_2$ ve Tween80, incelenmesi gereken faktörler olarak belirlenmişlerdir. Seçilen bu faktörlerin alkali proteaz üretimi üzerine olan bağıl önemleri dört faktörlü (soya küspesi, mısır şurubu, $CaCl_2$, Tween 80) 2_{IV}^{4-1} fraksiyonel faktöriyel tasarım metodu ile incelenmiştir. Bu inceleme sonucunda, soya küspesi, maltoz, Tween 80 konsantrasyonları ve başlangıç pH değerinin alkali proteaz üretimini en çok etkileyen faktörler olduğuna karar verilmiştir.

Değişkenler arasındaki karşılıklı etkileşimin saptanabilmesi ve bu değişkenlerin optimize edilmesi amacıyla Box-Behnken dizaynı ve merkezi bileşik dizayn ile yüzey yanıt metodundan faydalanılmıştır. Altı adet etkileşim terimi arasında en önemlileri soya küspesi ile maltoz (X_1*X_2) ve Tween 80 ile pH (X_3*X_4) arasındaki etkileşimlerdir. Ortam pH'ının nötral değerlerde ve Tween 80'in maksimum seviyelerinde bulunmasını proteolitik aktivite üzerine olumlu bir etki yaratmıştır. Bu modelin doğrulanması amacıyla yapılan ek deneylerde soya küspesi, maltoz, Tween 80 ve pH değişkenleri sırasıyla 2.5 g/L, 15 g/L, 0.35 g/L ve 8.5 değerlerinde tutulduğunda, 294.3 U/mL gibi bir gerçek proteaz aktivitesi elde edilirken, oluşturduğumuz model aynı değeri 338 U/mL olarak tahmin etmiştir. Uygulanan Box-Behnken dizaynı ve ek deneyler, seçilen faktörlerin gerçek optimum değerlerinin elde edilmesinde, oluşturulan yüzey yanıt grafiklerinin eyer benzeri bir tabiatta olması nedeniyle yetersiz kalmıştır. Bu nedenle, pH ve Tween 80'in sabit tutulduğu ve soya küspesi ile maltoz konsantrasyonlarının incelendiği, iki faktörlü bir merkezi bileşik dizayn kullanılmıştır. Bu modelin ayarlanmış R^2 değeri %93.3 olup, modelin uyumsuz olmadığı saptanmıştır (p-değeri=0.141). Merkezi bileşik dizaynın uygulanmasıyla birlikte soya küspesinin optimum değeri 3.0 g/L olarak belirlenirken, maltoz konsantrasyonu için bu değeri belirlemek amacıyla ek deneylere ihtiyaç duyulmuştur. Sonuç olarak, bu dört değişkenin optimum değerleri pH için 8.0, Tween 80 için 0.35 g/L, soya küspesi için 3.0 g/L ve maltoz konsantrasyonu için 30-40 g/L olarak belirlenmiştir. Bu şartlar altında elde edilen maksimum aktivite değeri 269.2 U/mL'dir.

Bu çalışmanın karakterizasyon bölümünde, *Bacillus* sp. L21'den elde edilen ham alkali proteazın optimum çalışma pH değeri 11.0 olup, stabilite çalışmaları sırasında pH 4.0 ve 11.0 aralığında orijinal aktivitesinin yaklaşık %73-78'ini koruduğu gözlenmiştir. Alkali proteazın optimum çalışma sıcaklığı 60ºC olarak tespit edilmiş ve 80ºC'de bile orijinal aktivitesinin yaklaşık %90'ını koruduğu belirlenmiştir. Termal stabilite analizlerinde alkali proteazın, Ca^{+2} 'nin varlığında ve yokluğunda, 30 dakika ve 1 saatlik inkübasyonlar sonucunda, 30°C ile 50°C sıcaklık aralığında stabilitesini koruduğu, fakat 60ºC'de aktivitesinin yaklaşık %40'ını kaybettiği bulunmuştur.

L21 suşunun ürettiği alkali proteaz bir ağartma ajanı olan H_2O_2'e karşı yüksek stabilite göstermiş ve %5 ve %15'lik (v/v) konsantrasyonlarına karşısında aktivitesinin sırasıyla, %82 ve %94'ünü korumuştur. Bu nedenle, ürettiğimiz alkali proteazın ağartmaya dayanıklı bir enzim olarak çeşitli deterjan formülasyonlarında

kullanılabileceği düşünülmektedir. EDTA'nın enzimi inhibite etmemiş olması, ürettiğimiz enzimin metalloproteaz olmadığına işaret etmiştir. Bunun yanında PMSF adlı inhibitör proteaz aktivitesini güçlü bir şekilde inhibe etmesi, ürettiğimiz enzimin bir serin proteaz olduğuna işaret etmektedir. Ek olarak, $CaCl_2$ aktiviteyi %10 kadar düşürdüğünden, inhibitör etki yaratmıştır ve bu da enzimin aktif veya stabil olabilmesi için Ca^{2+} iyonların ihtiyaç duymadığını göstermektedir.

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CHAPTER 1

INTRODUCTION

Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. More than 3000 different enzymes described to date the majority have been isolated from mesophilic organisms. These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known enzymes unrecommendable. Thus, the search for new microbial sources is a continual exercise, where one must respect biodiversity. The microorganisms from diverse and exotic environments called as extremophiles, are an important source of enzymes, whose specific properties are expected to result in novel process applications (Kumar and Takagi, 1999). The role of enzymes in many processes has been known for a long time. Their existence was associated with the history of ancient Greece where they were using enzymes from microorganisms in baking, brewing, alcohol production, cheese making etc.. With better knowledge and purification of enzymes the number of applications has increased manyfold, and with the availability of engineered enzymes a number of new possibilities for industrial processes have emerged (Beg et al., 2003). The current estimated value of the worldwide sales of industrial enzymes is $1 billion. However, proteases represent one of the three largest groups of industrial enzymes and account for about 60% of total worldwide enzyme sales. This dominance of proteases in the industrial market is expected to increase further by the year 2005 (Rao et al., 1998).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. They are essential constituents of all forms of life on earth, including prokoryates, fungi, plants and animals. They can be cultured in large quantities in relatively short time by established fermentation methods and produce an abundant, regular supply of the desired product. In recent years there has been a phenomenal increase in the use of alkaline protease as industrial catalysts. Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine

center (serine protease) or are of metallo- type (metalloprotease); and the alkaline serine proteases are the most important group of enzymes so far exploited (Gupta et al., 2002b). These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons; for example they exhibit high catalytic activity, a high degree of substrate specificity can be produced in large amounts and are economically viable. Microbial alkaline proteases dominate the worldwide enzyme market, accounting for two-third of the share of the detergent industry. Although production is inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. Alkaline proteases of *Bacillus* sp. origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Agrawal et al., 2004).

The scope of this dissertation was to optimize the environmental conditions during fermentation, developing an industrial media formulation using response surface methodology and evaluating the characteristic properties of the crude alkaline protease enzyme produced from *Bacillus* sp. isolated from natural habitat of İzmir region.

CHAPTER 2

PROTEASES

Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are enzymes that hydrolyse proteins via the addition of water across peptide bonds and catalyse peptide synthesis in organic solvents and in solvents with low water content (Sookkheo et al., 2000; Beg et al., 2003). The hydrolysis of peptide bonds by proteases as shown in Figure 2.1 is termed as proteolysis; the products of proteolysis are protein and peptide fragments, and free amino acids.

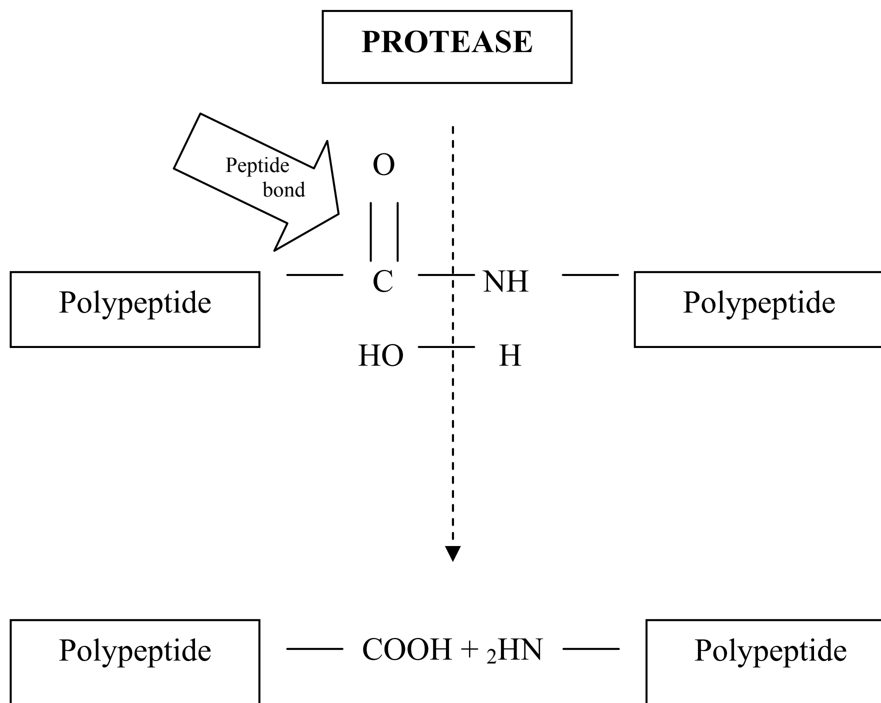


Figure 2.1. Protease catalysis of peptide bonds (Proteolysis)

Proteolytic enzymes are ubiquitous in occurrence, found in all living organisms, and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta et al., 2002b).

Proteases represent one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market

accounting for about 60% of total worldwide sale of enzymes (See Fig. 2.2) (Rao et al., 1998, Cowan, 1994). This dominance of proteases in the industrial market is expected to increase further by the year 2005 (Gupta et al., 2002b). The estimated value of the worldwide sales of industrial enzymes was $1 billion in 1998 (Rao et al., 1998).

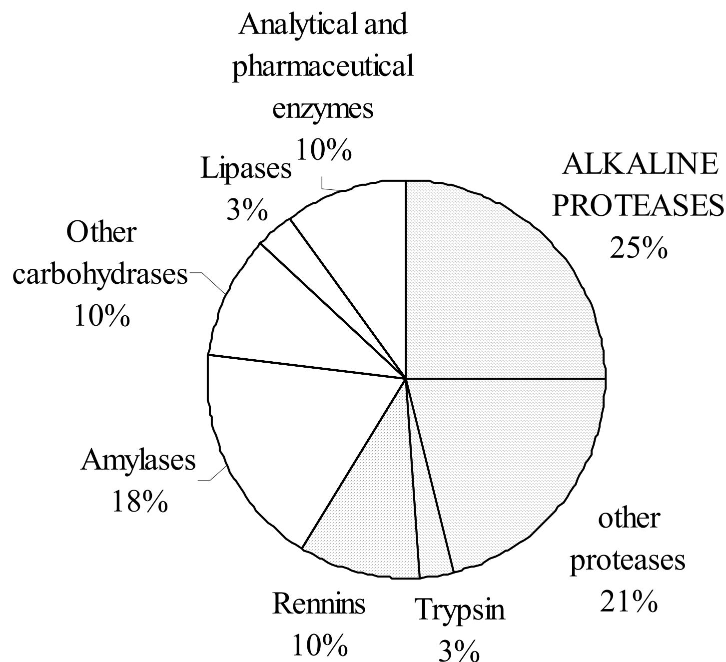


Figure 2.2. The contribution of different enzymes to the total sale of enzymes is indicated. The shaded portion indicates the total sale of proteases. (Rao et al., 1998)

Proteases the most important group of enzymes produced commercially are used in detergent, protein, brewing, meat, photographic, leather and dairy industries (Anwar et al., 1998). Proteases have a long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals, is a relatively new development and has conferred added biotechnological importance (Rao et al., 1998). These enzymes have also become widely used in the detergent industry, since their introduction in 1914 as detergent additives (Gupta et al., 2002b).

Proteases of commercial importance are produced from microbial, animal and plant sources. They constitute a very large and complex group of enzymes with different properties of substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles. Industrial proteases have application in a range of process taking advantage of the unique physical and catalytic properties of individual proteolytic enzyme types (Ward, 1991). This vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Rao et al., 1998).

2.1. Classification of Proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (Rao et al., 1998).

Proteases can be classified according to 3 major criteria. Such as;

i) the reaction catalysed,

ii) the chemical nature of the catalytic site,

iii) the evolutionary relationship, as revealed by the structure (Rao et al., 1998).

Proteases as shown in Table 2.1 are broadly classified as endo- or exoenzymes on the basis of their site of action on protein substrates (Rao et al., 1998).

They are further categorized as serine proteases, aspartic proteases, cysteine proteases, or metallo proteases depending on their catalytic mechanism. They are also classified into different families and clans depending on their amino acid sequences and evolutionary relationships. Based on the pH of their optimal activity, they are referred to as acidic, neutral, or alkaline proteases (Rao et al., 1998).

2.1.1. Exoproteases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively (Rao et al., 1998).

Table 2.1. Classification of proteases (Rao et al., 1998)

Protease	EC no.
Exopeptidases	3.4.11
Aminopeptidases	3.4.14
Dipeptidyl peptidase	3.4.14
Tripeptidyl peptidase	3.4.16-3.4.18
Carboxypeptidase	3.4.16
Serine type protease	3.4.17
Metalloprotease	3.4.18
Cysteine type protease	3.4.15
Peptidyl dipeptidase	3.4.13
Dipeptidases	3.4.19
Omega peptidases	3.4.19
Endopeptidases	3.4.21-3.4.34
Serine protease	3.4.21
Cysteine protease	3.4.22
Aspartic protease	3.4.23
Metallo protease	3.4.24
Endopeptidases of unknown catalytic mechanism	3.4.99

2.1.1.1. Aminopeptidases

Aminopeptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. They are known to remove the N-terminal Met that may be found in heterologously expressed proteins but not in many naturally occurring mature proteins. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi. In general aminopeptidases are intracellular enzymes, but there has been a single report on an extracellular peptidase produced by *A. oryzae*. (Rao et al., 1998)

2.1.1.2. Carboxypeptidases

The carboxypeptidases act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallo carboxypeptidases, and cysteine carboxypeptidases, based on the nature of the amino acid residue at the active site of the enzymes (Rao et al., 1998).

2.1.2. Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine and (iv) metalloproteases (Rao et al., 1998).

2.1.2.1. Serine Proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms (Rao et al., 1998).

Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCl), L-3 carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E-64), diisopropylfluorophosphate (DFP), phenyl methyl sulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity. Their molecular masses range between 18 and 35 kDa. The isoelectric points of serine proteases are generally between pH 4 and 6. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases (Rao et al., 1998).

Serine alkaline proteases are produced by several bacteria, molds, yeast, and fungi. They hydrolyse a peptide bond, which has tyrosine, phenylalanine, or leucine at the carboxyl site of the splitting bond. The optimal pH of alkaline proteases is around pH 10, and their isoelectric point is around pH 9. Their molecular weights are in the range at 15 and 30 kDa. Although serine alkaline proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces*, and *Flavobacterium* spp., subtilisins produced by *Bacillus* spp. are the best known ones (Rao et al., 1998).

Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg, and subtilisin Novo or bacterial proteases Nagase (BPN'), have been identified. Subtilisin Carlsberg produced by *Bacillus licheniformis* was discovered in 1947 by Linderstrom, Lang and

Ottesen at the Carlsberg laboratory and is widely used in detergents. Less commercially important Subtilisin Novo or BPN’ is produced by *B. amyloliquefaciens*. Both subtilisins have a molecular weight of 27.5 kDa but differ from each other by 58 amino acids. They have similar properties such as an optimal temperature of 60ºC and an optimal pH of 10 (Rao et al., 1998). Some of these properties are given in Table 2.2.

Table 2.2. Some properties of Subtilisin Carlsberg and Subtilisin BPN’ (Cowan, 1994)

Property	***Carlsberg***	**BPN’**
Number of peptide chains	1	1
Number of amino acids	274	275
Number of homologous amino acids	217	217
Number of cysteine residues	0	0
Isoelectric point	9.4	9.1
Dependence on calcium for stabilization at high temperatures or pH extremes	Less	More
pH optimum	10-11	10-11
% of maximum activity at pH 7.0	40-70	20
Inhibitors	DFP, PMSF, cereal and legume inhibitors, organophosphorus compounds	DFP, PMSF, cereal and legume inhibitors
Relative activities on BTEE[a]	3-4	1
Number of bonds hydrolysed in insulin B chain	7	7

2.1.2.2. Aspartic Proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin, retropepsin and enzymes from pararetroviruses. Most aspartic proteases show maximal activity at low pH and have isoelectric points in the range of pH 3 to 4.5. Their molecular weights are in the range of 30 to 45 kDa. The aspartic proteases are inhibited by pepstatin (Rao et al., 1998).

2.1.2.3. Cysteine / thiol Proteaeses

Cysteine proteases occur in both prokaryotes and eukaryotes. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. In catalytic mechanism of cysteine peptidases the thiol group of a single cysteine residue plays an essential role. This group is susceptible to oxidation and can react with a variety of reagents; heavy metals, iodoacetate, N-ethyl-maleimide etc. (Kenny, 1999). Based on their side-chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin-like, (iii) specific to glutamic acid and (iv) others. Papain is the best-known cysteine proteases. Cysteine proteases have neutral pH optima.

2.1.2.4. Metalloproteases

Metallo proteases are characterized by the requirements for a divalent metal ion for their activity. Based on the specificity of their action, metalloproteases can be divided into 4 groups, (i) neutral, (ii) alkaline, (iii) Myxobacter I and (iv) Myxobacter II. They are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP.

2.2. Sources of Proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, found in a wide diversity of sources such as plants, animals and microorganisms (Rao et al., 1998). Fortunately, enzymes can be separated from living cells and perform catalysis independent of their physiological environment. Commercial proteases are derived from animal tissues, plant cells and microbial cells via fermentation.

2.2.1 Plant Proteases

The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process.

Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin (Rao et al., 1998).

Papain and ficin are prepared by water extraction of crude material from Carica papaya and Ficus carica respectively. Bromelain is usually obtained from the stems of the pineapple plant by extraction and fractional solvent precipitation (Ward, 1985).

Bromelain and papain are plant-derived proteases with a longstanding history of use in a diverse range of food applications. As plant-derived products, they are perceived as safe and “natural” ingredients for use in the food applications and may offer unique benefits and functionality. However in applications where increasing the concentration of soluble solids or simple viscosity reduction are the primary objectives, bromelain and papain are usually not cost effective.

2.2.2. Animal Proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennin. These are prepared in pure form in bulk quantities. However their production depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies (Rao et al., 1998).

Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins (Rao et al., 1998).

Chymotrypsin is found in animal pancreatic extract. Pure chymotrypsin is an expensive enzyme and is used only for diagnostic and analytical applications (Rao et al., 1998).

Pepsin is an acidic protease that is found in the stomachs of almost all vertebrates (Rao et al., 1998). Pepsin is prepared from the fundus portion of hag stomachs, by acid extraction and filtration (Ward, 1985). Pepsin was used in laundry detergents as early as 1913, but is now being replaced by a mixture of serine and metal microbial proteases that appear to be less degradable by soaps, alkaline conditions and high temperatures (Adinarayana and Ellaiah, 2002).

Rennet is a pepsin-like protease that is produced as an inactive precursor in the stomachs of all nursing mammals. It is converted to active rennin by the action of pepsin. It is used extensively in the dairy industry to produce a stable curd with good flavour (Rao et al., 1998).

2.2.3. Microbial Proteases

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases (Rao et al., 1998). Proteases of bacteria, fungi and viruses are increasingly studied due to its importance and subsequent applications in industry and biotechnology. Commercial application of microbial proteases is attractive due to the relative ease of large-scale production as compared to proteases from plant and animals.

Microbial proteases account for approximately 40% of the total worldwide enzyme sales. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they posses almost all the characteristics desired for their biotechnological applications (Rao et al., 1998). Microorganisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods producing an abundant, regular supply of the desired product. Besides they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Rao et al., 1998, Gupta et al., 2002a). In general microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals (Gupta et al., 2002a).

Microbial proteases, especially from *Bacillus* sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergent formulations (Beg et al., 2003).

2.3. Alkaline Proteases

Alkaline proteases (EC.3.4.21-24, 99) are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine centre (serine protease) or are of metallo-type (metalloprotease); and they are the most important group of enzymes exploited commercially (Gupta et al., 2002b).

Alkaline proteases are most active at pH values of about pH 10. They are sensitive to DFP and a potato inhibitor but not to TLCK or tosyl-L-phenylalanine chloromethyl ketone (TPCK). They are all specific against aromatic or hydrophobic amino acid residues at the carboxyl side of the splitting point (Ward, 1985). These

enzymes also offer advantages over the use of conventional chemical catalysts for numerous reasons. For example they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts and are economically viable (Anwar et al., 1998).

Alkaline proteases being a physiologically and commercially important group of enzymes are used primarily as detergent additives. These enzymes have broad substrate specificities and will function to some extent under the rather extreme conditions encountered in domestic washing temperatures of 20 to 70ºC, a pH up to 11 and at high concentrations of detergents, polyphosphates, chelating agents such as EDTA and oxidizing agents such as sodium perborate (Cowan, 1994).

In recent years there has also been a phenomenal increase in the use of alkaline protease as industrial catalysts. In Japan, 1994 alkaline protease sales were estimated at $116 million. There is expected to be an upward trend in the use of alkaline proteases so that by the turn of the decade the total value for industrial enzymes is likely to reach $700 million or more (Kumar and Takagi, 1999).

Especially, alkaline proteases of microbial origin, which dominate the worldwide enzyme market, possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Agrawal et al., 2004, Gupta et al., 2002a).

Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues (Singh et al., 2001a). Despite this interest in other microbial sources, survey of the literature conclusively shows that bacteria are by far the most popular source of commercial alkaline proteases to date. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10 and their broad substrate specificity. Their optimal temperature is around 60ºC. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao et al., 1998).

From all the alkaliphilic bacteria that have been screened for use in various industrial applications, members of the genus *Bacillus*, mainly strains *B. subtilis* and *B. licheniformis* were found to be predominant and a prolific source of alkaline proteases (Kumar and Takagi, 1999).

2.3.1. Alkaline Proteases of Alkaliphilic *Bacillus* Strains

Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from many different exotic environments have been explored and exploited for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, and *B. majovensis* (Gupta et al., 2002a). Alkaline proteases produced by thermophilic and alkaliphilic bacilli can withstand high temperature, pH, chemical denaturing agents and in non-aqueous environments (Johnvesly and Naik, 2001).

Bacteria belonging to *Bacillus* sp. are by far the most important source of several commercial microbial enzymes. They can be cultivated under extreme temperature and pH conditions to give rise to products that are in turn stable in a wide range of harsh environments. *Bacillus* is a rod-shaped, gram positive, spore forming, aerobic, usually catalase positive, chemoorganotropic bacterium. Alkaliphilic *Bacillus* can be found mostly in alkaline environments such as soda soils, soda lakes, neutral environments and deep-sea sediments. Animal manure, man-made alkaline environments such as effluents from food, textile, tannery, potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources (Akbalık, 2003).

The first report concerning an alkaline enzyme was published by Horikoshi in 1971. Horikoshi reported the production of an extracellular alkaline serine protease from alkaliphilic *Bacillus* strain 221. This strain, isolated from soil, produced large amounts of alkaline protease that differed from the subtilisin group. The optimum pH of the purified enzyme was 11.5 and 75% of the activity was maintained at pH 13.0. The enzyme was completely inhibited by diisopropylfluorophosphate or 6 M urea but not by EDTA or *p*-chloromercuribenzoate. The molecular weight of the enzyme was 30,000 Da, which is slightly higher than those of other alkaline proteases. The addition of a 5mM solution of calcium ions increased the activity by 70% at the optimum temperature of 60ºC (Horikoshi, 1999). Moreover, various types of alkaline protease have been characterized and their potential industrial applications have been explored. The major applications of these enzymes are in detergent formulation, the food industry, leather processing, chemical synthesis and waste management (Gupta et al., 2002a). Some industrially important alkaline proteases produced from various *Bacillus* sp. are tabulated in Table 2.3.

Table 2.3. Some industrially important alkaline proteases produced from various *Bacillus* sp. (Anwar et al., 1998)

Species	pH optimum/stability	Industrial application(s)
Bacillus stearothermophilus	9.5	Detergents and heavy duty laundry powders
Bacillus sp. Y. (BYA)	10.0-12.5	Detergent formulations
Bacillus licheniformis	8.2	Catalyst for N-protected amino acids
Bacillus sp. (AH-101)	12.0-13.0	Dehairing/leather industry
Bacillus firmus	8.0	Detergent industry
Bacillus sp. (P-001A)	9.5	Production of biomass from natural waste
Bacillus sp. (Savinase/Durazym)	9.0-11.0	Detergent formulations
Bacillus licheniformis (Alcalase)	8.2	Synthesis of biologically active peptides
Bacillus subtilis	8.5	Bating agent in leather industry

2.4. Properties of Alkaline Proteases

2.4.1. Optimum Temperature and Thermostability of Alkaline Proteases

The heat stability of enzymes is affected by at least two factors alone or in combination. The first one is the primary structure of the enzyme. A high content of hydrophobic amino acids in the enzyme molecule provides a compact structure, which is not denatured easily by a change in the external environment. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Secondly, specific components such as polysaccharides and divalent cations, if any, can stabilize the molecule (Öztürk, 2001).

Even though there is no firm evidence to suggest that thermostable enzymes are necessarily derived from thermophilic organisms, nevertheless there is a greater chance of finding thermostable proteins from thermophilic bacteria (Rahman et al., 1994). Therefore, a wide range of microbial proteases from thermophilic species has been extensively purified and characterized. These include *Thermus* sp., *Desulfurococcus*

strain $Tok_{12}S_1$ and *Bacillus* sp.. Among them alkaline proteases derived from alkaliphilic bacilli, are known to be active and stable in highly alkaline conditions (Rahman et al., 1994). The earliest thermophilic and alkaliphilic *Bacillus* sp. was *B. stearothermophilus* strain F1 isolated by Salleh and friends in 1977, which was stable at 60ºC (Haki and Rakshit, 2003). Further studies on microbial alkaline proteases have been done in view of their structural-function relationship and industrial applications, as they needed stable biocatalysts capable of withstanding harsh conditions of operation (Rahman et al., 1994).

Table 2.4. Optimum temperature and thermostabilities of various alkaline proteases produced from *Bacillus* sp.

Source	**Optimum temperature**	**Thermostability**	**Reference**
Bacillus sp. No. AH-101	80ºC	n.d.	Takami et al., 1989
Bacillus sp. NCDC 180	55ºC	n.d.	Kumar et al., 1999
B. pumilus UN-31-C-42	55ºC	50ºC/30min	Huang et al., 2003
B. stearothermophilus F1	85ºC	70ºC/24h	Rahman et al., 1994
B. brevis	60ºC	60ºC/4days	Banerjee et al., 1999
B. subtilis IIQDB32	55ºC	30ºC/4h	Varela et al., 1997
Bacillus sp. P-2	90ºC	90ºC/1h	Kaur et al., 2001
Bacillus sp. SSR1	40ºC	n.d.	Singh et al., 2001a
Bacillus sp. SB5	70ºC	60ºC/1h	Gupta et al., 1999
Bacillus sp. B18'	85ºC	n.d.	Fujiwara et al., 1991
Bacillus sphaericus	55ºC	n.d.	Singh et al., 1999
Bacillus sp. RGR-14	60ºC	45ºC/1h	Oberoi et al., 2001
Bacillus clausii I-52	60ºC	50ºC/1h	Joo et al., 2003
Bacillus horikoshii	45ºC	50ºC/1h	Joo et al., 2002
Bacillus licheniformis	70ºC	n.d.	Manachini and Fortina, 1998
Bacillus JB-99	70ºC	80ºC	Johnvesly and Naik, 2001
Bacillus mojavensis	60ºC	60ºC/1h	Gupta and Beg, 2003

Generally alkaline proteases produced from alkaliphilic *Bacillus* are known to be active over a wide range of temperature. The optimum temperatures of alkaline proteases range from 40 to 80ºC. In addition, the enzyme from an obligatory alkaliphilic *Bacillus* P-2 showed an exceptionally high optimum temperature of 90ºC. The protease

has also good thermostability at high temperatures, being thermostable at 90ºC for more than 1 h and retained 95 and 37% of its activity at 99ºC (boiling) and 121ºC (autoclaving temperature), respectively. *Bacillus* P-2 was the only mesophile reported until 2001, which produced a proteolytic enzyme that was stable for so long even at autoclaving (121ºC) and boiling temperatures (Kaur et al., 2001).

In some studies it has also been observed that the addition of Ca^{2+} further enhanced enzyme thermostability (Takami et al., 1989, Gessesse, 1997, Rahman et al., 1994).

The optimum temperature and thermal stability of some alkaline proteases from *Bacillus* sp. are summarized in Table 2.4.

2.4.2. Optimum pH of Alkaline Proteases

Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly located on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This will affect the total net charge of the enzymes and the distribution of charges on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighborhood of the active sites, which will overall affect the activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990).

In general, all currently used detergent-compatible proteases are alkaline in nature with a high pH optimum; therefore they fit the pH of laundry detergents, which is generally in the range of 8 to 12. Therefore, most of the commercially available subtilisin-type proteases are also active in the pH range of 8-12 (Gupta et al., 2002a). A good example for this is the well-known detergent enzymes, subtilisin Carlsberg and subtilsin Novo or BPN' which show maximum activity at pH 10.5 (Banerjee et al., 1999).

Alkaline proteases of the genus *Bacillus* show an optimal activity and a good stability at high alkaline pH values (Margesin et al., 1992). The optimum pH range of *Bacillus* alkaline proteases is generally between pH 9 and 11, with a few exceptions of higher pH optima of 11.5 (Fujiwara and Yamamoto, 1987), 11-12 (Kumar et al., 1999), 12-13 (Takami et al., 1989, Fujiwara et al., 1991, Ferrero et al., 1996) (Kumar and Takagi, 1999).

The pH optima of some alkaline proteases produced from *Bacillus* sp. are given in Table 2.5.

Table 2.5. pH optimum of various alkaline proteases produced from *Bacillus* sp.

Source	Substrate	Optimum pH	Reference
Bacillus sp. No. AH-101	Casein	12-13	Takami et al., 1989
Bacillus sp. NCDC 180	Casein	11-12	Kumar et al., 1999
B. pumilus UN-31-C-42	Casein	10.0	Huang et al., 2003
Bacillus licheniformis MIR29	Casein	12.0	Ferrero et al., 1996
B. brevis	Azocasein	10.5	Banerjee et al., 1999
Bacillus sp. AR-009	Casein	10	Gessesse, 1997
Bacillus sp. P-2	Casein	9.6	Kaur et al., 2001
Bacillus sp. SSR1	Azocasein	10.0	Singh et al., 2001a
Bacillus sp. SB5	Casein	10.0	Gupta et al., 1999
Bacillus sp. B18'	Casein	13.0	Fujiwara et al., 1991
Bacillus sphaericus	Azocasein	10.5	Singh et al., 1999
Bacillus sp. RGR-14	Casein	11.0	Oberoi et al., 2001
Bacillus clausii I-52	Casein	11.0	Joo et al., 2003
Bacillus horikoshii	Casein	9.0	Joo et al., 2002
Bacillus licheniformis	Casein	9.0	Manachini and Fortina, 1998
Bacillus sp. JB-99	Casein	11.0	Johnvesly and Naik, 2001
Bacillus mojavensis	Casein	10.5	Gupta and Beg, 2003
Bacillus sp. B21-2	Casein	11.5	Fujiwara and Yamamoto, 1987

2.4.3. The Isoelectric Point

The pH referred as isoelectric point (pI) at which the net charge on the molecule is zero, is a characteristic of each enzyme, where solubility in aqueous solutions is generally minimum. In aqueous solution, charged groups interact with polar water molecules and stabilize the protein, which is intrinsically hydrophobic. A low number of charged groups and a high number of aliphatic or aromatic side chains characterize a protein that is less soluble in water. As one moves farther from pI, the number of

ionized groups increases therefore the solubility tends to increase. Hence the isoelectric point is important as it affects the solubility of proteins as well as interaction between them (Öztürk, 2001). Isoelectric pH values of various alkaline proteases produced from *Bacillus* sp are given in Table 2.6.

Table 2.6. Isoelectric pH values of various alkaline proteases produced from *Bacillus* sp.

Microorganism	**pI**	**Reference**
Bacillus sp. PS719	4.8	Hutadilok-Towatana et al., 1999
Bacillus sp. NKS-21	8.2	Tsudchida et al., 1986
Bacillus sp. No. AH-101	9.2	Takami et al., 1989
Bacillus sp. GX6638	4.2	Durham et al., 1987
B. pumilus UN-31-C-42	9.0	Huang et al., 2003
Bacillus subtilis no. 16	3.9	Kato et al., 1992
Bacillus sphaericus	8.6	Singh et al., 2001b

2.4.4. The Molecular Weight

The molecular weights of alkaline proteases generally range from 15 to 30 kDa (Kumar and Takagi, 1999) with few reports of higher molecular weights of 32.0 kDa (Huang et al., 2003), 33.5 kDa (Rahman et al., 1994), 36.0 kDa (Durham et al., 1987). These are tabulated in Table 2.7.

Table 2.7. Molecular weights of various alkaline proteases produced from *Bacillus* sp.

Source	**Molecular weight (kDa)**	**Method**	**Reference**
Bacillus sp. No. AH-101	30	SDS-PAGE	Takami et al., 1989
B. pumilus MK6-5	28	SDS-PAGE	Kumar, 2002
B. pumilus UN-31-C-42	32	SDS-PAGE	Huang et al., 2003
B. stearothermophilus F1	33.5	SDS	Rahman et al., 1994
Bacillus licheniformis MIR 29	25/40	SDS-PAGE	Ferrero et al., 1996
Bacillus sp. NKS-21	31	SDS-PAGE	Tsudchida et al., 1986
Bacillus mojavensis	30	SDS-PAGE	Gupta and Beg, 2003
Bacillus sp. SSR1	29	SDS-PAGE	Singh et al., 2001a
	35	Native PAGE	
Bacillus sp. GX6638	36	SDS-PAGE	Durham et al., 1987
Bacillus pseudofirmus AL-89	24	SDS-PAGE	Gessesse et al., 2003
Bacillus sp. B18’	30	SDS-PAGE	Fujiwara et al., 1993
	28	Gel filtration	

2.4.5. Metal Ion Requirement and Inhibitors of Alkaline Proteases

Alkaline proteases require a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations, for maximum activity. These cations were also found to enhance the thermal stability of a *Bacillus* alkaline protease. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Kumar and Takagi, 1999).

Inhibition studies give insight into the nature of the enzyme, its cofactor requirements, and the nature of the active site. Alkaline proteases are completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP). In this regard, PMSF sulfonates the essential serine residue in the active site, results in the complete loss of activity. This inhibition profile classifies these proteases as serine hydrolases. In addition, some of the alkaline proteases were found to be metal ion dependent in view of their sensitivity to metal chelating agents, such as EDTA. Thiol inhibitors have little effect on alkaline proteases of *Bacillus* spp., although they do affect the alkaline enzymes produced by *Streptomyces* sp. (Kumar and Takagi, 1999).

2.5. Industrial Applications of Alkaline Proteases from *Bacillus* Strains

2.5.1. Food Industry

Alkaline proteases can hydrolyse proteins from plants and animals to produce hydrolysates of well-defined peptide profiles. These protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations specific therapeutic dietary products and the fortification of fruit juices and soft drinks. In recent years there has been substantial interest in developing enzymatic methods for the hydrolysis of soya protein, gelatin, casein, whey and other proteins in order to prepare protein hydrolysates of high nutritional value. In developing commercial products from these proteins, emphasis is placed on achieving a consistent product in high yields, having desirable flavour, nutritional and/or functional properties (Ward, 1991).

Alkaline protease from *B. licheniformis* is used for the production of highly functional protein hydrolysates (Ward, 1991). This commercial alkaline protease, Alcalase, has a broad specificity with some preference for terminal hydrophobic amino acids. Using this enzyme, a less bitter hydrolysate and a debittered enzymatic whey protein hydrolysate were produced (Kumar and Takagi, 1999). Soluble meat hydrolysate can also be derived from lean meat wastes and from bone residues after mechanical deboning by solubilization with proteolytic enzymes. Alcalase has been found to be the most appropriate enzyme in terms of cost, solubilization, and other relevant factors. In an optimized process with Alcalase at a pH of 8.5 and temperature of 55-60ºC, a solubilization of 94 % was achieved. The resulting meat slurry was further pasteurised to inactivate the enzyme and found wide application in canned meat production, soups and seasoning (Kumar and Takagi, 1999).

Very recently, another alkaline protease from *B. amyloliquefaciens* resulted in the production of a methionine-rich protein hydrolysate from chickenpea and soy protein, which found major application in hypoallergenic infant food formulations (Kumar and Takagi, 1999). In another study, Rebecca et al., (1991) reported the production of fish hydrolysate of high nutritional value, using *B. subtilis* proteases. Perea et al., (1993) on the other hand used alkaline protease for the production of whey protein hydrolysate, using cheese whey in an industrial whey bioconversion process.

2.5.2. Detergent Industry

Enzymes have long been of interest to the detergent industry for their ability to aid the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies (Gupta et al., 2002b). The use of enzymes in detergent formulations is now common in developed countries, where more than half of the presently available ones contain enzymes (Chaplin and Bucke, 1990). Detergent enzymes account for approximately 30% of total worldwide enzyme production (Horikoshi, 1996) and 89% of the total protease sales in the world; a significant share of the market is captured by subtilisins and/or alkaline proteases from many *Bacillus* species (Gupta et al., 2002b).

Ideally alkaline proteases used in detergent formulations should have high activity and stability over a broad range of pH and temperature, should be effective at low levels (0.4-0.8%) and should also be compatible with various detergent components

along with oxidizing and sequestering agents. They must also have a long shelf life (Kumar and Takagi, 1999).

Alkaliphilic *Bacillus* strains are good sources of alkaline proteases with the properties that fulfil the essential requirements to be used in detergents; therefore the main industrial application of alkaliphilic proteases has been in the detergent industry since their introduction in 1914 as detergent additives. (Ito et al., 1998; Horikoshi, 1996).

The major use of detergent–compatible alkaline proteases is in laundry detergent formulations. Detergents available in the international market such as Dynamo®, Eraplus® (Procter & Gamble), Tide® (Colgate Palmolive), contain proteolytic enzymes, the majority of which are produced by members of the genus *Bacillus* (Anwar et al., 1998).

The main producers of alkaline proteases using species of *Bacillus* are the companies such as Novo Industry A.I.S. and Gist Brocades. Novo produces three proteases, Alcalase from *B. licheniformis*, Esperase from an alkalophilic strain of a *B. licheniformis* and Savinase from an alkalophilic strain of a *B. amyloliquefaciens*. Gist Brocades on the other hand produces and supplies Maxatase from *B. licheniformis*. Alcalase and Maxatase (both mainly subtilisin) are recommended to be used at 10-65ºC and pH 7-10.5. Savinase and Esperase can be used at up to pH 11 and 12 respectively (Chaplin and Bucke, 1990). The commercial alkaline proteases used in detergent formulations can also be found in Table 2.8.

Table.2.8. Commercial *Bacillus* sp. alkaline proteases used in detergent formulations

Trade names	**Source organism**	**Optimum pH**	**Optimum temperature (ºC)**	**Manufacturer**
Alcalase	B. licheniformis	8-9	60	Novo Nordisk
Savinase	Alkaliphilic *Bacillus* sp.	9-11	55	Novo Nordisk
Esperase	Alkaliphilic *Bacillus* sp.	9-11	60	Novo Nordisk
Maxacal	Alkaliphilic *Bacillus* sp.	11	60	Gist-Brocades
Maxatase	Alkaliphilic *Bacillus* sp.	9.5-10	60	Gist-Brocades
Opticlean	Alkaliphilic *Bacillus* sp.	10-11	50-60	Solvay Enzymes
Optimase	Alkaliphilic *Bacillus* sp.	9-10	60-65	Solvay Enzymes
Protosol	Alkaliphilic *Bacillus* sp.	10	50	Advanced Biochemicals
Wuxi	Alkaliphilic *Bacillus* sp.	10-11	40-50	Wuxi Synder Bioproducts

Conventionally, detergents have been used at elevated washing temperatures, but at present there is considerable interest in the identification of alkaline proteases, which are effective over wider temperature ranges (Oberoi et al., 2001). For example there is considerable current interest on the exploration of proteases that can catalyse reactions in cold water. This allows their use in detergents, which can be used in normal tap water without the requirement for increasing the temperature of the water. The search for such enzymes is very much a challenge at this time (Haki and Rakshit, 2003).

Banerjee and his colleagues (1999) have studied on an alkaline protease from a facultative thermophilic and alkalophilic strain of *Bacillus brevis*. The alkaline protease from *B. brevis* having maximum activity at pH 10.5, showed a high level of thermostability at 60ºC. The enzyme showed compatibility at 60ºC with all of the commercial detergents tested. It could also remove blood strains completely when used with detergents. All the tests were studied in the presence of Ca^{2+} and glycine and the data obtained in this study implies that the protease of *B. brevis* has most of the properties to be used as a detergent enzyme.

In another study, Gupta and his friends (1999) reported a bleach-stable & thermotolerant alkaline protease from a new variant of *Bacillus* sp., having potential application in detergent formulations.

The alkaline protease from newly isolated *Bacillus* SB5 displayed stability in the presence of 10% (v/v) H_2O_2 (oxidizing agent) and 1% SDS (sodium dodecyl sulphate, surfactant). The enzyme had an optimum activity at pH 10 and 60ºC to 70ºC, where this was further increased in the presence of all ionic and non-ionic detergents, surfactants and commercial detergents tested (Gupta et al., 1999).

Oberoi and his colleagues (2001) also produced an alkaline, SDS-stable protease from *Bacillus* sp. RGR-14 that was also suggested to be an ideal detergent additive for detergent formulations. The enzyme was active over a wide temperature range in alkaline conditions. In addition being SDS-stable it was also stable towards oxidizing agents such as H_2O_2 and sodium perborate.

The alkaline protease from *B. clausii* I-52 is significant for industrial perspective because of its ability to function in broad pH and temperature ranges in addition to its tolerance and stability in presence of an anionic surfactant like SDS and oxidants like peroxides and perborates. The enzymatic properties of this protease therefore suggest its suitable application as additive in detergent formulations (Joo et al., 2003).

The in place cleaning of ultrafiltration (UF) and reverse osmosis (RO) membranes forms one of the most important aspects of modern dairy and food industries. However, the major limitation in the application of UF in the dairy industry is the decline in flux with time due to fouling of the membranes. Thus, protein deposition and precipitation of minerals were inferred as major contributors to this phenomenon of fouling. An effective protease-based cleaning that can hydrolyze milk protein into fragments small enough to be rinsed off from the UF system, is required to overcome fouling problem (Kumar and Tiwari, 1999).

Kumar and Tiwari produced an alkaline protease from an alkalophilic *Bacillus* strain MK5-6. The ability of this alkaline protease to clean UF membranes fouled during milk processing was determined in combination with two alkaline UF membranes cleaner, Alconax and Ultrasil. The alkaline enzyme that is extremely stable in Alcanox was optimal at 5 g/L in cleaning the fouled membranes when added to the same membrane cleaner. For these reasons, they believe the alkaline protease preparation from alkalophilic *Bacillus* strain MK5-6 as an attractive candidate to be used as membrane cleaner additive (Kumar and Tiwari, 1999).

Durham produced Subtilisin GX from alkaliphilic *Bacillus* sp. GX6644 (ATCC 53441) that was found to possess properties suitable as a detergent additive (Durham, 1987).

2.5.3. Leather Industry

The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and safety hazards. Thus, for environmental reasons, the biotreatment of leather using enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced (Gupta et al., 2002b). Alkaline proteases with elastolytic and keratinolytic activity can be used in leather processing industries. During bating, the hide is softened by partial degradation of the interfibrillar matrix proteins (elastin & keratin). Therefore enzyme preparations with low levels of elastase and keratinase activity but no collagenase activity are particularly applicable for this process. (Cowan, 1994). Bating is traditionally an enzymatic process involving

pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular. The substitution of chemical depilatory agents in the leather industry by proteolytic enzymes produced by *Bacillus* sp. could have important economical and environmental impacts (Anwar et al., 1998) where the dehairing process is accelerated by the use of alkaline proteases.

2.5.4. Medical usage

Alkaline proteases are also used for developing products of medical importance. It was stated in Gupta et al., (2002b) that Kudrya and Simonenko (1994) exploited the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kim et al., (2001) reported the use of alkaline protease from *Bacillus* sp. strain CK 11-4 as a thrombolytic agent having fibrinolytic activity (Gupta et al., 2002b). Furthermore, *Bacillus* sp. has been recognized as being safe to human. (Kumar and Takagi, 1999).

2.5.5. Management of Industrial and Household Waste

Alkaline proteases provide potential application for the management of wastes from various food processing industries and household activities.

Feather is composed of over 90% protein, the main component being keratin, a fibrous and insoluble protein. Worldwide several million tons of feather is generated annually as waste by poultry-processing industries. Feathers constitute approximately 5% of the body weight of poultry and can be considered a high protein source for food and feed, provided their rigid keratin structure is completely destroyed. Pretreatment with NaOH, mechanical disintegration, and enzymatic hydrolysis results in total solubilization of the feathers. The end product is a heavy, greyish powder with a very high protein content, which could be used as a feed additive (Kumar and Takagi, 1999).

Considering its high protein content, this waste could have a great potential as a source of protein and amino acids for animal feed as well as for many other applications. In some countries, feather is used as animal feed supplement in the form of feather meal. Development of enzymatic and/or microbial methods for the hydrolysis of feather to soluble proteins and amino acids is extremely attractive, as it offers a cheap

and mild reaction condition for the production of valuable products (Gessesse et al., 2003). Gessesse et al. (2003) isolated an organism from an alkaline soda lake in the Ethiopian Rift Valley Area and identified as *Bacillus pseudofirmus*. *B. pseudofirmus* AL-89, and the protease it produces offers an interesting potential for the enzymatic and/or microbiological hydrolysis of feather to be used as animal feed supplement (Gessesse et al., 2003). Dalev (1994) reported also an enzymatic process using a *B. subtilis* alkaline protease in the processing of waste feathers from poultry slaughterhouses.

A formulation containing proteolytic enzymes from *B. substilis*, *B. amyloliquefaciens* and *Streptomyces* sp. and a disulfide reducing agent (thioglycolate) that enhances hair degradation, helps in clearing pipes clogged with hair-containing deposits and is currently available in the market. It was prepared and patented by Genex (Gupta et al., 2002b).

2.5.6. Photographic Industry

Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. These waste films contain 1.5-2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Conventionally, this silver is recovered by burning the films, which causes undesirable environmental pollution. Furthermore, base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also in obtaining polyester film base that can be recycled (Gupta et al., 2002b). Fujiwara and co-workers studied on this interesting application of alkaline proteases. They reported the use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered (Horikoshi, 1999). Protease B18' had a higher optimum pH and temperature, around 13.0 and 85ºC. The enzyme was most active toward gelatin on film at pH 10 (Fujiwara et al., 1991).

Singh et al., (1999) isolated an obligate alkaliphilic *Bacillus sphaericus* strain from alkaline soils in the Himalayas, which produced an extracellular alkaline protease. The alkaline protease of this strain efficiently hydrolysed the gelatin layer of used X-ray films within 12 min at 50ºC and at pH 11.0 (Singh et al., 1999).

Enzymatic decomposition of gelatin layers on X-ray films and repeated utilization of enzyme for potential industrialization were also investigated using thermostable alkaline protease from the alkaliphilic *Bacillus* sp. B21-2 by Masui and his co-workers (1999). They concluded that the decomposition of gelatin layers at 50ºC with the mutant enzyme (Ala187Pro) was higher than those of the wild-type and the mutant enzyme could also be satisfactorily used five times.

2.5.7. Peptide Synthesis

Amino acids are of increasing importance as dietary supplements for both humans and domestic animals. Only the L-amino acids can be assimilated by living organisms, since the chemical synthesis of amino acids produces a racemic mixture, it is necessary to separate the isomers before commercial use.

Alcalase is a proteolytic enzyme isolated from a selected strain of *B. licheniformis*, its major component being subtilisin Carslberg. It was determined that Alcalase was stable in organic solvents and could be of use as a catalyst in the resolution of *N*-protected amino acids having unusual side chains (Anwar et al., 1998).

2.5.8. Silk Degumming

One of the least explored areas for the use of proteases is in the silk industry where only a few patents have been filed describing the use of proteases for the degumming process of silk. The conventional silk degumming process is generally expensive and therefore an alternative method suggested, is the use of protease preparations for degumming the silk prior to dyeing. In a recent study, the silk degumming efficiency of an alkaline protease from *Bacillus* sp. RGR-14 was studied. After 5h of incubation of silk fiber with protease from *Bacillus* sp., the weight loss of silk fiber was 7.5%. Scanning electron microscopy of the fibers revealed that clusters of silk fibers had fallen apart as compared with the smooth and compacted structure of untreated fiber (Gupta et al., 2002b).

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

G40 (glucose syrup, 40%), G95 (glucose syrup, 95%), F42 (fructose corn syrup, 42%), M50 (maltose, 50%), GF30 (glucose-fructose, 30%), natural corn starch (NCS) was supplied by Cargill Sweeteners and Starches Inc.. Soybean meal and corn meal were supplied by Cargill Soy Protein Solutions (Park Food Ltd. Co.). Pınar Milk Products Inc. and Pakmaya Co. supplied whey and molasses, respectively.

3.2. Methods

3.2.1. Bacterial Strain and Cultivation of Microorganism

L18 and L21 strains were isolated from the by products of a leather factory, by IZTECH (İzmir Institute of Technology) Biology department, whereas strain coded I18 was isolated from the soil of the Ege University Campus by the Biology department of the same University. They were also identified as *Bacillus* sp. by IZTECH (İzmir Institute of Technology) Biology department. These three strains that showed potential for high proteolytic activity were selected among 85 strains. The stock cultures of the strains were maintained on glycerol stocks (20% (v/v)) and stored at -80ºC for long-term preservation.

These microorganisms were cultivated in a basal media containing 10g/L glucose (Applichem), 5g/L yeast extract (Acumedia), 5g/L pepton (Acumedia), 1g/L potassium dihydrogen phosphate (Applichem), 0.2g/L magnesium sulfate heptahydrate (Applichem) and 10g/L sodium carbonate (Applichem) where sterile sodium carbonate was added after the sterilization process aseptically. This media formulation was called as Horikoshi-I media (Horikoshi, 1996). For the solid media preparation 15g/L of agar was added to this formulation. Cultures were regenerated every 2-3 weeks on a fresh plate from the frozen stock culture.

3.2.2. Growth Curve Determination of the First Subculture

A loop-full of 48h-old single colony of each strain was transferred from a fresh Horikoshi agar plate into 25 mL Horikoshi broth media and incubated at 37°C and 500

rpm on Ikalabortechnik KS125 basic orbital shaker for 24 hours. During this time period, samples were taken for viable cell count and optical density. Samples were diluted in dilution tubes of 0.85% NaCl and inoculated on plates by spread plate technique. The cell densities were determined by reading the optical densities at 600 nm by using Varian Cary Bio 100 spectrophotometer.

3.2.3. Enzyme Production

The actual fermentation was carried out in 250 mL Erlenmayer flask containing 50 mL of the Horikoshi-I media inoculated with 5% of the inoculum of the first subculture and incubated at 37ºC and 180 rpm for 4 days. At certain time intervals samples were taken for the determination of cell density, protease activity, carbon utilization, and protein contents.

3.2.4. Determination of Total Protein Content

The total protein contents of the samples were determined according to the method described by Lowry (Lowry et al., 1951); the protein standard used was Bovine Serum Albumin (Sigma). Protein standard solution, in the range of 0-300 μg/mL was prepared in triplicate to obtain a standard curve (see Appendix A.1). Samples (cell-free supernatant) were diluted to 500μl with water so that the protein content would be within the range of the standards. 2.5 mL Lowry reagent solution was added to each tube and mixed well. The solutions were kept at room temperature for 10 minutes followed by the addition of 0.25 mL Folin & Ciocalteu's Phenol reagent (Merck) working solution. Each tube was rapidly mixed, and incubated in dark for 30 minutes. Absorbances of the samples were measured spectrophotometrically at 660 nm using Varian Cary Bio 100 spectrophotometer.

3.2.5. Determination of Proteolytic Activity

Alkaline protease activity was determined by applying a modified form of the method given by Takami et al. (1989). According to this procedure 0.25 mL of Glysin:NaCl:NaOH (50 mM, pH 10.5) buffer was incubated with 2.5 mL of 0.6% casein (Merck) dissolved in the same buffer at 30˚C until equilibrium. An aliquot of

0.25 mL of the enzyme solution was added to this mixture and incubated for 20 minutes. The reaction was stopped by adding 2.5 mL TCA solution (0.11M trichloroacetic acid, 0.22M sodium acetate, 0.33M acetic acid). After 30 more minutes the whole mixture was centrifuged at 5000 *g* for 15 minutes. 0.5 mL of the supernatant was mixed with 2.5 mL of 0.5 M Na_2CO_3 and 0.5 mL of Folin-Ciocalteu's Phenol solution and was kept for 30 minutes at room temperature. The optical densities of the solutions were read against the sample blanks at 660 nm using Varian Cary Bio 100 spectrophotometer. Readings were completed within 30 minutes. Tyrosin standard solution, in the range of 0-1000 mg/L was prepared in triplicate to obtain a standard curve (see Appendix A.2). One alkaline protease unit (U) was defined as, the enzyme amount that could produce 1 μg of tyrosine in one minute under the defined assay conditions.

3.2.6. Total Carbohydrate Assay

Total carbohydrate content was determined according to the phenol-sulfuric acid method (DuBois, 1956). Standard curve was prepared by using 0-50 μg/mL of glucose solutions (see Appendix A.3). The assay was, performed as followings: 1.0 mL of 5% (w/v) phenol solution was pipetted to each tube. Samples (cell-free supernatant) were diluted with deionized water to contain 0-50 μg/mL total carbohydrate. 1.0 mL of the prepared sample dilution was added into each phenol-containing tube and tubes were vortexed. 5.0 mL of concentrated sulfuric acid was added quickly and directly into the center of each test tube and tubes were again immediately mixed by using the vortex mixer. The absorbance of each sample (or standard solution) was determined by using Varian Cary Bio 100 spectrophotometer at 490nm against the blank after the solutions cooled to room temperature.

3.2.7. Effect of Culture Conditions on Enzyme Production

The prescreening of the media components was done using the Horikoshi-I media as reference media and changing the individual components one at a time. The actual fermentation was carried out in 50 mL of the modified media inoculated with 5% of the inoculum of the first subculture and incubated at 37ºC and 180 rpm for 96 hours.

3.2.7.1. Effect of Various Carbon Sources

In the prescreening of various carbon sources, glucose (10g/L) in the Horikoshi-I media was substituted by G40 (glucose syrup, 40%), G95 (glucose syrup, 95%), F42 (fructose corn syrup, 42%), M50 (maltose, 50%), GF30 (glucose-fructose, 30%), natural corn starch (NCS), soluble starch, molasses and whey in the same ratio, individually.

3.2.7.2. Effect of Various Nitrogen Sources

In the prescreening of various nitrogen sources, pepton and yeast extract (10g/L totally) in the Horikoshi-I media was replaced with casein, corn steep liquor, yeast extract, corn meal, soybean meal (fine) and whey at a concentration of 10g/L, individually.

3.2.7.3. Effect of Various Elements

In the prescreening of various elements, magnesium sulfate heptahydrate (0.2g/L) in the Horikoshi-I media was substituted by magnesium sulfate heptahydrate (0.1g/L and 1g/L). Also Horikoshi-I media was enriched with the addition of ammonium nitrate (5g/L), Tween 80 (0.25g/L), $CaCl_2$ (0.1g/L and 1g/L), individually. In another formulation, Horikoshi-I media was modified where the concentration of KH_2PO_4 was changed from 1g/L to 0.5 and 2 g/L respectively.

3.2.8. Effect of pH on Alkaline Protease Activity and Stability

The pH values investigated were 4.0, 7.0, 9.0, 10.5, 11.0 and 13.0. In these analyses, pH was adjusted using the following buffer systems: acetate (pH 4.0), phosphate (pH 7.0), Tris-HCl (pH 9.0), glycine-NaOH (pH 10.5-11) and KCl-NaOH (pH 13.0). The concentration of each buffer was 0.1M. The optimum pH of alkaline protease was determined by standard alkaline protease assay with casein 0.6% (w/v) as substrate dissolved in the buffer systems mentioned. To study stability as a function of pH, 2 mL of the crude enzyme was mixed with 2 mL of the buffer solutions mentioned above and aliquots of the mixture were taken to measure the residual protease activity (%) under standard assay conditions after incubation at 30ºC for 2 hours.

3.2.9. Effect of Temperature on Alkaline Protease Activity and Stability

The optimum temperature of the alkaline protease was determined by incubation of the reaction mixture (pH 10.5) for 20 minutes, at different temperatures ranging from 30 to 80ºC (30, 40, 50, 60, 70 and 80ºC) and measuring the activity with standard proteolytic activity assay. Before the addition of enzymes, the substrate (0.6% (w/v) casein) was pre-incubated at the respective temperature for 10 minutes.The thermostability of the crude alkaline protease was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 30 to 60ºC (30, 40, 50 and 60ºC) for 30 and 60 minutes, respectively, in the presence and absence of 0.05 M Ca^{2+}. The incubation medium used was 0.05 M (pH 10.5) glycine:NaCl:NaOH buffer system.

3.2.10. Effect of Various Reagents on Alkaline Protease Activity

The effects of Ca^{2+} ion 10 mM as a metal ion, H_2O_2 (5 and 15% (v/v)) as an oxidizing agent, EDTA (ethylene diamine tetra acetic acid, 0.01M) and PMSF (phenyl methyl sulphonyl flouride, 0.01M) as inhibitors on alkaline protease activity were investigated as a part of enzyme characterization studies. Crude alkaline protease was pre-incubated with the chemicals above for 1 hour at 30ºC and then the residual activity (%) was tested by standard proteolytic activity assay.

3.2.11. Experimental Design and Statistical Analysis

The optimization of medium components (the carbon source (maltose), the nitrogen source (soybean meal), surfactant (Tweeen 80)) and pH of the medium, which have been predicted to play an important role in the production of alkaline protease were selected as main variables to optimize the process. Therefore, a Box-Behnken design with four factors and central composite design with two factors were used in the design of experiments and Minitab Statistical Software Release 13 in the analysis of data and generation of response surface graphics.

Total of 27 experiments in three blocks were performed in the media optimization part of the study. Block variables were three different incubators to be able to run three different runs at the same time. Additional 12 media optimization

experiments were designed in CCD. The response variable in two sets of experiments was protease activity. The actual factor levels corresponding to the coded factor levels are presented in Table 3.1. After running the experiments and measuring the activity levels, a second order full model (Eq. 3.1) including interactions was fitted to the protease activity data:

$$Y= \beta_o + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_3X_3^2 + \beta_4X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 \quad -3.1-$$

Where Y is the predicted response, β_o is the model constant, β_1, β_2, β_3, β_4 are linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} are the quadratic coefficients, β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are the interaction coefficients. X_1, X_2, X_3, X_4 are the independent variables in their coded forms. The following equation (Eq. 3.2) was used for coding the actual experimental values of the factors in a range of [-1 +1]:

$$X = [actual – (low\text{-}level + high\text{-}level)/2] / (high\text{-}level – low\text{-}level)/2 \quad -3.2-$$

Table 3.1. Actual factor levels corresponding to coded factor levels

Factor (unit)	**Symbol**	**Actual factor levels at coded levels**		
		-1	**0**	**+1**
Soybean Meal (g/L)	X_1	2.5	5	7.5
Maltose (g/L)	X_2	5	10	15
Tween80 (g/L)	X_3	0.15	0.25	0.35
pH	X_4	7	9.5	12

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Determination of Specific Growth Rates

A loop-full of a single colony of each strain was transferred from a fresh Horikoshi agar plate into 25 mL Horikoshi broth media and incubated at 37°C and 180 rpm at pH 10.5 for 24 hours. During this time period, samples were taken for viable cell count and optical density measurements. These were used to estimate the specific growth rates which are presented in Figure 4.1 and its corresponding cell density in Figure 4.2. This was the first subculture, which was used as inoculum into the actual fermentation media for further experiments. The inoculum amount for each strain was based on the optical density at the end of the exponential phase (Fig. 4.2) corresponding to 5% (v/v). The aim here was to standardize the inoculum amount to each experiment based on the growth rate of the first subculture. As it is observed from the Figure 4.1 and 4.2, the specific growth rates were calculated from the slope of the logarithmic phase as 0.49h^{-1}, 0.60 h^{-1}, 0.70 h^{-1} for I18, L18 and L21 respectively. According to these data L21 was the strain with highest specific growth rate.

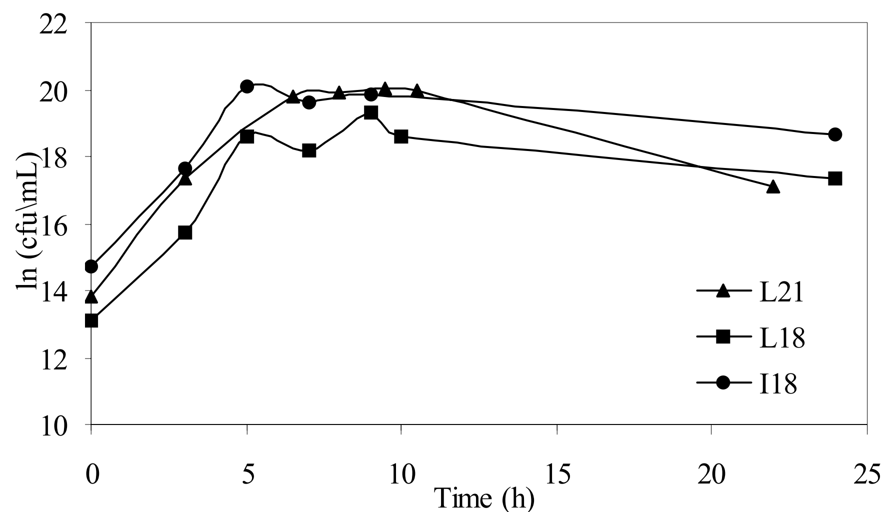


Figure 4.1. Growth curves of L21, L18 and I18 strains (37°C, 180rpm, pH 10.5)

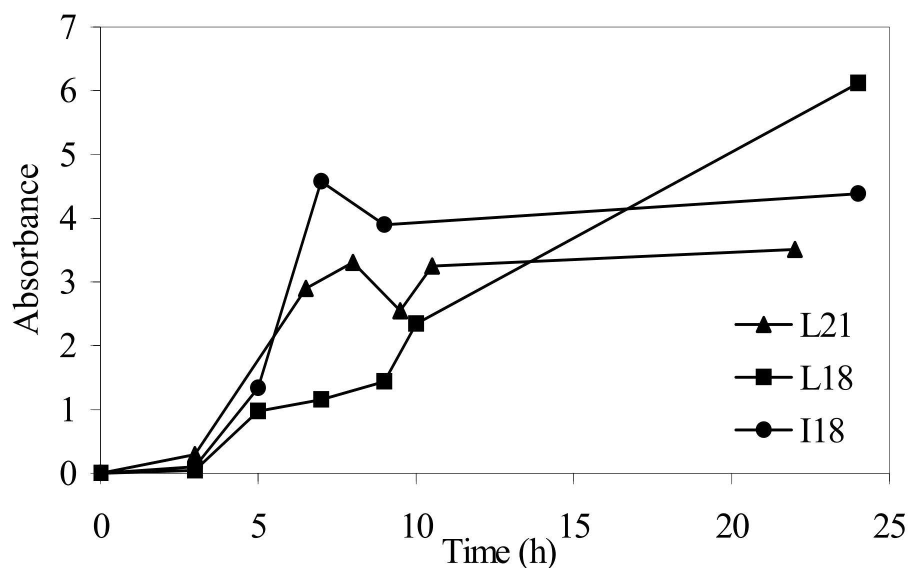


Figure 4.2. Optical density profiles of L21, L18 and I18 at 600nm

4.2. Effect of Environmental Conditions on Alkaline Protease Production

In this section, three strains (L21, L18 and I18) with high protease activities were optimized with respect to inoculum concentration, temperature, agitation speed, media pH and incubation time.

4.2.1. Effect of Incubation Temperature

In order to determine the effect of the incubation temperature on the proteolytic activity, 50 mL of Horikoski media in 250 mL of Erlenmeyer flask was inoculated with 5% of inoculum using the first subculture of each strain and incubated at 30°C, 37°C, 45°C and 55°C at 180 rpm for 96 hours. At certain time intervals samples were taken for optical cell density, total protein, protease activity and substrate utilization. The results in Fig 4.3 and Table 4.1 indicated that all three strains have different temperature requirements. According to Table 4.1, strain I18 has temperature optima at 30°C whereas strains L18 and L21 have at 37°C. Similarly, from Figure 4.3, it is observed that even so L18 and L21 reach almost the same final enzyme activity; the rate of enzyme synthesis is much faster with strain L21 compared to the other two strains. This is also supported with the specific activity data (Table 4.1) where, L21 has 18.4% more specific activity than L18 and 75.3% more than I18 if taken the same incubation

temperature (37°C) into account. However if the optimum temperature (30°C) of I18 is taken as basis, this value will change to 54.6%. Considering either total or specific enzyme activities, in both cases strain I18 is the less performing one. A comparison with the literature review done on the characteristics of alkaliphilic *Bacillus* strains producing alkaline proteases, it is determined that most of the alkaliphilic *Bacillus* strains have a temperature optima between 30-37°C and are mostly of mesophilic type. With this regard our strains are in agreement with the literature reported data (Takami et al., 1989; Mabrouk et al., 1999; Banerjee et al., 1999; Çalık et al., 2002; Puri et al., 2002; Kanekar et al., 2002; Joo et al., 2003). Therefore it is thought that the proteases produced by these strains may have a high possibility to have temperature optima around these temperatures, and being a good potential source for the pretreatment of heat sensitive food items as well as detergent and other industrial products where high temperatures are not desired.

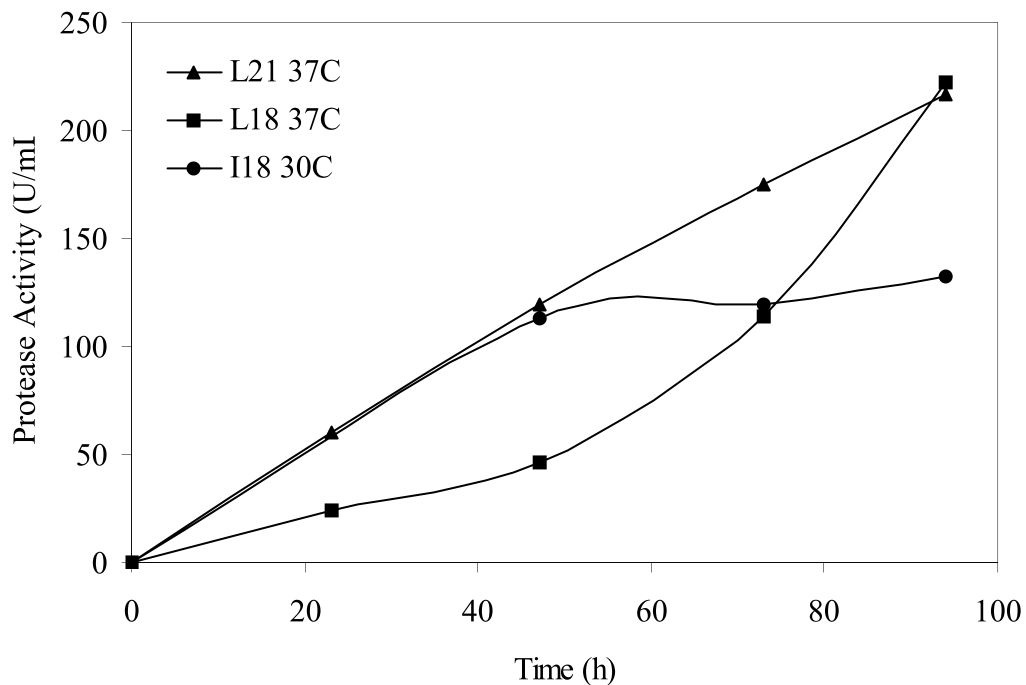


Figure 4.3. Protease enzyme profiles of I18, L18 and L21 at optimum temperatures

Table 4.1. Total and specific enzyme activities of strains I18, L18 and L21 at different incubation temperatures after 96 hours of incubations

Strain	Temperature (°C)	Protease activity (U/mL)	Specific Protease Activity (U/mg protein)
I18	30	132.2	27.3
	37	128.0	14.9
	45	127.1	16.3
	55	No growth	No growth
L18	30	133.8	28.9
	37	222.1	49.0
	45	85.0	18.3
	55	No growth	No growth
L21	30	163.0	42.7
	37	216.3	60.1
	45	92.1	14.6
	55	No growth	No growth

4.2.2. Effect of Agitation Speed

The effect of the agitation speed on the protease enzyme production was determined by inoculating 5% of each first subculture into 50 mL of Horikoshi media and incubated at 37°C and 100, 180, 250 and 325 rpm under the same fermentation conditions. The total and specific protease activities after 96 hours of fermentation at different agitations speeds are presented in Table 4.2. According to these results, 100 rpm was determined to be the optimum agitation speed for strain I18 and 180 rpm for the other two strains L18 and L21. Based on the total and specific protease activities, one can conclude (Table 4.2), that even L18 has 2.6 % more total protease activity than L21 and 28.1 % more than I18, based on specific activities this picture changes, where L21 becomes the dominant strain with 18.4 % and 46.9 % more specific activity than the strains I18 and L18 respectively. This is also supported with the enzyme profiles at optimum agitation speeds shown in Figure 4.4. In the reported literature data, one can determine that most of the agitation speeds used for this type of strains is between 180-

220 rpm. Therefore our results support these findings. (Liu and Tzeng, 1998; Gupta et al., 1999; Banerjee et al., 1999; Joo et al., 2002).

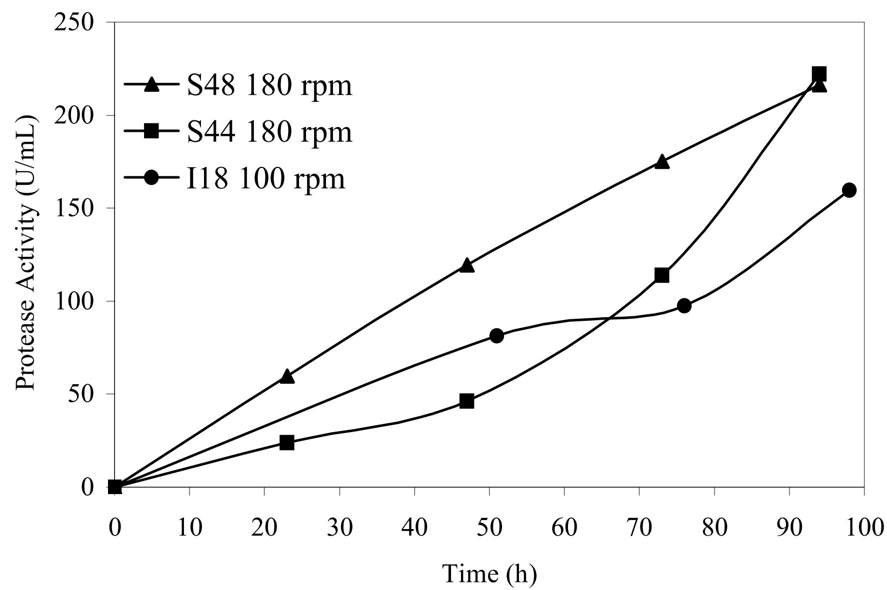


Figure 4.4. Protease enzyme profiles of L21, L18 and I18 at optimum agitation speeds

Table 4.2. Total and specific enzyme activities of strains L21, L18 and I18 at different agitation speeds after 96 hours of incubation

Strain	**Agitation Speed (rpm)**	**Protease activity (U/mL)**	**Specific Protease Activity (U/mg protein)**
I18	100	159.5	31.97
	180	128.0	14.87
	250	141.4	20.73
	325	134.6	15.42
L18	100	161.2	19.49
	180	222.1	49.02
	250	142.3	16.55
	325	135.3	13.61
L21	100	106.7	16.94
	180	216.3	60.09
	250	94.2	8.91
	325	186.0	30.24

4.2.3. Effect of Inoculation Ratio

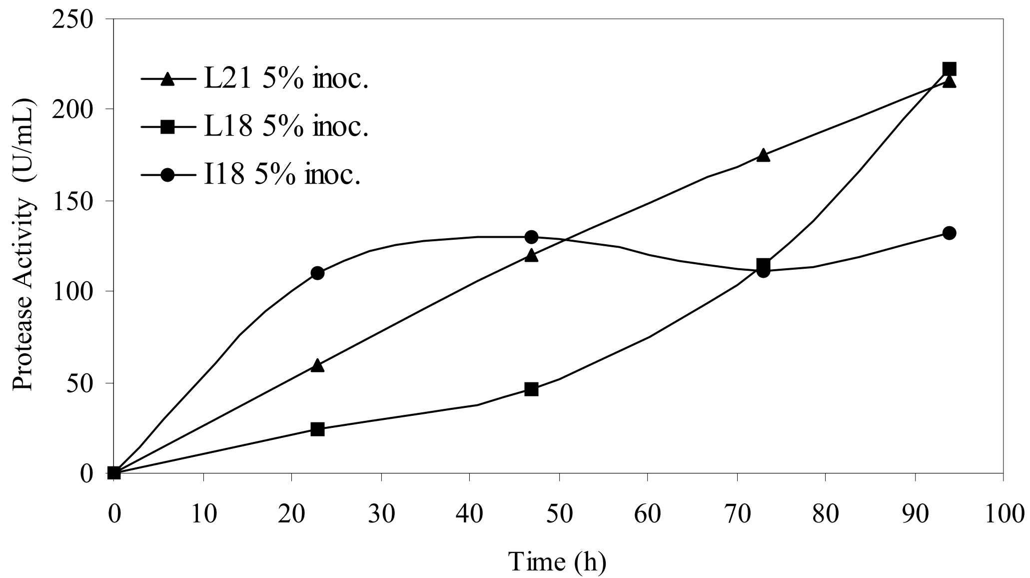


Figure 4.5. Protease enzyme profiles of L21, L18 and I18 at optimum inoculation rates

To determine the effect of inoculation ratio on the enzyme synthesis, 50 mL of the Horikoshi media was inoculated, with 1%, 2.5%, 5% and 10% (v/v) of the first subculture of each strain. I18 was incubated at 100 rpm and 30°C; L18 and L21 were incubated at 180rpm and 37°C, since these were the predetermined optimum conditions. The total and specific enzyme activities obtained after 96 hours of incubation are presented in Table 4.3. As it is seen from these data inoculation ratio has very high impact on the enzyme synthesis. The optimum inoculation ratio was determined as 5% (v/v) for all three strains. In the comparison of the strains with regard to total and specific enzyme activities at this optimum ratio, a similar conclusion as mentioned above, as L21 being the better performing strain was withdrawn. Since the specific enzyme activity gives an indirect indication on the purity of the enzyme, this value is desired to be as high as possible. Therefore L21 is thought to produce less side by-products than the other two strains. In the literature it is well documented that an inoculation ratio of 2% to 5% is an optimum value for *Bacillus* type of strains (Mabrouk et al., 1999; Kanekar et al., 2002). Therefore our strains are in good agreement with these data.

Table 4.3. Total and specific enzyme activities of strains L21, L18 and I18 at different inoculation ratios after 96 hours of incubation

Strain	Inoculation ratio (%, v/v)	Protease activity (U/mL)	Specific Protease Activity (U/mg protein)
I18	1	51.9	6.9
	2.5	26.7	3.7
	5	132.2	27.3
	10	28.3	3.8
L18	1	61.3	7.4
	2.5	42.7	4.9
	5	222.1	49.0
	10	36.8	3.7
L21	1	161.6	21.8
	2.5	167.7	22.3
	5	216.3	60.0
	10	176.8	25.1

4.2.4. Effect of Incubation Time

In order to determine the optimum incubation time for maximum enzyme production, 50 mL of Horikoshi media inoculated with 5% (v/v) of the first subculture of each strain, was incubated at 30°C and 100 rpm for I18 and 37°C at 180 rpm for L18 and L21 for 73, 96 and 125 hours. According to the results taken at different time intervals, it was determined that the optimum incubation time for I18 and L21 was 96 hours and for L18, 125 hours. Comparing the results to the literature there is a broad incubation time ranging from 24-120 hours reported for *Bacillus* strains (Singh et al., 2001b; Chu et al., 1992; Mabrouk et al., 1999; Gupta and Beg, 2003). Based on these data our strains fit in this interval.

Table 4.4. Total protease activity of L21, L18 and I18 at different incubation times

Strain	73 h	96 h	125 h
L21	175.2	216.3	166.5
L18	122.1	222.1	243.7
I18	97.5	159.5	81.3

4.2.5. Effect of Media pH

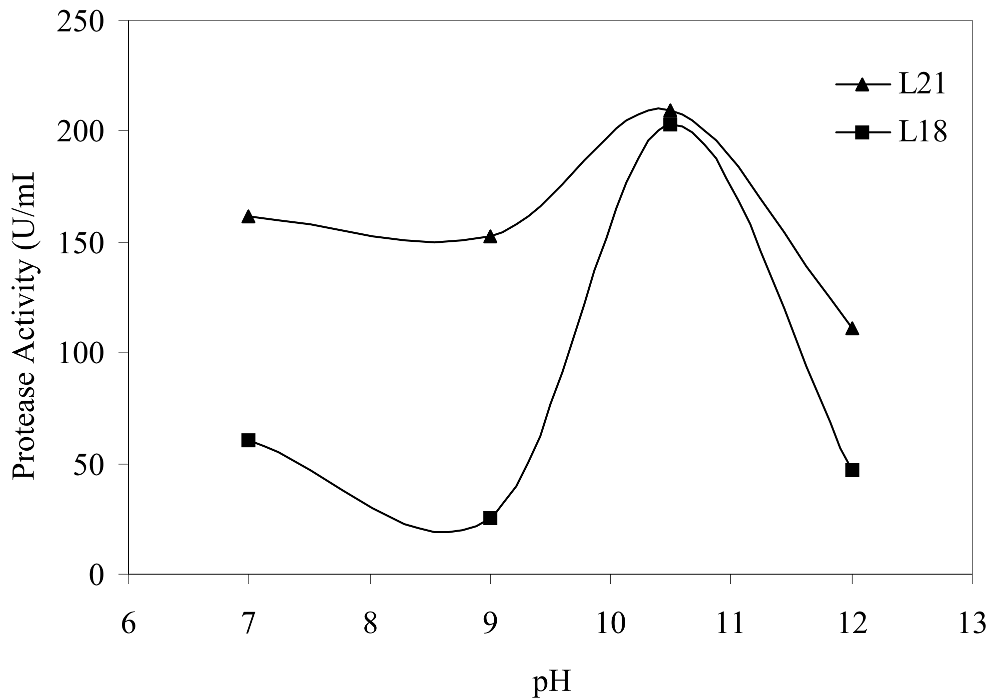


Figure 4.6. pH Profiles of L18 and L21 at 37°C and 180 rpm in standard Horikoshi media incubated for 96 hours

The effect of initial media pH was determined for strains L18 and L21, where the Horikoshi media was adjusted to different initial pH using 6N NaOH and 6N HCl, and inoculated with 5% (v/v) inoculum of each subculture and incubated at 37°C and 180 rpm for 96 hours. I18 was not taken in this set of experiment since, L18 and L21 strains were the promising strains for high protease activities and further study was decided to be done on these. As it is well seen from the activity profiles (Fig. 4.6) at different pH, L21 has a much broader pH range than L18. This graph presents the main difference between these two strains where one can conclude that L18 is a strict alkaline with a narrow pH range, whereas L21 might range to be a neutral to alkaline strain. L21

is much more stable against pH changes, whereas it cannot be said for L18. With this regard L21 seems to be a better candidate for further study.

4.2.6. Yield Factors at Optimum Conditions

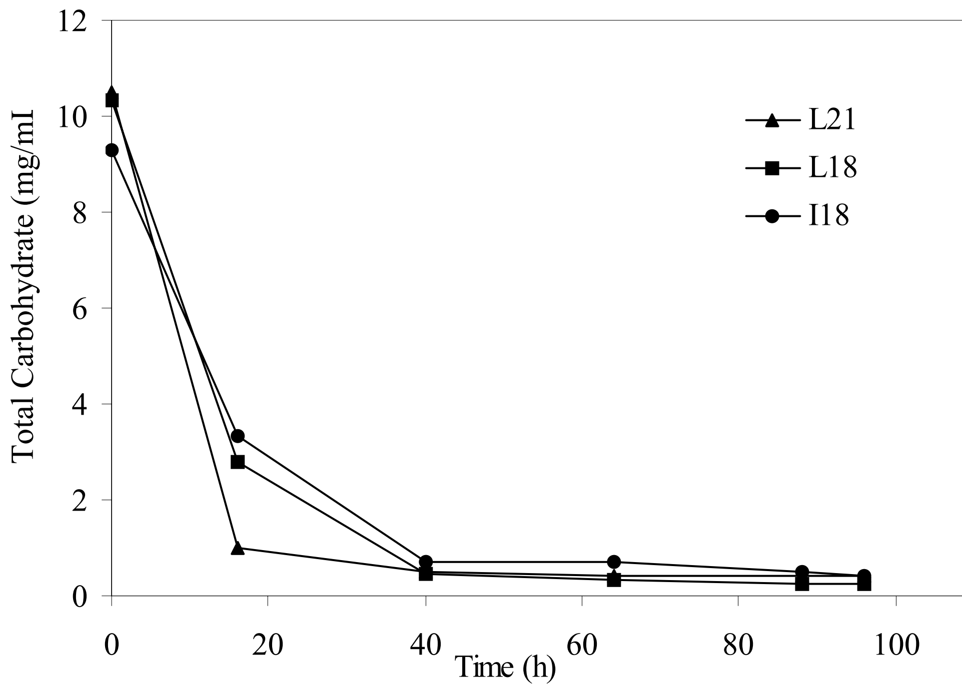


Figure 4.7. Carbon utilization profiles at optimum conditions determined for each strain after 96 hours of incubation

Biomass and product yield factors were estimated using the protease activities reported in Table 4.1, 4.2 and 4.3 and carbon utilization profiles (Fig. 4.7) at optimum conditions determined for each strain after 96 hours of incubation. These were for I18 at 100 rpm, 30°C, and for strains L18 and L21 at 180 rpm, 37°C. The optimum inoculation ratio for all three strains was 5% (v/v). The initial pH of the Horikoshi media was 10.5, which was already determined to be the optimum pH for L18 and L21. In the calculations of these yield factors maintenance effects and endogenous metabolism were neglected. According to the given results (Table 4.5) there is almost an inverse relationship between the biomass and product yields. This relation is very much pronounced in strain L18 than the other two. Therefore one can conclude that the enzyme production is not growth related in this strain and if the aim is to produce more enzyme, then one should set up the environmental conditions, which favor more product

formation than growth of this organism. However for the other two strains I18 and L21, the product formation is closer related to growth of the organism. Thus for these two organisms environmental conditions favoring the growth seem not to adversely effect the product formation that much. These results are very much confirmed with carbohydrate utilization profiles (Fig. 4.7) which shows that the carbohydrate is faster utilized in strain L21 compared to the other two strains. This brings up the issue, controlled carbohydrate supply at the end of 40 hours would increase product formation of this strain (L21) further. Therefore this will be task to be studied in future. Another drawback would be that faster carbohydrate utilization would increase raw material cost, however a balance between the cost of the raw material and the gain from the product yield has to be established. With this respect strain L18 seems to be a more efficient strain with less carbohydrate consumption but still with higher product yield. But since this strain resulted in lower specific activity with a less pH stability, it was not considered to be a candidate for future study. Comparison among the strains demonstrated that, strain L21 is the strain with high biomass and product yield.

Table 4.5. Biomass and product yields of I18, L18 and L21 at optimized conditions after 96 hours of incubation

Strain	Biomass ($Y_{X/S}$) (g biomass/g substrate)	Product yield ($Y_{P/S}$) (U product/g substrate)
L21	0.023	0.021
L18	0.011	0.022
I18	0.017	0.014

4.3. Optimization of Media Formulation for Alkaline Protease Production by *Bacillus* sp. L21

4.3.1. Screening of Media Components

In the preliminary screening studies on the development of the production medium, various industrially low-cost carbon and nitrogen sources and elements were screened and as a result, maltose, soybean meal, Tween 80 and pH of the medium were found to be important factors in enhancing the alkaline protease formation.

4.3.1.1. Effect of Carbon Sources on the Alkaline Protease Production

Different carbon sources obtained from a Cargill Sweeteners and Starches Inc. were screened as main industrial carbon sources in the formulation of the reference media Horikoshi-I. The concentration used was 10 g/L as indicated in the reference media.

As it is seen from Table 4.6, except for whey, molasses and potato starch, the rest of the carbon sources gave satisfactory specific protease activity results if compared with the control. However if total protease enzyme activities were considered Glucose–Fructose (30%) and Maltose (50%) were taken as best carbon sources. These two sources were also very much satisfactory with respect to their specific protease activities. The product yield factor $Y_{p/s}$, neglecting endogenous metabolism and maintenance effect, were calculated and presented, (Table 4.6). As it is seen here, only potato starch resulted in approximately 40 % higher yield factor compared to control, maltose 50 and corn starch, however its specific and total proteolytic activities were lower. Considering all these factors, maltose (50%) due to its lower cost, was preferred as the carbon source for further optimization study.

Table 4.6. Effect of various carbon sources on the total and specific protease activity and product yield factor after 70 hours of fermentation

Carbon Sources	Protease Activity (U/mL)	Specific Protease Activity (U/mg protein)	Product Yield (U/g substrate)
Glucose (40 %)	93.8	20.4	0.023
Glucose syrup (95%)	127.3	19.9	0.019
Fructose corn syrup (42%)	130.6	20.3	0.014
Maltose (50%)	152.8	22.8	0.035
Glucose-Fructose (30%)	163.9	22.1	0.027
Natural corn starch	106.5	20.9	0.038
Potato Starch	139.2	14.5	0.057
Molasses	113.5	14.8	No data
Whey	73.6	8.5	0.015
Control	152.4	22.4	0.034

4.3.1.2. Effect of Nitrogen Sources on the Alkaline Protease Production

Different nitrogen sources were screened which were obtained from Cargill Soy Protein Solution Inc. as main nitrogen sources used industrially. The concentration used was total 10 g/L as given in the reference media formulation. As it is seen from Table 4.7, the lowest total and specific activities were obtained with casein, corn meal and whey. Corn steep liquor, yeast extract and soybean meal gave much more satisfactory result compared to the other sources used. Since yeast extract was a much more expensive source compared to the other two sources (corn steep liquor and soybean meal), which are the industrial byproducts of the corn and soybean industry, it was eliminated from the further study. However since it was difficult to decide which of these nitrogen sources should be taken for the optimization study, a preliminary study on their interaction if used together was performed. Therefore, Resolution IV fractional factorial design with single replicate of treatment combinations of four factors was designed and analysed by Minitab. Design matrix of experiments and corresponding protease activity measurements are given in Table 4.9.

Table 4.7. Effect of various nitrogen sources on the total and specific protease activity and product yield factor after 70 hours of fermentation.

Nitrogen Sources	Protease Activity (U/mL)	Specific protease activity (U/mg protein)
Casein	7.4	0.46
Corn steep liquor	128.7	36.1
Yeast extract	114.9	29.8
Corn meal	49.9	12.6
Soybean meal (fine)	96.4	15.8
Soybean meal	102.5	14.0
Whey	28.4	3.4
Control	152.4	22.4

In these experiments the carbon source was taken as Maltose (50%) at a constant level of 10 g/L. A fractional factorial design with the variables as corn steep liquor, soybean meal, Tween 80 and $CaCl_2$ were established and run at the same time. According to these results, soybean meal or corn steep liquor could be used as a single nitrogen source, provided that there is Tween 80 at 0.15 g/L (minimum) and $CaCl_2$ at 0.5 g/L (maximum) concentrations. Therefore since soybean meal resulted in slightly higher activity than corn steep liquor, we decided on using soybean meal as the nitrogen source in the experimental design set up for response surface method analysis.

4.3.1.3. Effect of Various Elements on the Alkaline Protease Production

In order to study the effects of different elements on the protease activity, the reference media was enriched with the additions of these components at the given concentrations (Table 4.8). Tween 80 was also used in these experiments in order to determine if it had a promoting or inhibiting effect on the enzyme synthesis. Tween 80 is a well-known industrial surfactant; therefore it was thought that its presence in the media would affect the homogeneity of the broth and facilitate the nutrient and oxygen transfer to the microorganism. From Table 4.8, $CaCl_2$ at 0.1g/L concentration and Tween 80 (0.25 g/L) were the two components, which had promoting effect on the synthesis. The positive effect of Tween 80 and $CaCl_2$ at 0.1g/L concentration was pronounced with respect to the specific protease activities as well. The higher $CaCl_2$ concentration (1 g/L) however had inhibitory effect. As expected the presence of Tween 80 in the medium increased the activity by 16%. The presence of the other components at the given concentrations did not influence the enzyme synthesis very much. Since it was difficult to decide which of the component Tween 80 or $CaCl_2$ should be taken as a variable for optimization process, and to see their interaction, if presented in the same media formulation, same experimental design with single replicate presented in Table 4.9 was performed. From the results of Table 4.9, the presence of $CaCl_2$ (0.5 g/L) at its maximum concentration together with Tween 80 (0.15 g/L) at minimum concentration gave satisfactory result. Using Tween 80 at its maximum concentration with $CaCl_2$ at minimum level decreased the protease activity by at least 15.8 %. Therefore according to this preliminary study, we decided to keep the $CaCl_2$ concentration constant at the maximum concentration of 0.5 g/L and use Tween 80 as the main variable to be used in the response surface.

Table 4.8. Effect of various elements and surfactant on the total and specific protease activity and product yield factor after 70 hours of fermentation

Elements	Concentration (g/L)	Protease Activity (U/mL)	Specific protease activity (U/mg protein)
Mg_2SO_4	0.1	145.2	19.9
Mg_2SO_4	1.0	134.7	21.0
KH_2PO_4	0.5	149.7	22.3
KH_2PO_4	2.0	143.1	20.7
$CaCl_2$	0.1	171.7	27.3
$CaCl_2$	1.0	77.4	8.7
Tween 80	0.25	180.9	29.4
Control	-	152.4	22.4

Table 4.9. 2_{IV}^{4-1} fractional factorial design and protease activity results after 96 hours

Run	Concentrations (g/L)				Protease activity (U/mL)
	CSL	SoyB	Tween 80	$CaCl_2$	
1	5	5	0.35	0.5	148.7
2	0.0	5	0.35	0.1	200.7
3	5	5	0.15	0.1	108.5
4	5	0.0	0.15	0.5	229.8
5	5	0.0	0.35	0.1	194.1
6	0.0	0.0	0.35	0.5	105.5
7	0.0	5	0.15	0.5	238.4
8	0.0	0.0	0.15	0.1	107.9

4.3.2. Optimization of Media Formulation for Alkaline Protease Production by *Bacillus* sp. L21 with Response Surface Methodology

The conventional method of optimization involves variation of one parameter at a time and keeping the others constant. This is a extremely time consuming and expensive method when a large number of variables are considered and also does not often bring up the effect of interaction of various parameters as compared to factorial design (Adinarayana and Ellaiah, 2002). These limitations of a single factor optimization process were eliminated by optimizing all the affecting parameters collectively by statistical experimental design using response surface methodology (RSM). Response surface methodology consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria. This study involves the optimization of the fermentation medium for maximum alkaline protease production from *Bacillus* sp. L21 as a result of the interaction between four variables (soybean meal, maltose, Tween 80 concentrations, pH of the medium) by employing Box-Behnken design which, proposes a three level design for fitting response surfaces. The reason of using Box-Behnken design was reducing the number of experiments by the method that involves 2^k factorial design with incomplete blocks.

The medium constituents and the process parameters were optimized by single factor optimization keeping the other factors constant (See Section 4.2 and 4.3.1). Therefore, strain L21 was fermented with 5% (v/v) inoculation ratio at 37°C and 180 rpm for 88 hours. Peptone (5 g/L), KH_2PO_4 (1 g/L), $Mg_2SO_4.7H_2O$ (0.02 g/L), and Na_2CO_3 (10 g/L) were the constant components of the medium formulation. $CaCl_2$ concentration (0.5 g/L) was determined by a preliminary experimental design as mentioned in Section 4.3.1. It was also determined that soybean meal, maltose, Tween 80 and pH of the medium had the most profound effects on alkaline protease production and therefore these four factors were selected for statistical optimization by response surface methodology (RSM).

In order to search for the optimum formulation of the medium, Box-Behnken design with four factors, which indicated that 27 experiments were required for this procedure, was employed to fit a second order polynomial model. The experimental design and corresponding alkaline protease yields are shown in Table 4.10.

Table 4.10. Box-Behnken design and experimental results of protease activity

Blocks	Soybean Meal (g/L)	Maltose (g/L)	Tween80 (g/L)	pH	Activity (U/mL)	
					Observed	Predicted
2	5	5	0.25	12	151.6	161.0
2	5	10	0.25	9.5	160.0	177.7
2	5	15	0.25	7	252.2	238.3
2	5	5	0.25	7	192.8	172.3
2	2.5	10	0.15	9.5	227.0	261.6
2	7.5	10	0.15	9.5	226.5	199.1
2	2.5	10	0.35	9.5	224.3	247.2
2	5	15	0.25	12	158.7	174.7
2	7.5	10	0.35	9.5	239.6	200.6
3	7.5	10	0.25	7	126.0	160.3
3	5	15	0.15	9.5	238.8	214.6
3	5	5	0.15	9.5	178.0	177.5
3	7.5	10	0.25	12	100.3	94.7
3	5	10	0.25	9.5	141.0	144.6
3	2.5	10	0.25	7	182.0	186.7
3	5	5	0.35	9.5	145.1	168.3
3	5	15	0.35	9.5	211.4	210.9
3	2.5	10	0.25	12	212.5	177.3
1	5	10	0.35	12	128.5	130.1
1	5	10	0.15	12	177.0	190.7
1	5	10	0.35	7	230.1	221.7
1	7.5	5	0.25	9.5	158.5	168.8
1	2.5	15	0.25	9.5	268.2	263.2
1	5	10	0.15	7	170.4	174.1
1	2.5	5	0.25	9.5	194.0	171.9
1	7.5	15	0.25	9.5	129.8	157.2
1	5	10	0.25	9.5	176.9	155.6

At the end of the analysis of response data and treatment combinations, the least square estimate of parameters (β_i) (Table 4.11) of the final model were determined, therefore it was concluded that among six interaction terms only that of Tween 80 and pH (X_3*X_4) was significant. Linear terms of soybean meal and maltose concentrations and pH, and quadratic term of Tween 80 were also the significant terms as can be seen in p-values in Table 4.11. However, all the linear, quadratic and interaction terms were included in the model since the removal of the insignificant terms caused a decrease in the adjusted R^2 term.

Therefore, the regression equation (Eq. 4.1), which gives the empirical relationship between alkaline protease activity (Y) and the four test variables in coded unit was;

$$Y= 159.3 + 18.40 - 27.275X_1 + 19.925X_2 - 3.225X_3 - 18.742X_4 + 17.967X_1^2 + 16.767X_2^2 + 31.467X_3^2 - 7.858X_4^2 - 25725X_1X_2 - 27.05X_3X_4 \quad \text{-4.1-}$$

where X_1, X_2, X_3 and X_4 stands for soybean meal, maltose, Tween 80 and pH, respectively.

Table 4.11. Estimated regression coefficients for activity using data in coded units

Terms	Parameter Estimates	p-value
Constant	159.3	0.000
Block1	-3.74	0.653
Block2	18.40	0.040
X_1	-27.28	0.007
X_2	19.93	0.037
X_3	-3.22	0.714
X_4	-18.74	0.048
X_1^2	17.97	0.187
X_2^2	16.77	0.216
X_3^2	31.47	0.029
X_4^2	-7.86	0.553
X_1*X_2	-25.73	0.107
X_3*X_4	-27.05	0.092

The three-dimensional response surface and contour presentations were plotted to study the interaction among various media formulation factors used and to find out the

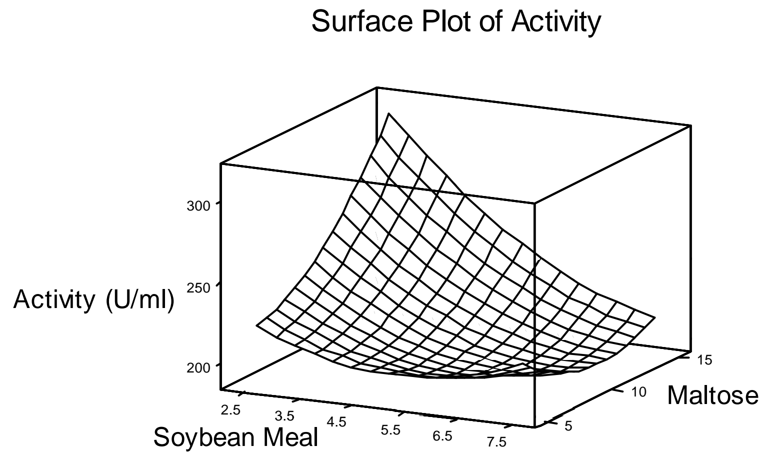
optimum level of each factor for maximum alkaline protease production from Bacillus sp. L21. However, to understand the interaction behaviors of parameters, the response surfaces were investigated for each couple of variables, keeping the other factors constant.

Figure 4.8 (a) constitutes the surface plot of activity versus soybean meal and maltose concentrations when all the factors kept constant at their optimum levels. It can be observed from the Figure 4.8 (a) that the maximum alkaline protease activity can be achieved at low values of soybean meal and high values of maltose, yielding an activity value of 300 U/mL as clarified in the contour plot (Fig. 4.8 (b)) with significant interaction. Figure 4.9 (a) shows the relative effects of Tween 80 and pH in combinations when all the factors kept constant at their optimum levels. It can be observed from the Figure 4.9 (a) that initial pH values close to neutral has a positive effect on activity. At the neutral pH values of the medium, keeping Tween 80 around its maximum level causes an increase in activity. As there is a significant interaction between the two parameters, Tween 80 concentration was adjusted according to pH of the medium since it is an effective parameter. The surface and contour plots indicating the interactions between Tween 80 and soybean meal, pH and soybean meal, pH and maltose, and, Tween 80 and maltose can be seen in Appendix B.

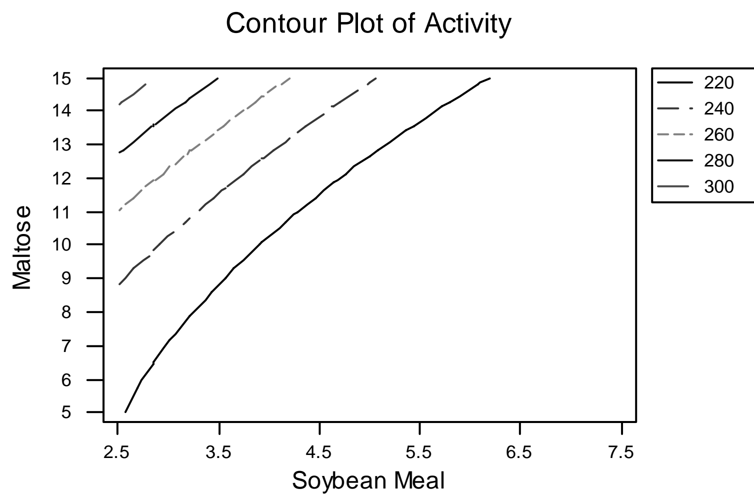
According to the observations of experimental design, the experiments given in Table 4.12 were employed in order to affirm the regression model and select the optimum experimental conditions for the maximum protease activity. Experiments A, B and C were carried out to investigate the effect of pH variation on activity when other factors were kept at the optimum levels. Experiment D aimed an economical approach by decreasing the maltose consumption. The conditions in experiment B, which state soybean meal, maltose, Tween 80 and pH as 2.5 g/L, 15 g/L, 0.35 g/L and 8.5, respectively, yielded an actual protease activity of 294.3 U/mL, where the model estimated a value of 338 U/mL.

Table 4.12. Additional experiments (A, B, C, D) for validation of Box-Behnken design

	Soybean (g/L)	M50 (g/L)	Tween80 (g/L)	pH	Activity (U/mL)
A	2.5	15	0.35	7.0	283.3
B	2.5	15	0.35	8.5	294.3
C	2.5	15	0.35	9.5	276.3
D	2.5	12	0.35	9.5	265.6

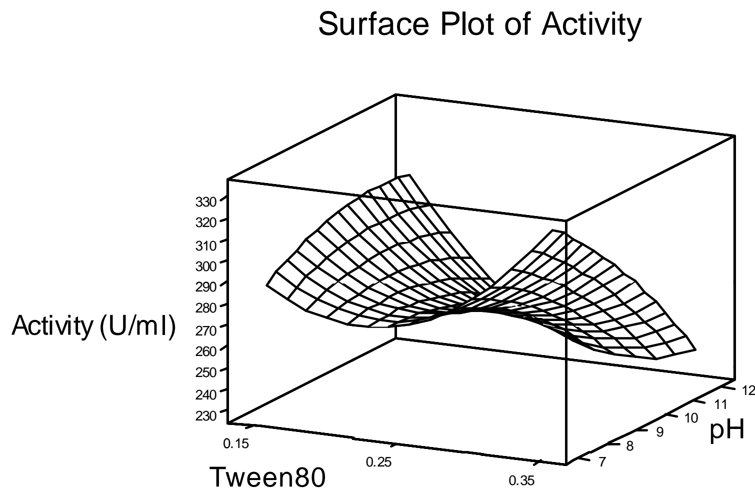


(a)



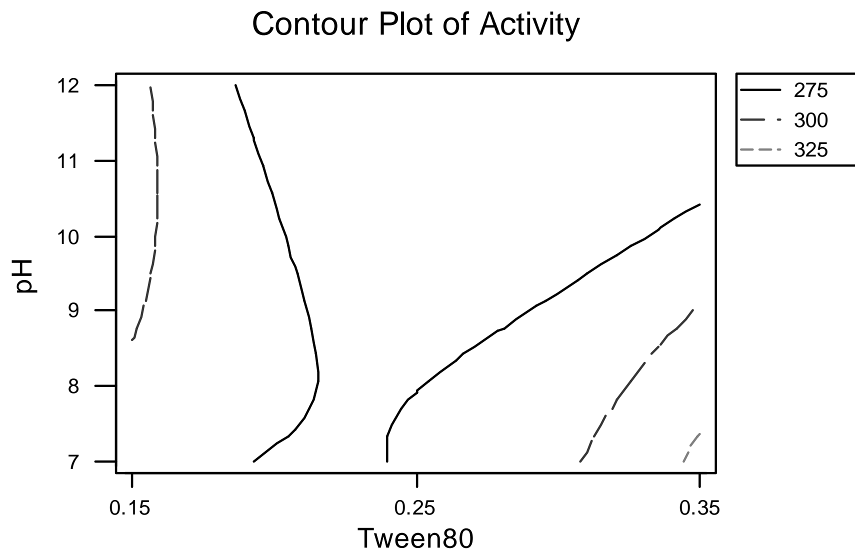
(b)

Figure 4.8. (a) Response surface plot; (b) contour plot of alkaline protease production showing the interaction between maltose and soybean meal at the constant values of all other parameters.



Hold values: Soybean: 2.5 Maltose: 15.0

(a)



Hold values: Soybean: 2.5 Maltose: 15.0

(b)

Figure 4.9. (a) Response surface plot; (b) contour plot of alkaline protease production showing the interaction between pH and Tween 80 at the constant values of all other parameters.

At the end of Box-Behnken design, true optimum values for the significant factors could not be determined because of the saddle nature of the response surfaces (Figure 4.8 (a) and Figure 4.9 (a)). Therefore it was decided to conduct 2-factor Central Composite Design (CCD) at constant pH of 8.0 and 0.35 g/L of Tween 80, where maltose concentration at the levels of 12, 20 and 28 g/L and soybean meal concentration at the levels of 0, 2 and 4 g/L were taken as the main factors. In an industrial process it is important to reduce the cost of maltose and soybena meal since they play an economical role. Hence, in CCD, two factors –maltose and soybean meal- were chosen and the others –pH and Tween 80- were kept constant. The protease activity results of the CCD of 12 different runs are presented in Table 4.13. At the end of the analysis of response data and treatment combinations, it was concluded that the quadratic term of maltose and the interaction term were neglected. All the linear terms, and quadratic term of soybean meal were included in the model since these were the significant terms as observed from the p-values in Table 4.14. Therefore, the final empirical model for protease activity using the least square estimate of parameters, β_i, (Table 4.14), in terms of two factors in coded units is described as;

$$Y = 284.68 + 83.77X_1 + 12.72X_2 - 92.32X_{12} \qquad -4.2-$$

Table 4.13. Central composite design and experimental results of protease activity after 88 hours of fermentation

Blocks	Soybean Meal (g/L)	Maltose (g/L)	Activity (U/mL)
1	2	20	293.5
1	2	20	306.5
1	4	12	273.8
1	4	28	297.9
1	2	20	284.7
1	0	28	149.2
1	2	12	280.9
1	0	12	80.6
1	2	20	278.0
1	4	20	256.7
1	2	28	264.5
1	0	20	96.0

Table 4.14 Parameter estimates of model coefficients of 2-factor CCD in coded units

Terms	Parameter Estimates
Constant	284.68*
X_1	83.77*
X_2	12.72
X_1^2	-92.32*

*p value is less than 0.01

The adjusted R^2 of the model is 93.3% with an insignificant lack of fit (p value = 0.141). According to Figure 4.10 and the derivative of Y with respect to X_1, maximum activity can be achieved at 3.0 g/L of soybean meal. Since we could not determine an optimum maltose concentration, additional experiments at 3.0 g/L of soybean meal, 0.35 g/L of Tween 80 and pH of 8.0 were conducted at different maltose concentrations of 20, 30, 40, 50, 60, and 80 g/L. After, an increase in the activity level at maltose concentration of 30g/L, a drop was observed at 40 g/L of maltose (Table 4.15). It was concluded that an optimum maltose concentration could be achieved between 30-40 g/L. The maximum activity under these conditions was determined as 269.2 U/mL. It was thought that these newly optimized conditions would even enhance the activity; compared to the previous activity data of 294.3 U/mL obtained in the validation experiments of Box-Behnken design. This decreasing trend in the activity values can be explained by some unknown factors resulting possibly from strain instability and experimental errors.

On the other hand, comparison of response results of Box-Behnken design and CCD shows that protease activity values are higher in the second set of experiments (Table 4.10 and Table 4.13). In CCD, media composition and pH value was adjusted according to the first set of experiments (Box-Behnken design) to be able to get an optimum region for a better protease activity. For example, the maximum activity value, 268.2 U/mL, in the first set was achieved at 2.5 g/L soybean, 15 g/L maltose, 0.25 g/L Tween 80 and pH of 9.5 (Table 4.10). The closest composition to this combination in the second set is 2 g/L soybean, 20 g/L maltose, 0.35 g/L Tween 80 and pH of 8.0, which produces activity values in the range of 278 – 306.5 U/mL (Table 4.13). This adjustment caused an increasing trend in the overall activity results.

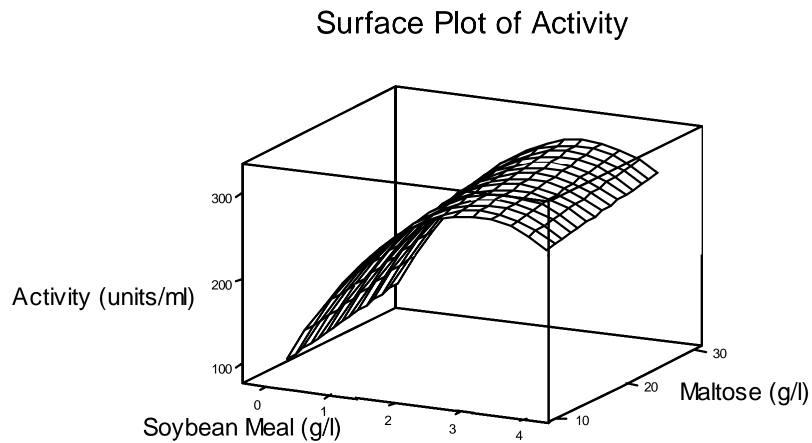


Figure 4.10. Response surface plot of 2-factor CCD alkaline protease production showing the interaction between soybean meal and maltose

Table 4.15. Protease enzyme activity results after 88 hours of fermentation at optimum soybean meal concentration of 3.0 g/L, pH 8.0, Tween 80 at 0.35 g/L and at various maltose concentrations

Maltose (g/L)	Activity (U/mL)
20	251.4
30	269.2
40	254.0
50	251.2
60	224.2
80	215.8

4.4. Characterization of Alkaline Protease

In this chapter, experimental results about the characteristic properties of the crude enzyme preparation produced by *Bacillus* strain L21 were presented. Production of protease from *Bacillus* strain L21 for characterization studies was carried out in standard Horikoshi-I medium at temperature of 37°C and 180 rpm for 88 hours. Experimental results include the effects of temperature and pH on activity and stability of the enzyme. In addition, the effects of metal ion, surfactant and inhibitor on the enzyme activity were also investigated. All experiments were done in duplicates the mean values were reported here.

4.4.1. Effect of pH on Alkaline Protease Activity and Stability

The effect of pH on the proteolytic activity of alkaline protease produced from strain L21 was determined by assaying enzyme activity at different pH values with buffers at pH range of 4.0 – 13.0 and the results of this study are given in Figure 4.11.

The enzyme was active in a broad pH range, displaying over 50% of its maximum activity in the pH range of 4.0-11.0. However, the activity decreased to 50% of its maximal value under acidic conditions (below pH 7). Therefore, the enzyme found to be a typical alkaline protease, displaying its activity for casein predominantly in the alkaline region of pH 8.0-11.0 (Huang et al., 2003; Banerjee et al., 1999; Joo et al., 2002). Optimum protease activity was observed at pH 11.0 and an increase in pH beyond 11.0 brought about a rapid decline in protease activity resulting in 16% relative activity at pH 13.0. These findings are in accordance with several earlier reports showing pH optima of 11.0 for protease from *Bacillus* sp. NCDC 180 (Kumar et al., 1999), *Bacillus* sp. RGR-14 (Oberoi et al., 2001), *Bacillus* sp. KSM-K16 (Kobayashi et al., 1996) and *Bacillus pseudofirmus* AL-89 (Gessesse et al., 2003). Strain L21 produced an alkaline protease that is active up to 11.0, which is a very important characteristic for the use of alkaline proteases as detergent components, because the pH of laundry detergents is generally in the range 9.0-12.0. The important detergent enzymes, Subtilisin Carlsberg and Subtilisin Novo or BPN also showed maximum activity at pH 10.5, which is a close value to that of L21 protease (Banerjee et al., 1999).

Enzymatic hydrolysis of gelatin layer of X-ray films at an elevated pH is preferable to prevent microbial contamination and degrade the gelatin layer in a short time. Therefore, alkaline protease produced from strain L21 can be a potential enzyme for the recovery of silver from used X-ray films process.

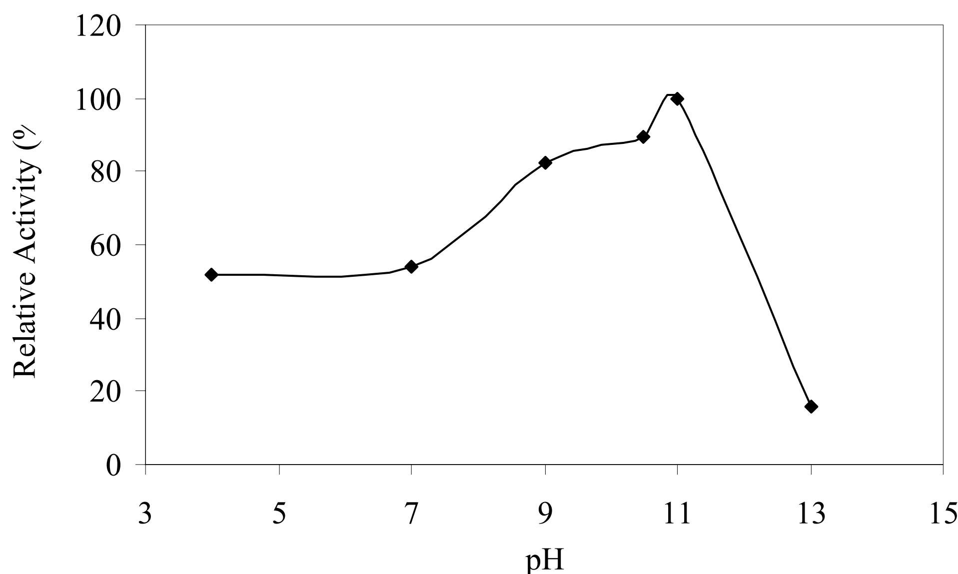


Figure 4.11. Effect of pH on the activity of *Bacillus* sp. L21 protease

The pH stability of *Bacillus* sp. L21 protease was investigated in the range of 4.0-13.0 and results are given in Figure 4.12. The stability of the enzyme was expressed as the % activity remaining after 2 hours at the corresponding pH, at 30°C, based on the original enzyme activity. The experimental procedure was explained in Section 3.2.8.

Investigations on pH stability showed the enzyme to be stable in the pH range of 4.0 to 11.0 with maximum stability at pH 9.0. As shown in Figure 4.12 the enzyme was very stable in a broad pH range, maintaining over 73-78% of its original activity between pH 4.0 and 11.0. The enzyme was stable at both acidic and alkaline pH, but declined sharply with about 1% of the initial activity being retained at pH 13.0.

The results of this study was similar with that of Oberoi et al. (2001), who found alkaline protease from *Bacillus* sp. RGR-14 to be fully stable under alkaline conditions (between pH 6.0 to 12.0). In another study Singh et al. (1999) reported a protease produced by *Bacillus sphaericus* that showed stability in the pH range of 7.5 to 11.0. Similarly, pH 12.0 and 13.0 were found to be the pHs where the enzyme lost its activity.

In addition, the broad pH stability range of the enzyme indicated its suitability for application(s) requiring a wide pH stability range, as in case of detergents.

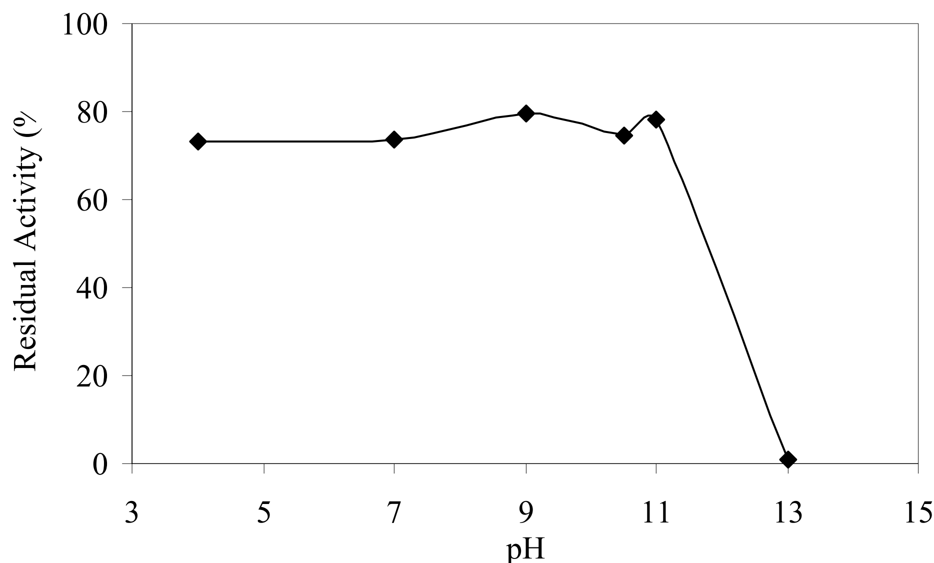


Figure 4.12. Effect of pH on the stability of *Bacillus* sp. L21 protease

4.4.2. Effect of Temperature on Alkaline Protease Activity and Stability

The effect of temperature on the proteolytic activity of alkaline protease produced from strain L21 was determined by incubation of the reaction mixture (pH 10.5) for 20 minutes, at different temperatures ranging from 30 to 80ºC. The results of this experiment can be seen in Figure 4.13.

The protease was active over a broad temperature range of 20-80ºC. The optimum temperature for protease activity was found to be 60ºC and retained about 90% of the original activity even at 80°C. Several investigators have reported the optimum temperature of *Bacillus* sp. alkaline protease as 60ºC (Gupta and Beg, 2003; Joo et al., 2003; Oberoi et al., 2001). As can be seen from Table 2.4 this value of optimum temperature is one of the highest among *Bacillus* sp. alkaline proteases.

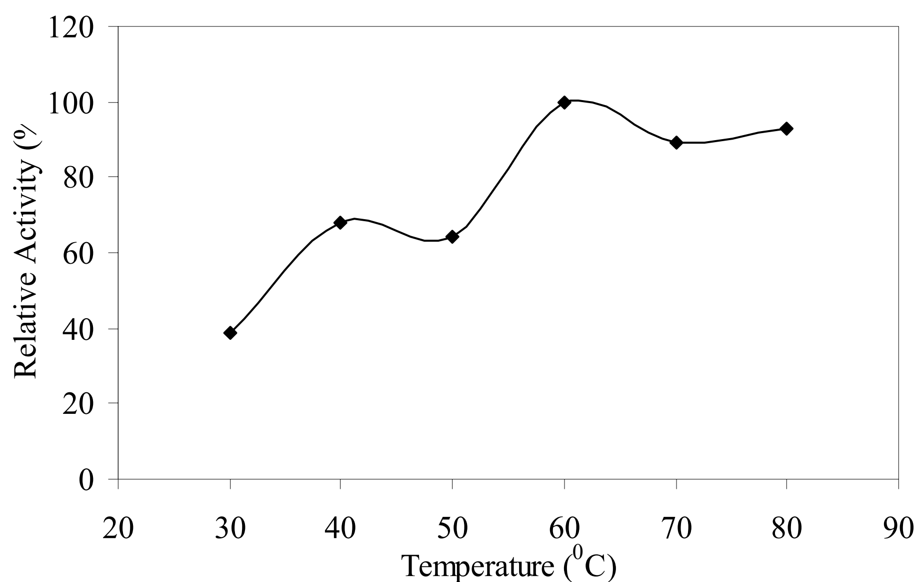


Figure 4.13. Effect of temperature on the activity of *Bacillus* sp. L21 protease

Experiments in order to determine the thermostability of the crude alkaline protease was conducted by measuring the residual activity after incubating the enzyme at various temperatures ranging from 30 to 60ºC for 30 and 60 minutes, respectively, in the presence and absence of 0.05 M Ca^{2+}. The experimental procedure is given in detail in Section 3.2.9. The results obtained from this study can be seen in Figure 4.14 (a) and (b).

As shown in Figure 4.14 (a), after 30 minutes incubation, the enzyme was stable at 30, 40 and 50ºC both in the presence and absence of Ca^{2+} with a residual activity ranging from 52% to 71%, however residual activity about 40% and 50% was retained at 60ºC, in the presence and absence of Ca^{2+}, respectively. After 1h incubation, the enzyme was also stable at 30-40ºC both in the presence and absence of Ca^{2+} and residual activity about 30% and 40% was retained at 60ºC, in the presence and absence of Ca^{2+}, respectively (Figure 4.14 (b)). The effect of temperature on stability of the enzyme was similar for 30 minutes and 1 hour incubated proteases.

The increased rate of proteolysis of proteases at elevated temperatures is one of the factors responsible for the rapid thermal inactivation of these enzymes (Ghorbel et al., 2003). Most alkaline proteases have been reported to be significantly stabilized by the addition of Ca^{2+} at higher temperatures (Ghorbel et al., 2003; Rahman et al., 1994; Singh et al., 2001a). The improvement in protease thermostability against thermal inactivation in the presence of Ca^{2+} may be explained by the strengthening of

interactions inside protein molecules and by binding of Ca^{2+} to autolysis sites. The present protease was affected positively by the presence of Ca^{2+} ions, almost at all temperatures (30, 40 and 60ºC) studied, with an increasing trend where the effect was pronounced more at 60 minutes of incubation. A 12% increase in stability, which was determined to be the highest, was obtained at 40ºC, after 60 minutes of incubation in the presence of Ca^{2+}. The incubation time did not influence the effect of Ca^{2+} ion on the enzyme stability at 60°C, which was determined to be 9.6% increase in both cases. No significant Ca^{2+} interference or effect was observed when enzyme was incubated in the presence of Ca^{2+} at 50ºC, which could be explained by the effect of unknown factors or experimental error.

In the process of detergent formulation, where alkaline proteases are commonly added, chelating agents are included to overcome the problem of water hardness. In the presence of such chelating agents, however, the Ca^{2+} from the weak binding site of the alkaline protease can easily be stripped off thus greatly affecting the thermal stability of the detergent enzyme under application conditions. Therefore, enzymes such as alkaline protease produced from strain L21, which do not require Ca^{2+} for stability, especially at temperatures below 40°C, could offer tremendous potential for detergent application.

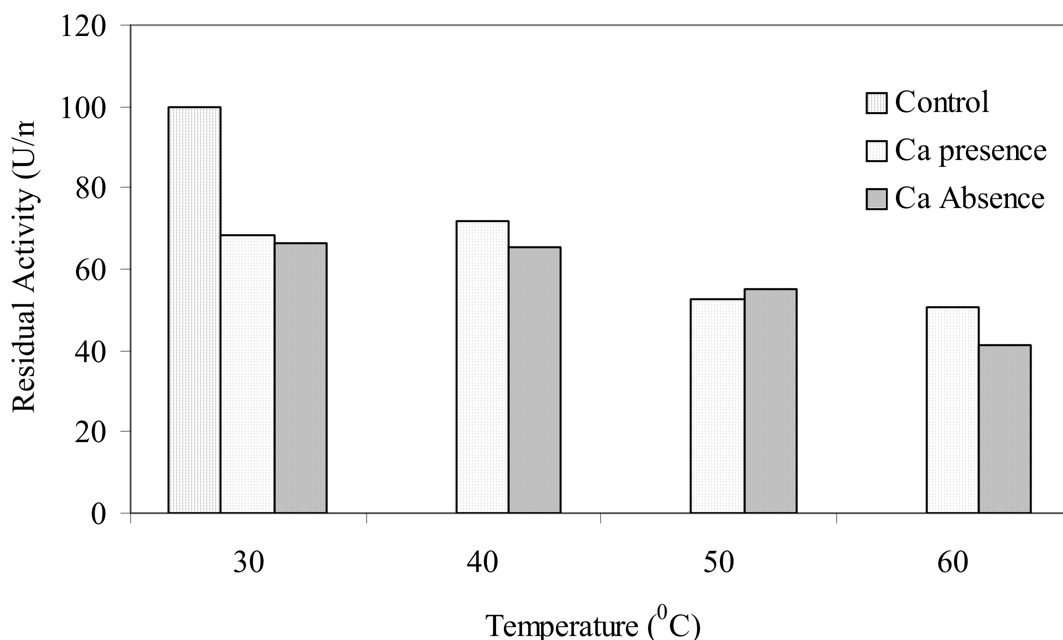


Figure 4.14 (a). Effect of temperature on the stability of *Bacillus* sp. L21 protease (30 min. incubation).

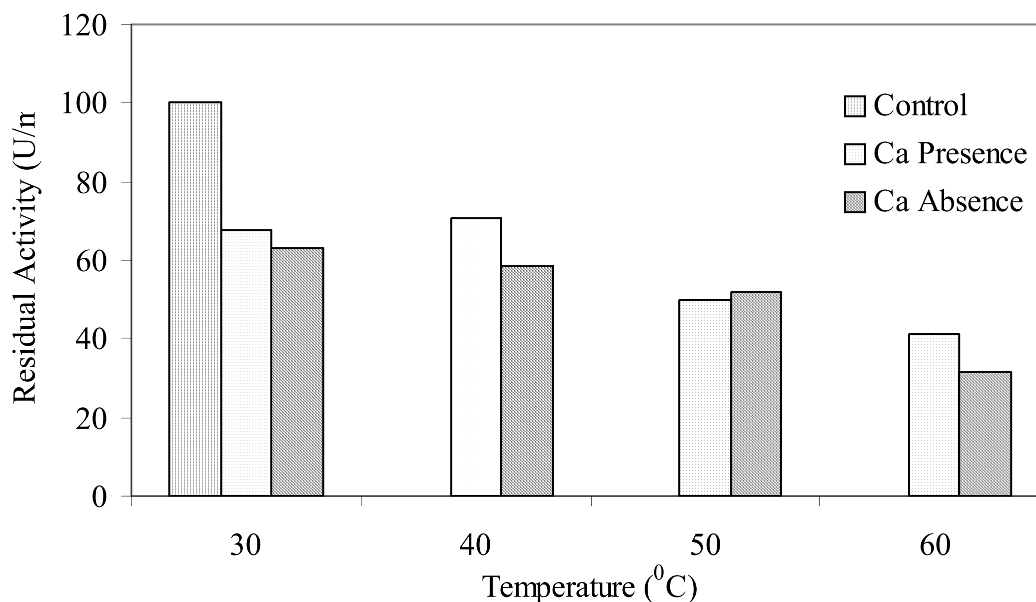


Figure 4.14 (b). Effect of temperature on the stability of *Bacillus* sp. L21 protease (60 min. incubation).

4.4.3. Effect of Various Reagents on Alkaline Protease Activity

The effects of Ca^{2+} ion as a metal ion, H_2O_2 as an oxidizing agent, PMSF and EDTA as inhibitor on alkaline protease activity were investigated to further characterize the enzyme. The results of this study can be found in Table 4.16.

Table 4.16. Effect of various reagents on activity of alkaline protease

Agent	Residual Activity (%)
Control	100.0
H_2O_2 (5%, v/v)	81.8
H_2O_2 (15%, v/v)	93.6
EDTA (0.01 M)	92.8
$CaCl_2$ (0.01 M)	89.7
PMSF (0.01 M)	1.5

The protease from strain L21 showed high stability against both 5% and 15% (v/v) concentrations of H_2O_2, which is a bleaching agent, and retained approximately

82% and 94% of its activity, respectively. Gupta et al. (1999) reported that the protease from *Bacillus* sp. SB5 retained about 95% of its activity on treatment for 1 h with 5% H_2O_2, while the *Bacillus* sp. JB-99 protease retained more than 100% of its activity on treatment for 1 h with 5% H_2O_2 (Johnvesly and Naik, 2001). The present alkaline protease is thought to be a bleach-stable enzyme, so that it can be used in detergent formulations.

The enzyme retained 93% of its activity after incubation with EDTA, which is a specific inhibitor of metallo-type proteases. In this study, there was no inhibitory effect observed from EDTA that further show that the enzyme is not a metalloprotease. In addition, the stability of the enzyme in presence of EDTA is advantageous for its use as a detergent additive. PMSF is known to sulphonate the essential serine residue in the active site of the protease, resulting in a total loss of enzyme activity (Kumar, 2002). After 1-hour incubation the enzyme activity was strongly inhibited by 0.01 M PMSF, therefore this inhibition profile suggested that the protease produced from strain L21 belongs to the family of serine proteases. Many of the *Bacillus*-derived alkaline proteases reported so far belong to the class of serine proteases (Gessesse, 1997; Joo et al., 2003; Singh et al., 2001b).

$CaCl_2$ of 0.01 M concentration showed inhibitory effect on the enzyme, decreasing the activity to 90% of the control. This can be explained that the enzyme does not require the presence of Ca^{2+} ions to be active and stable. The results of this study is similar with that of Oberoi et al. (2001), who found that Ca^{2+} ions did not show influence on proteolytic activity of alkaline protease from *Bacillus* sp. RGR-14.

Chapter 5

CONCLUSION

The first goal of this study was to optimize the alkaline protease production from *Bacillus* strains L21, L18 and I18 by investigating the effect of environmental conditions during fermentation. Secondly, an industrial media formulation optimized by using response surface methodology was developed for alkaline protease production from strain *Bacillus* sp. L21. Moreover, the characteristic properties of the crude alkaline protease of strain L21 were also studied.

Strains L21 and L18 were isolated from the by products of a leather factory, by IZTECH (Izmir Institute of Technology) Biology department, whereas strain coded I18 was isolated from the soil of the Ege University Campus by the Biology department of the same University. They were also identified as *Bacillus* sp. and as potential alkaline protease producers by IZTECH Biology department.

Specific growth rates calculated from the slope of the logarithmic phase were as $0.49h^{-1}$, $0.60\ h^{-1}$, $0.70\ h^{-1}$ for I18, L18 and L21 respectively, L21 being the strain with highest specific growth rate.

The fermentation conditions (temperature, agitation speed, inoculum concentration, incubation time and initial media pH) were optimized in order to produce alkaline protease with maximum activity from the three strains.

The studies to determine the effect of the incubation temperature on the proteolytic activity indicated that all three strains have different temperature requirements (working conditions: 180 rpm, 5% inoculum, Horikoshi-I medium, 96h fermentation, 10.5 initial pH). According to the results strain I18 has temperature optima at 30°C whereas strains L18 and L21 have at 37°C, which means that all three strains were mesophilic like most of the alkaliphilic *Bacillus* strains in nature. The alkaline protease production profiles showed that the rate of enzyme synthesis is much faster with strain L21 compared to the other two strains.

According to the result of the study based on agitation speed, 100 rpm was determined to be the optimum agitation speed for strain I18 and 180 rpm for the other two strains L18 and L21 (working conditions: 37°C, 5% inoculum, Horikoshi-I medium, 96h fermentation, 10.5 initial pH). L21 was the dominant strain with 18.4 %

and 46.9 % more specific activity than the strains I18 and L18 respectively, which is also supported with the enzyme profiles at optimum agitation speeds.

Inoculation ratio was also an important parameter in fermentation of the three strains. The optimum inoculation ratio was determined as 5% (v/v) for all three strains (working conditions: 37°C and 180 rpm for L21 and L18, 30°C and 100 rpm for I18, 180, Horikoshi-I medium, 96h fermentation, 10.5 initial pH). The specific activity data indicates that L21 produced less side by-products than the other two strains.

In addition, according to the results taken at different time intervals, it was determined that the optimum incubation time for I18 and L21 was 96 hours and for L18, 125 hours (working conditions: 37°C and 180 rpm for L21 and L18, 30°C and 100 rpm for I18, 180, Horikoshi-I medium, 5% inoculum, 10.5 initial pH).

pH profiles of L18 and L21 revealed that L21 has a much broader pH range than L18, whereas the optimum pH of growth was 10.5 for both of the strains indicating that they are alkaliphilic microorganisms hours (working conditions: 37°C, 180 rpm, Horikoshi-I medium, 5% inoculum, 96 h fermentation).

According to the results of biomass and product yield determination experiments, there was almost an inverse relationship between the biomass and product yields. Therefore, it can be concluded that the enzyme production is not growth related in the strain L18 and if the aim is to produce more enzyme, one should set up the environmental conditions, which favor more product formation than growth of this organism. However, for the strains L21 and I18, environmental conditions favoring the growth seem not to adversely effect the product formation that much. The carbohydrate is faster utilized in strain L21 compared to the other two strains. This brings up the issue, if controlled carbohydrate supply at the end of 40 hours would increase product formation of this strain (L21) further. Therefore this will be task to be studied in future. Another drawback would be that faster carbohydrate utilization would increase raw material cost, however a balance between the cost of the raw material and the gain from the product yield has to be established. With this respect strain L18 seems to be a more efficient strain with less carbohydrate consumption but still with higher product yield. But since this strain resulted in lower specific activity with a less pH stability, it was not considered to be candidate for future study. Comparison among the strains demonstrated that, strain L21 is the strain with high biomass and product yield.

When we evaluate all these results strain L21 was chosen to be the strain for further optimization study and a potential candidate with alkaline proteolytic activity for possible future industrial applications.

This part of the study contents the screening of various carbon, nitrogen sources and elements having low-costs and selection of the ones that enhance alkaline protease production from strain L21. Moreover, 27 experiments and additional 12 experiments were designed to investigate the optimum levels of four important variables i.e. soybean meal, maltose, Tween 80 concentrations and initial pH of the media. At the end of experiments of the design, results were evaluated by using response surface methodology.

At the end of screening experiments, soybean meal and corn steep liquor as nitrogen source, maltose as carbon source, $CaCl_2$ and Tween80 as elements were selected to be used in media formulation. But, a further study was required to select the exact variables of media optimization process. Therefore a resolution IV fractional factorial design with single replicate of treatment combinations of four factors (soybean meal, corn steep liquor, $CaCl_2$, Tween 80) was employed. As a result of these experiments, it was decided to keep the $CaCl_2$ concentration at the maximum concentration 0.5 g/L constant and use Tween 80 as the main variable to be used in the response surface analysis. It was also decided on using soybean meal as the nitrogen source in the experimental design set up for response surface method analysis, since it resulted in slightly higher activity than corn steep liquor. Finally, soybean meal, maltose, Tween 80 and pH of the medium were selected for statistical optimization by response surface methodology.

A regression equation implying the least square estimate of parameters of the final model was determined at the end of the analysis of response data and treatment combinations obtained from Box-Behnken design of experiments. However, to understand the interaction behaviors of parameters, the response surfaces were investigated for each couple of variables, keeping the other factors constant. As can be observed from these response surfaces that the interactions between maltose and soybean meal, and Tween 80 and pH factors were significant. Since then at the neutral pH values of the medium, keeping Tween 80 around its maximum level causes an increase in activity.

In order to affirm the regression model and select the optimum experimental conditions for the maximum protease activity 4 sets of additional experiments were

employed. The conditions in experiment B, which state soybean meal, maltose, Tween 80 and pH as 2.5 g/L, 15 g/L, 0.35 g/L and 8.5, respectively, yielded an actual protease activity of 294.3 U/mL, where the model estimated a value of 338 U/mL.

At the end of Box-Behnken design and validation experiments, true optimum values for the significant factors could not be determined because of the saddle nature of the response surfaces. Therefore a central composite design with two factors - maltose and soybean meal -, keeping pH and Tween 80 constant was conducted. All the linear terms, and quadratic term of soybean meal were included in the model according to their p-values. The adjusted R2 of the model was 93.3% with an insignificant lack of fit (p value=0.141). According to the response surface plot of 2-factor CCD alkaline protease production showing the interaction between soybean meal and maltose the optimum value of soybean meal was determined as 3.0 g/L, however it can not be determined for maltose concentration. Therefore, additional experiments at 3.0 g/L of soybean meal, 0.35 g/L of Tween 80 and pH of 8.0 were conducted at different maltose concentrations and it was concluded that an optimum maltose concentration could be achieved between 30-40 g/L. Consequently, as an optimum region of protease activity, pH of 8.0, 0.35 g/L of Tween 80, 3.0 g/L of soybean meal and a maltose concentration of 30-40 g/L was determined. The maximum activity under these conditions was 269.2 U/mL. It was observed that these newly optimized conditions did not enhance the activity; compared to the previous activity data of 294.3 U/mL obtained in the validation experiments of Box-Behnken design, which can be explained by some unknown factors resulting possibly from strain instability and experimental errors. On the other hand, comparison of response results of Box-Behnken design and CCD shows that protease activity values are higher in the second set of experiments.

The last part of the study includes the experimental results about the characteristic properties of the crude enzyme preparation produced by *Bacillus* strain L21.

As a result of pH characteristics study, the enzyme was found to be a typical alkaline protease, displaying its activity for casein predominantly in the alkaline region of pH 8.0-11.0. Optimum protease activity was observed at pH 11.0 and an increase in pH beyond 11.0 brought about a rapid decline in protease activity resulting in 16% relative activity at pH 13.0. Alkaline protease of strain L21 having pH optima of 11.0 is thought to be a potential detergent additive, since the pH of laundry detergents is

generally in the range 9.0-12.0 and also a potential enzyme for the recovery of silver from used X-ray films process.

Investigations on pH stability showed the enzyme to be stable in the pH range of 4.0 to 11.0 with maximum stability at pH 9.0. The enzyme was stable at both acidic and alkaline pH, but declined sharply with about 1% of the initial activity being retained at pH 13.0. This broad pH stability range of the enzyme indicated its suitability for application(s) requiring a wide pH stability range, as in case of detergents

The protease was active over a broad temperature range of 20-80ºC. The optimum temperature for protease activity was found to be 60ºC and retained about 90% of the original activity even at 80°C. In thermostability studies, after 30 minutes incubation, the enzyme was stable at 30-40ºC both in the presence and absence of Ca^{2+} and residual activity about 40% and 50% was retained at 60ºC, in the presence and absence of Ca^{2+}, respectively. However, after 1h incubation, the enzyme was also stable at 30-40ºC both in the presence and absence of Ca^{2+} and residual activity about 30% and 40% was retained at 60ºC, in the presence and absence of Ca^{2+}, respectively. The effect of temperature on stability of the enzyme was similar for 30 minutes and 1 hour incubated proteases. In addition, enzymes such as alkaline protease produced from strain L21, which do not require Ca^{2+} for stability, especially at low temperatures, could offer tremendous potential for detergent application.

The present alkaline protease is thought to be a bleach-stable enzyme, showing high stability against both 5% and 15% (v/v) concentrations of H_2O_2, so that it can be used in detergent formulations. In this study, there was no inhibitory effect observed from EDTA that further show that the enzyme is not a metalloprotease. In addition, the stability of the enzyme in presence of EDTA is advantageous for its use as a detergent additive. 0.01 M of PMSF completely inactivated the enzyme, indicating it to be a serine protease. $CaCl_2$ of 0.01 M concentration showed inhibitory effect on the enzyme, decreasing the activity to 90% of the control. This can be explained that the enzyme do not require the presence of Ca^{2+} ions to be active and stable.

It was supported with the results of this study that among the three microorganisms isolated, strain L21 is the best performing strain in the case of alkaline protease production.

The screening and statistical optimization procedures have lead to the development of a new low-cost industrial media formulation for alkaline protease production. Statistical optimization of media formulation using Box-Behnken design

and response surface methodology appears to be a valuable tool for the production of alkaline protease by *Bacillus* sp. L21.

The alkaline protease from *Bacillus* sp. L21 is significant for an industrial perspective because of its ability to function in broad pH and temperature ranges in addition to its tolerance and stability in presence of a bleaching agent, like H_2O_2 and chelating agent EDTA. The enzymatic properties of the alkaline protease also suggest its suitable application as additive in detergent formulations.

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APPENDIX A

A.1. Protein Determination Calibration Curve

During the determination of protein content of *Bacillus* proteases, following calibration was used with the regression coefficient of 0.9968.

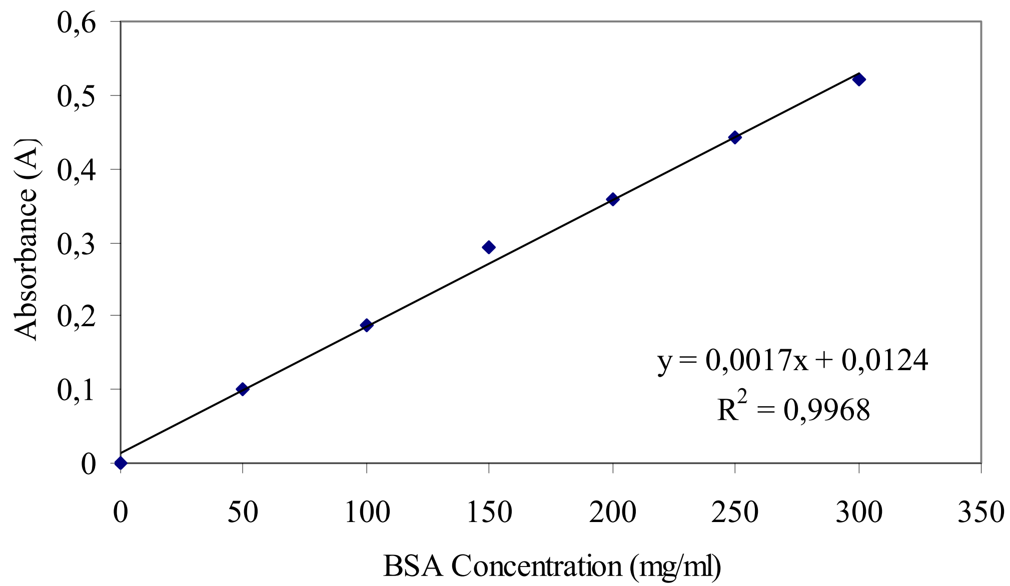


Figure A.1. Calibration curve for determination of protein content

A.2. Proteolytic Activity Determination Calibration Curve

During the determination of proteolytic activity of *Bacillus* proteases, following calibration was used with the regression coefficient of 0.9978.

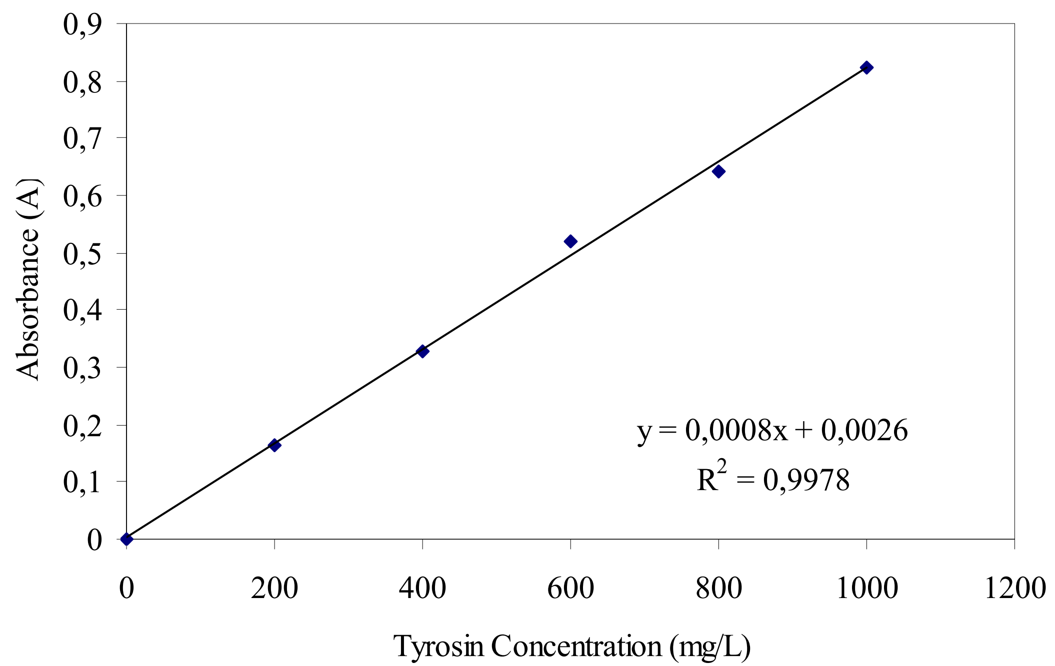


Figure A.2. Calibration curve for determination of proteolytic activity

A.3. Calibration Curve of Total Carbohydrate Content Determination

During the determination of total carbohydrate content of the culture broth, following calibration was used with the regression coefficient of 0.9865.

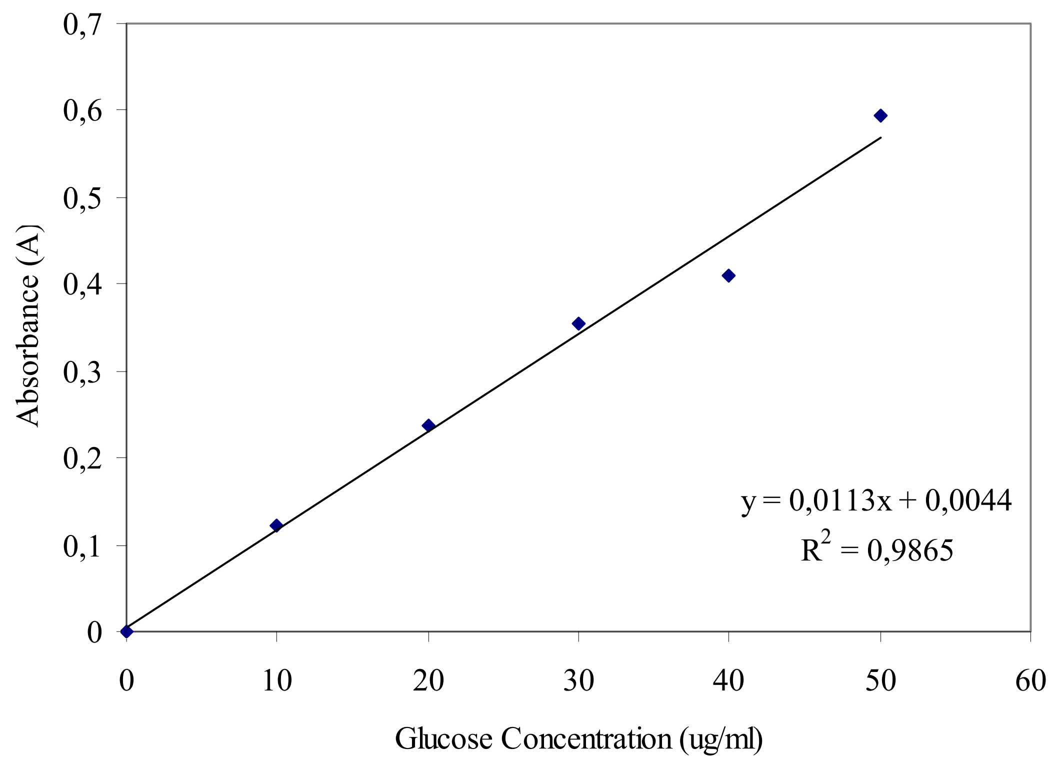
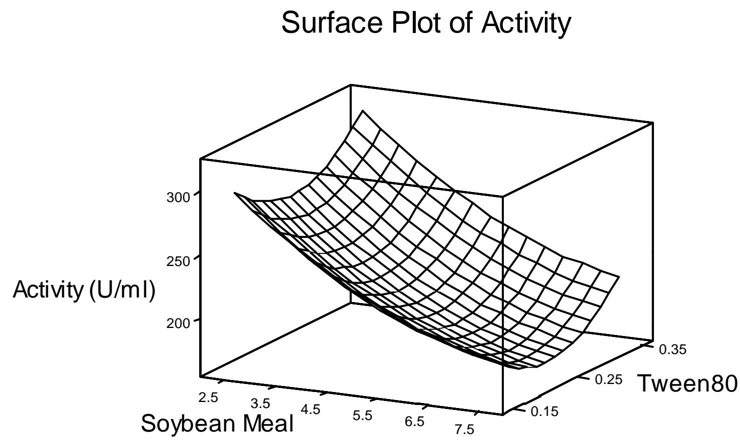


Figure A.3. Calibration curve for determination of total carbohydrate content

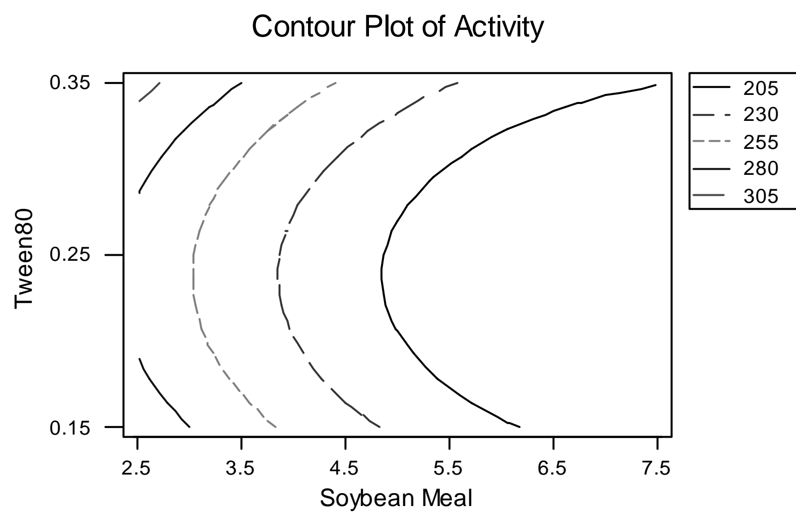
APPENDIX B

RESPONSE SURFACE AND CONTOUR PLOTS



Hold values: Maltose: 15.0 pH: 8.5

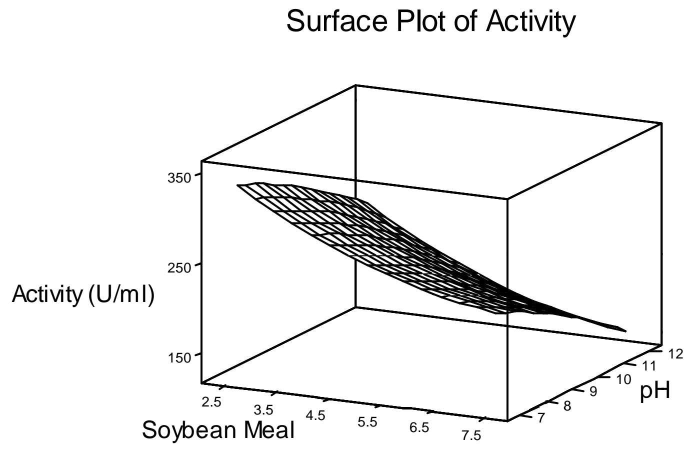
(a)



Hold values: Maltose: 15.0 pH: 8.5

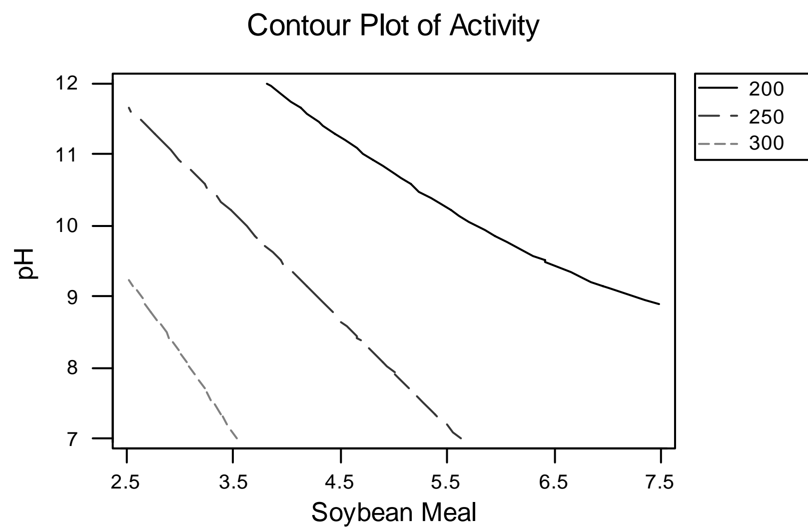
(b)

Figure B.1 (a) Response surface plot; (b) contour plot of alkaline protease production showing the interaction between Tween 80 and soybean meal at the constant values of all other parameters.



Hold values: Maltose: 15.0 Tween80: 0.35

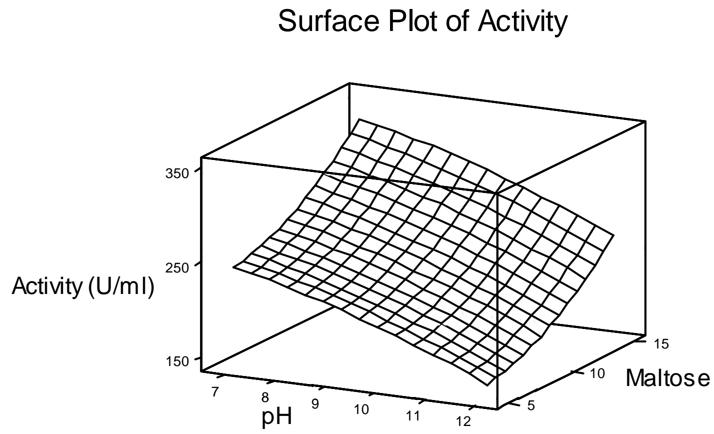
(a)



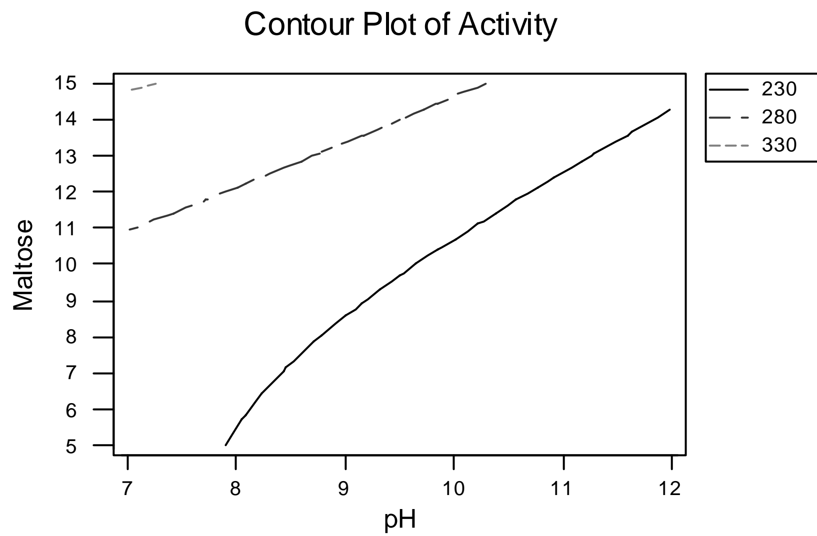
Hold values: Maltose: 15.0 Tween80: 0.35

(b)

Figure B.2. (a) Response surface plot; (b) contour plot of alkaline protease production showing the interaction between pH and soybean meal at the constant values of all other parameters.

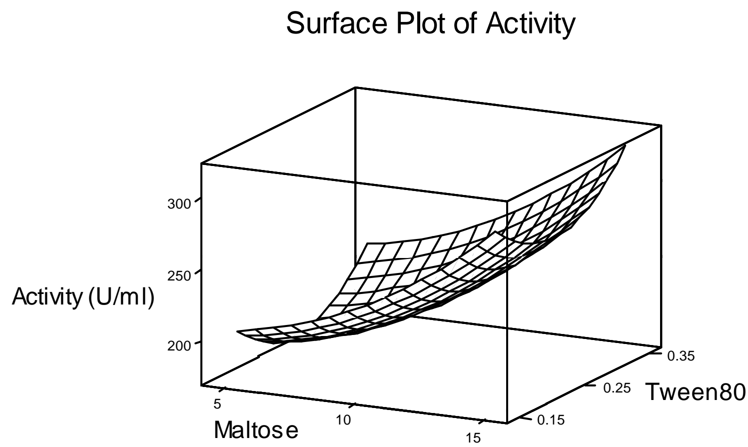


(a)

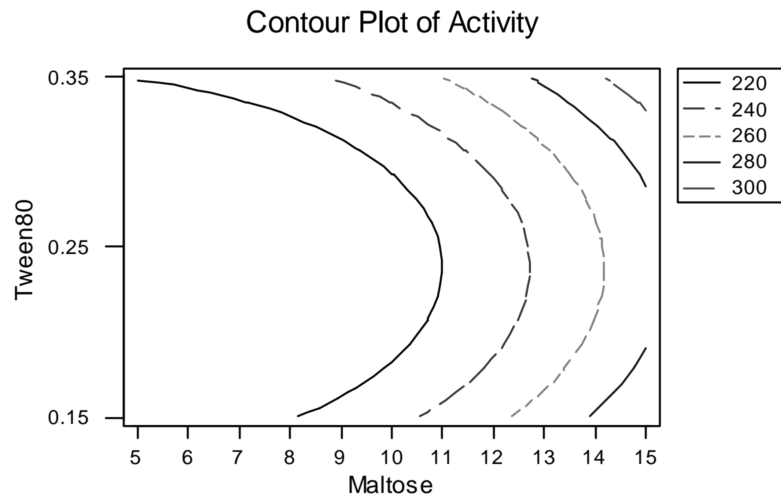


(b)

Figure B.3. (a) Response surface plot; (b) contour plot of alkaline protease production showing the interaction between pH and maltose at the optimum values of all other parameters.



(a)



(b)

Figure B.4. (a) Response surface plot; (b) contour plot of alkaline protease production showing the interaction between Tween 80 and maltose at the optimum values of all other parameters.