Gene Cloning of *Bacillus licheniformis* for Increased Production of Keratinase for Industrial Applications

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MUKITU NAHAR

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UNIVERSITY OF DHAKA

DHAKA-1000, BANGLADESH

CERTIFICATE OF APPROVAL

Name	Mukitu Nahar
Registration number and Session	Reg. no. 94, 2013-2014
Department	Microbiology, University of Dhaka
Degree	Doctor of Philosophy
Title of the thesis	Gene cloning of *Bacillus licheniformis* for increased production of keratinase for industrial applications

We approve the submission of the PhD Dissertation.

Date of Signature

.....

January 23, 2018

Prof. Dr. Md. Mozammel Hoq
Supervisor
Department of Microbiology,
University of Dhaka, Dhaka-1000,
Bangladesh

.....

January 23, 2018

Prof. Dr. Shakila N. Khan
Department of Microbiology.
University of Dhaka, Dhaka-1000,
Bangladesh

.....

January 23, 2018

Prof. Humaira Akhter
Chairman
Department of Microbiology,
University of Dhaka, Dhaka-1000,
Bangladesh.

ABSTRACT

Keratinases are a special group of proteases which are well recognized for their action on recalcitrant proteins such as feather, nail, hair, hoof etc. They have also great applications in eco-friendly dehairing processing of leathers, production of poultry feed, medical treatment and cosmetics products. So it is important to increase the production level of these enzyme. Therefore, present study was aimed at improving the production level of the enzyme through cloning of keratinase encoded gene of *Bacillus licheniformis* MZK05 and its expression in *E.coli* BL21.

Bacillus licheniformis strain MZK05, isolated from feather-decomposed soil and was identified by 16S rRNA gene sequencing. The primers were designed from the alignment of the sequences obtained for *ker*A genes from different *Bacillus licheniformis* strains. PCR amplification with the designed primers revealed a distinctive 1156 bp fragment from *B. licheniformis* MZK05. PCR product was purified and cloned into two vectors viz: pGEX-6P-2 induced on IPTG and pET-30a(+) induced on lactose expressed in *E.coli* BL21. *Escherichia coli* BL21 cells were transformed with the respective recombinant vector for the expression of protein. In pGEX-6P-2, GST-KerA fusion protein was expressed and optimized in different IPTG concentrations and the maximum expression of the desired protein was exhibited on 3 hours with 0.3 mM IPTG. From SDS-PAGE analysis, the molecular weight of the GST-KerA fusion protein was determined to be 58 kDa. Its purification by Glutathione Sepharose 4B followed by PreScission protease cleavage produced the KerA protein of about 39 kDa. The yield of purified protein was approximately 119 mg/L with the corresponding keratinase activity of 312 U/ml. A 4-fold increase in keratinase activity was obtained by heterologous expression of keratinase over the wild-type strain.

The pET-30a(+)-kerA protein expression was observed within 1-3 hours of induction at 2 mM lactose concentration. The time course induction produced gradual increase of expressed protein and was achieved into highest level after 3 hours of post-induction. A 40 kDa His-tag protein was expressed upon lactose induction which upon successful purification using Ni^{2+}-NTA resin, gave a 39 kDa KerA protein. The purified KerA protein showed 4.5 fold higher keratinase activity than that of wild strain of *B. licheniformis* MZK05.

The keratinase expression by the recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA were influenced by temperature, pH, metal ions and inhibitors in the liquid culture. The activity of both the recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA appeared highest at pH 8.0 and temperature at 40°C. While the keratinolytic activity was augmented following addition of metal ions: Ca^{2+}, Mg^{2+} and Mn^{2+}, it were decreased with Co^{2+}, Zn^{2+} and Cu^{2+}. Both pGEX-6P-2-kerA and pET-30a(+)-kerA were strongly inhibited by PMSF and slightly inhibited by EDTA and DDT indicating it serine like protease.

The whole genome sequencing of *Bacillus licheniformis* MZK05 was performed using Illumina MiSeq technique. The genome of strain *Bacillus licheniformis* MZK05 is 4,145,737 bp long with a GC content of 43.05%, containing 85 tRNA genes and 6 rRNA operons. The number of known protein coding gene is 2796 and hypothetical gene is 1282. The position of *Ker*A gene in whole genome sequencing of *Bacillus licheniformis* MZK05 is (1211369-1212344) bp.

Keratinase productivity was compared between shake flask and bioreactor cultivations. These recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA showed highest keratinase expression at 5 hrs which were 240 U/ml and 310 U/ml respectively in bioreactor cultivation. The result showed that the productivity of pGEX-6P-2-kerA and pET-30a(+)-kerA in bioreactor were 1.5 and 1.9 times higher as compared to the shake flask.

The application of keratinase enzyme in human hair rebonding test demonstrated that the hair treated with enzyme applied alone showed better performance to only rebonding cream used hair. This suggest that the keratinase produced can successfully be applied in the business of beauty parlour. In whole feathers hydrolysis test, after 48 hrs, both the enzymes used completely hydrolyzed the whole feathers comparing to wild MZK05. In dehairing of goat skins, processing demonstrated that the treatment with purified recombinant keratinase removed 95% of hair from goat skins after 26 hrs whereas the treatment with the enzyme (keratinase) and 5% lime together resulted in 85% dehairing under similar condition. Sodium sulfide along with lime removed 100% hair as done in conventional chemical methods. This test also suggest the enzyme can also be used in dehairing processing of leathers in tanneries. The overall findings of the present study thus will be an useful basis for developing a bioprocess for commercial production and application of keratinase in Bangladesh.

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ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
(v/v)	Volume/volume e.g. (mL/mL)
(w/v)	Weight per volume e.g. (g/mL)
APPB	Alkaline Protease Producing Broth
Amp	Ampicillin
bp	Base pair
Bl	*Bacillus licheniformis*
cm	Centimeter
DNA	deoxyribonucleic acid
dNTP	deoxy Nucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra-acetic acid
et al.	And others
Fig.	Figure
GST	Glutathione S-transferase
g	gram
hrs	hour
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside
Kg	Kilo gram
kb	Kilobase pair
kDa	kilo Dalton
L	Liter
LB	Luria Bertani
M	Molar
mg	milligram
min	minute
mm	millimeter
ml	milliliter
mM	millimole
μL	Micro liter
Mcs	Multiple Cloning Site
MW	Molecular weight
NA	Nutrient Agar
ng	Nano gram
nm	Nano meter
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pH	Negative logarithm of hydrogen ion concentration
RNA	Ribonucleic acid
rRNA	ribosomal RNA
rpm	Rotation per minute

RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	Second
spp.	Species
Taq	*Thermus aquaticus*
TCA	Tri-chloro acetic acid
TBE	Tris-borate EDTA
TE	Tris EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
UV	Ultra violet
vol.	Volume

CHAPTER 1

Introduction

1.0 INTRODUCTION

Microorganisms and their enzymes have many biotechnological applications but their potentialities are yet to be fully explored. One of the prospective application of microorganisms and their enzymes is in field of biodegradation or deterioration of biological waste in an eco-friendly manner. An essential biological waste which has gained huge importance in the last few decades is accumulation of keratin in nature. Keratins, the most abundant proteins in epithelial cells of vertebrates, represent the major constituents of skin and its appendages such as nail, hair, feather and wool. Keratins have major characteristics, that is, they have high mechanical stability and resistance to proteolytic degradation, which depends on the disulfide and hydrogen bonds, salt and other cross linkings (Korkmaz *et al*., 2004; Hoq *et al*., 2005). The phenomenon of shedding and molting of animal appendages, death of animals or as by-product of poultry and leather industry, causes keratin to be added to our environment. Particularly, chicken feather constitute a troublesome waste product that is produced in large quantities in commercial poultry processing plants (Onifade *et al.*, 1998). Feathers contain around 90% keratin protein and the traditional methodology to degrade them lead to the destruction of valuable amino acids needed to prepare protein rich feather meal (Cai *et al.*, 2008). Keratin, an unusually hard protein to decompose, is reportedly degraded mainly by bacteria, fungi and actinomycetes which are abundant in nature and thrive on keratin (Kunert, 1989; Malviya *et al.*, 1992; Anbu *et al.*, 2008; Casarin *et al.*, 2008). These organisms produce proteolytic enzymes having keratinolytic ability which naturally degrade keratin wastes. Such proteolytic enzymes are commonly known as keratinase responsible for keratin degradation and find applications in various industries.

Keratinase [EC 3.4.21/24/99.11] is an extracellular enzyme produced by different groups of microorganisms namely, bacteria, fungi and actinomycetes (Gupta and Ramnani, 2006). Keratinase belongs to the subtilisin family of serine proteases and has a high homology with subtilisin Carlsberg from *Bacillus subtilis* (Evans *et al.*, 2000). The keratinous wastes which are increasingly accumulating in the environment can be efficiently degraded by the keratinases.

Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates which are fibrous, extensively cross-linked with disulphide, hydrogen and hydrophobic bonds that result in mechanical stability and resistance to common proteolytic enzymes such as pepsin, trypsin and papain generally on broad range of protein substrates (Lin *et al*., 1995). Keratinases have great importance in eco-friendly dehairing processing of leathers in leather industry, feed and bio fertilizers production from feather and treatment of keratin-rich wastes from poultry etc. This enzyme has long been studied for dehairing processes in the leather industry and it was demonstrated that keratinase from *Bacillus* spp. was able to perform the dehairing by removing various proteins of hide especially the keratin, elastin and matrix protein (Takami *et al.*, 1989). Dehairing of goat skin was performed by using keratinase from *Streptomycetes* spp. (Mukhopadhyay *et al.*, 1993) also.

Keratinase have many significant applications (Gupta and Ramnani, 2006). For example, the feather hydrolysates of *Bacillus licheniformis* PWD-1 and *Vibrio* spp. strain kr2 (Williams *et al*., 1991; Grazziotin *et al*., 2006) have feed additive values, while the keratinase from *Bacillus subtilis* S14 exhibits extraordinary dehairing capabilities (Macedo *et al*., 2005). Moreover, keratinase from *B. licheniformis* PWD-1 can degrade the infectious form of prion PrPsc, in the presence of detergents and heat treatment (Langeveld *et al*., 2003), which is very important for the utilization of animal meal as feed. Since feathers are almost pure keratin, feather wastes represent a potential alternative to more expensive dietary ingredients for animal feed stuffs (Shih, 1993). *Bacillus* (Williams *et al*., 1990; Riffel *et al*., 2003; El-Refai *et al*., 2005), fungi (Gradisar *et al*., 2000) and *Actinomycetes* (Gousterova *et al*., 2005) have previously been shown to be able to produce feather-degrading keratinases. “Hydrolyzing the feathers (with keratinase) makes them highly digestible,” says Shih (1993). Feathers become a value-added product, a higher-quality and thus less expensive feed that may be more profitable for producers.

Keratinolytic enzymes have found important utilities in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes. The generation of pollution is significantly high in the pre-tanning operations compared to the post-tanning operations (Puvanakrishnan and Dhar, 1988).

Tanneries are constantly concerned about the obnoxious odor and pollution caused by the extremely toxic sodium sulfide used in the dehairing process. Other chemicals responsible for pollution in pre-tanning processes are lime, sodium sulphide and caustic soda. In fact, one third of the pollution caused by the leather industries results from the wastes generated during dehairing operations (Venkanta and Puvanakrishnan, 1993). The attention of tanneries is focused towards revamping the processing methods, recovery systems and effluent treatment techniques to make leather processing eco-friendly. In order to overcome the hazards caused by the tannery effluents, use of enzymes as a viable alternative has been reported to use in pre-tanning operations such as soaking, dehairing and bating. Keratinase enzymes could be promising in leather industries where they can be used to remove hairs in preference to traditional drastic chemical methods (Mukhopadhyay and Chandra, 1993). The dehairing of skin goat was performed by use of keratinase which reduced the use of lime and sulphide up to 50% required in non-enzymatic process (Kalisz *et al.*, 1988; Hoq *et al*., 2008). Now a days keratinase enzyme is being used in different types of hair treatment.

Bangladesh and other south Asian countries produce and export leather as one of the major items, which are mostly being processed by chemical treatment, resulting in inferior quality of product as well as environmental pollution. About 175 tanneries in Bangladesh still employ conventional method of lime sulfide process, which is environmentally hazardous and increase the pollution. More foreign exchange will be saved if keratinase and protease enzyme are employed in soaking, dehairing and bating process and it will also be a safe and sound to environment. Although the keratinase enzyme was found to be suitable in the above mentioned technical applications, the enzyme concentration produced by the wild strain MZK05 of *B. licheniformis* was not sufficient for the development of commercial bioprocess. For this, the wild strain of *B. licheniformis* was subjected to gene cloning for getting increased production of keratinase. So, the present study is aimed to clone the gene *ker*A encoding keratinase from the *B. licheniformis* MZK05 in suitable hosts such as *E.coli.* This will follow the investigation into expression of the keratinase enzymes, their characterization, production and applications. The whole genome analysis of the wild strain has also been done.

CHAPTER 2

Literature Reviews and Objectives

2. LITERATURE REVIEWS

2.1 General Characteristics of *Bacillus licheniformis*

Bacillus licheniformis is a bacterium commonly found in the soil. It is found on bird feathers, especially chest and back plumage, and most often in ground-dwelling birds (like sparrows) and aquatic species (like ducks). Veith *et al.*, (2004) reported that *B. licheniformis* is a rod-shaped, Gram-positive bacterium (Fig. 2.1). *Bacillus licheniformis* tends to form spores in soil which makes it desirable to be used for the industrial purposes such as the production of enzymes, antibiotics and small metabolites. It produces a variety of extracellular enzymes that are associated with the cycling of nutrients in nature. Its optimal growth temperature is 50°C, but it can also survive at much higher temperatures. Its optimal temperature for enzyme secretion is 37°C. High capacity of secretion of the alkaline serine protease has made *B. licheniformis* one of the most important bacteria in industrial enzyme production (Schallmey *et al*., 2004). A small antisense RNA against Subtilisin Carlsberg named BLi_r0872 was discovered in an RNA-seq based study. It may have a putative impact on protease production and serve as target for strain improvement (Wiegand *et al.*, 2013).

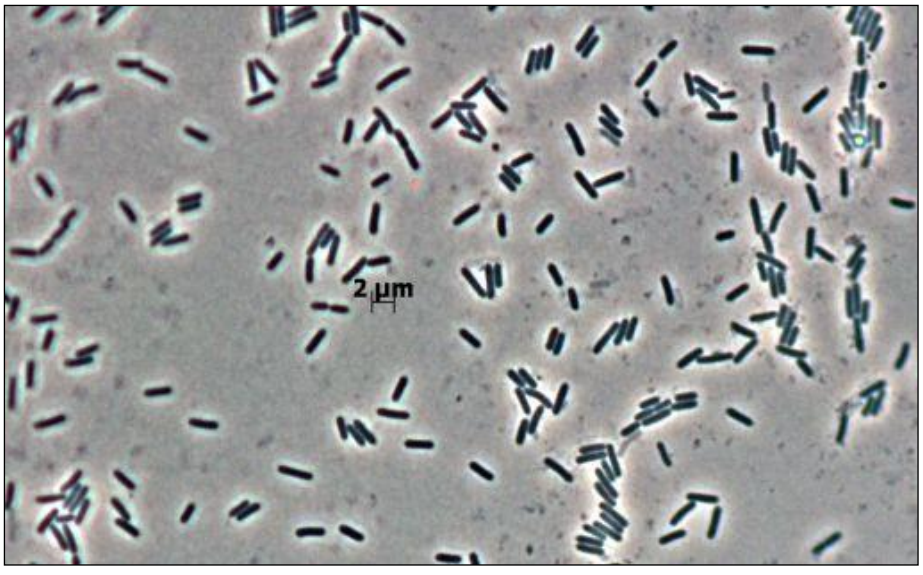

Figure 2.1: Microscopic observation of *Bacillus licheniformis* (Gleim *et al.*, 1980)

2.1.1 Genome structure

The complete nucleotide sequence of *Bacillus licheniformis* consists of the ATCC 14580 genome, which has a circular chromosome of 4,222,336 bp (base pairs) which contains 4,208 predicted protein-coding genes (average size of 873 bp), 7 rRNA operons, and 72 tRNA genes. The GC content is 46.2% and no plasmids were detected (Rey *et al.*, 2004). The chromosome of *B. licheniformis* has large regions that are similar to *Bacillus subtilis*. But, although similar to *B. subtilis*, they differ in the amount and location of prophages, transposable elements, extracellular enzymes and secondary metabolic pathway operons (Rey *et al.*, 2004).

2.1.2 Ecology

Bacillus licheniformis forms spores in soil. A pathway that leads to endospore formation is initiated when the bacterium is starved. Endospore formation is actually desired and serves as a great example of prokaryotic development and differentiation. These spores are quite tolerant of heat, cold, radiation and other environmental stresses. Under good conditions, the spores will germinate and produce vegetative cells. *B. licheniformis* produces a variety of extracellular enzymes that are associated with the cycling of nutrients in nature. It is an apathogenic soil organism that is mostly associated with plant and plant materials in nature. Although it is most common to isolate this bacterium from soil, it is believed that *B. licheniformis* can actually be isolated from practically anywhere since it produces highly resistant endospores that are spread around with dust.

2.1.3 Improvement of yield

Strain improvement plays a key role in the commercial development of microbial fermentation processes. As a rule, the wild strains usually produce limited quantities of the desired enzyme to be useful for commercial application (Glazer and Nikaido *et al*., 1995). The yield can be improved by using of mutagenesis. Shah *et al*., (1986) developed a cysteine auxotrophic mutant of *B. licheniformis* with improved protease production. Further, increased yields of alkaline proteases have been achieved by *Bacillus* mutants that were resistant to antibiotics such as vancomycin and ristocetin.

The advent of protein engineering and sophisticated molecular technologies has opened possibilities for screening variants of enzymes and tailor-made proteins from alkalophilic microorganisms with enhanced production yields which may be of interest for specific commercial applications. New constructions have been made by the transfer of genes between organisms to produce high yielding variants (Takagi *et al*., 1988; Takami *et al*., 1992).

Currently, two conceptionally different strategies are available for generation of protein-engineered variants: random and site-directed mutagenesis. With random mutagenesis, a large number of variants are produced, but the success of this approach largely depends on the availability of efficient screening procedures to identify variants with improved properties. Site-directed mutagenesis depends on the access to structural or biochemical data to reduce the number of variants to be constructed, as every protein variant is purified and individually tested for improvements. For producing mutated enzymes, the two approaches are optimally used in combination with each other. Promising variants generated and identified by random mutagenesis often can be improved by further site-directed introduction of known advantageous mutations.

Gene cloning is widely used as an yield improving tool. It is also used to over express a gene or a group of genes to change its property like yield or content etc. Gene cloning and targeted gene duplication becomes an important tool. Duplication of genes involved in rate limiting steps can be achieved to improve product yields by inserting the desired genes into neutral sites in the chromosome by homologous recombination or by site-specific integration (Richard, 2000).

2.2 Enzymes

Catalysts act by reducing the energy barrier of chemical reactions, therefore producing a dramatic increase in reaction rates, ranging from 106 to 1024 fold. Biocatalysts are *strictu sensu* the catalysts of cell metabolism, i.e. the enzymes. Enzymes are effective catalysts, responsible for the thousands of co-ordinated chemical reactions involved in biotechnological process of living systems. However, this concept has expanded beyond its physiological meaning, a biocatalysts being, in broader terms, any biological entity capable of catalyzing the conversion of a substrate into a product.

In comparison to chemical catalysts, some outstanding features of enzymes are substrate specificity and specificity in promoting of only one biochemical reaction with their substrate ensuring synthesis of a specific bimolecular product without the concomitant production of by products (Chaplin *et al.*, 1990). With the understanding of the nature, enzyme has gradually been extended in variety of fields, such as food production, brewing, pharmaceuticals, medicine textiles, leather and detergents.

2.2.1 Keratinase enzyme

From keratinophilic fungi, keratinolytic protein production was reported by Yu *et al.*, (1968), (Asahi *et al.*, 1985; Willams *et al.*, 1989). keratinase production by *Streptomyces* spp. was reported by Mukhopadhay *et al.*, (1990). He isolated an inducible extracellular homogenous enzyme, which shows 7.5 fold increases in its activity after DEAE cellulose column chromatography. At first Molyneux *et al.*, (1959) attempted to isolate some bacteria that are able to degrade keratin. He isolated organism from the contents of experimentally induced dermoid cysts from mid lateral region of sheep. The same year Noval *et al.*, (1959) published another article on enzymatic decomposition of native keratin by *Streptomyces fradiae*. They showed extracellular enzyme secreted by this bacteria capable of degrading the human hair in its native state. Williams *et al.*, (1990) continued his work on enriched feather degrading culture and characterized the organism to its species level for the first time. The microorganisms were identified as *Bacillus licheniformis*. Lin *et al.*, (1992) purified and characterized keratinase from feather degrading *Bacillus licheniformis* strain isolated by Williams *et al.*, (1990) with the help of membrane ultra-filtration and C-75 gel chromatography. He purified enzyme with 70 fold increased activity. SDS-PAGE analysis revealed that purified keratinase had a molecular weight of 33 kDa. The keratinolytic protease production by various microorganisms were reported by number of researchers and it was found that keratinolytic protease produced by bacteria, fungi, *Streptomysec* spp. in nearly alkaline pH and almost thermophilic temperature.

The enzyme also has a wide range of substrate specificity such as it can degrade fibrous protein fibrin, elastin, collagen and other non-fibrous protein like casein, bovine serum albumin etc. (Mukhopadhdhay *et al.*, 1993; Lin *et al.*, 1995; Letourneau *et al.*, 1998). Keratinases are proteolytic enzyme in nature.

It was classified as proteinase of unknown mechanism as recommended by the Nomenclature Committee on the International Union of Biochemistry (1978) with EC number 3.4.99 (Owen *et al.*, 1983).

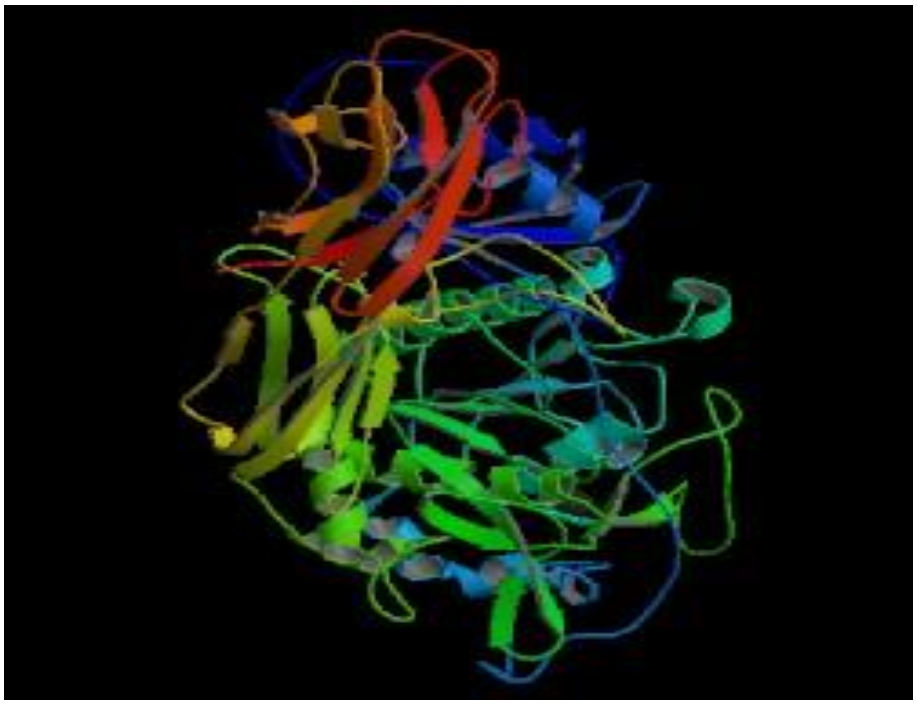

Figure 2.2: keratinase act as serine protease.

Recently, some of the worker defined keratinase as serine protease due to its 97% sequence homology with alkaline protease (Fig. 2.2) and it is also inhibited by the same inhibitor that inhibits serine protease (Taha *et al.*, 1998 and Bressollier *et al*., 1999). Keratinases are produced only in the presence of keratin containing substrate. It mainly attacks on the disulfide (-S-S-) bond of the keratin substrate (Bockel *et al.*, 1995). These enzymes have wide range of substrate specificity such as it can degrade other fibrous protein fibrin, elastin, collagen and other non-fibrous protein like casein, bovine serum albumin gelatin etc (Noval *et al.*, 1959; Dozie *et al.*, 1994; Lin *et al.*, 1995; Letourneau *et al.*, 1998; Bressollier *et al.*, 1999).

2.2.2 Diversity of keratinase producing microorganisms

Keratin degrading microorganisms are classified under protease producing microbes that are capable of degrading keratin containing materials. Keratinase producing microorganisms can belong to bacteria, fungi and actinomycetes.

Of these, keratinase producing bacteria are most numerous, followed by fungi and then actinomycetes (Table 2.1).

Table 2.1: Keratinase producing microorganisms (Bacteria, fungi and actinomycetes).

Microorganisms		**References**
Bacteria	*Micrococcus* spp., *Nisternkonia* spp., *Clostridium* spp., *Pseudomonas* spp., *Chryseobacterium* spp., *Xanthomonas* spp. and *Fervidobacterium* spp.	Williams *et al.*, 1990; Lin *et al.*, 1992; Cai *et al*., 2009; Ionata *et al.*, 2008; Sangali and Brandelli, 2000; Yamamura *et al.*, 2002.
Properties	Feather degradation	
Fungi	*Trichophyton, Aspergillus, Microsporum, Fusarium, Alternaria, Cladosporium,, Chrysosporium, Penicillium*	Gradisar *et al.*, 2000; Gupta and Ramnani, 2006.
Properties	Capable of utilizing varied keratinous substrates such as chicken feather, wool, human hair	
Actinomycetes	*Streptomyces fradiae, Streptomyces pactum, Streptomyces albidoflavus* and *Streptomyces thermoviolaceus.*	Mukhopadhyay and Chandra 1990; Bockle *et al*., 1995; Letourneau *et al*., 1998; Chitte *et al*., 1999; Ignatova *et al*., 1999; Gousterova *et al*., 2005
Properties	Keratin degradation	

2.2.3 Keratinase: Biotechnological approaches

Keratinolytic enzymes have important uses in biotechnological process for the development of non-polluting processes and for the treatment of keratin-containing wastes from poultry and leather industry (Sangali and Brandelli, 2000). After hydrolysis, the feathers can be converted to feed-stuffs, fertilizer, glues and films or selected amino acids such as serine, cysteine and proline (Riffel *et al.*, 2003). Keratinase treatment process replaces the use of sodium sulfide in the dehairing process in leather industry (Macedo *et al.*, 2005). Feather degrading keratinase activity was reported from *Bacillus licheniformis* (Cheng *et al*., 1995) and especially in Bangladesh first reported by Hoq *et al*., (2005). Keratinase enzymes could be promising in leather industries where they can be used to remove hairs in preference to traditional drastic chemical methods (Mukhopadhyay *et al.*, 1993). The bioconversion of poultry feather wastes into protein rich fodder (Williams *et al*., 1989; Taha *et al.*, 1998) is another important trend in the application of keratinase.

2.2.4 Keratinase production

The constituents of a medium must be sufficient enough to fulfill the elemental requirements for biomass production and metabolite production. There must be an adequate supply of energy for biosynthesis and cell maintenance. A typical production medium would contain certain nutrients which are required for biomass production, some for enzyme production and certain nutrients for both. This would include water, carbon source, nitrogen source, trace elements and substrate (Stanburry and Whittaker, 1997). The choice of these nutrients for designing a production medium depends upon the type of microorganism used for the process and the target product of the fermentation process. Optimum requirements for keratinase production has been presented below:

2.2.4.1 Carbon source

For microorganisms to release keratinase and exhibit keratinase activity, keratin would be required as a source of carbon and nitrogen. Thus, in many cases, keratin is preferred as a sole source of carbon and nitrogen for to enable maximum keratinase production by the isolates (Kim *et al.*, 2001).

The presence of another simpler carbon source would result in the suppression of enzyme production or inhibition, a result of catabolite repression, a common control mechanism used for biosynthesis of microbial proteases (Gupta *et al.*, 2002). The presence of glucose in the production medium had totally suppressed keratinase production in case of *B. licheniformis* PWD-1 (Williams *et al.*, 1990). This phenomenon is also reported for keratinases by Wang and Shih, (1999); Thys *et al*., 2004; Brandelli and Riffel, 2010; Kainoor *et al.*, 2010 in which presence of simpler carbon source like glucose, sucrose or lactose in the production medium has resulted in suppression or complete inhibition of keratinase production. Other studies have also mentioned about the presence of glucose accompanied with yeast extract resulting in increased keratinase production, in which glucose would serve as a source of carbon while yeast extract would serve as a source of nitrogen, growth factors and energy (Yamamura *et al.*, 2002).

2.2.4.2 Nitrogen Source

Most keratinase producing media contain keratinous substrate as a sole source of carbon and nitrogen as mentioned before. In some cases, additional nitrogen source is a requirement for the microorganisms to perform keratinase activity and release keratinase. Some examples of additional nitrogen sources required for keratinase production include- peptone (Ramnani and Gupta, 2005), NH_4Cl and yeast extract (Yamamura *et al.*, 2002; El-Refai *et al.*, 2005). Yeast extract in the production medium suggested that increased enzyme activity is associated with increased microbial growth as a result of yeast extract being an easily metabolizable nutrient source (Yamamura *et al.*, 2002). Feather degrading *B. subtilis*, for which, the presence of casein greatly increased the production of the enzyme while *B. pumilis* did not produce the enzyme in the presence of casein (Kim *et al.*, 2001).

2.2.4.3 Temperature and pH

Temperature and pH have a strong influence on microbial growth and therefore enzyme production. Physical parameters for keratinase production are species-specific and vary with respect to microorganism (Gupta and Ramnani, 2006). For example, *B. licheniformis* which carried out keratinase production at 50^{o}C (Williams *et al.*, 1990). Feather degrading isolate *Frevidobacterium pennavorans* carried out feather degradation at 70^{o}C. Feather degrading *Vibrio* spp. Kr2 performing feather degradation at 30^{o}C (Friedrich and Antranikian, 1996; Sangali and Brandelli, 2000).

For isolate *Chryseobacterium* spp. Kr6, the preferred temperature for keratinase production is 25-30^{o}C (Brandelli and Riffel, 2010). Thus, Temperature for keratinase production ranges from 28 to 50°C for most bacteria (Thys *et al.*, 2004). Certain actinomycetes and fungi also prefer temperatures as high as 70°C for keratinase production- *Thermoanaerobacter* and *Fervidobacterium* spp. (Friedrich and Antranikian, 1996; Nam *et al.*, 2002).

pH range of 6-9 supports keratinase production and most microorganisms carry out keratinase production at alkaline pH range. The possible explanation for this could be that alkaline pH modifies cysteine residues to lanthionine (Friedrich and Antranikian, 1996), making it available for keratinase action. Alkaliphilic strains *Nesternkonia* spp. (Gessesse *et al.*, 2003) *Nocardiopsis* spp. (Mitsuiki *et al.*, 2006) have been reported to show keratinase activity.

2.2.4.4 Substrates and their concentrations

The production of any enzyme by a microorganism would dependent upon the substrate. Most feather degrading microorganisms are capable of keratin utilization as a sole source of carbon and nitrogen (Williams *et al.*, 1990; El-Naghy *et al.*, 1998; Gousterova *et al.*, 2005). Microorganisms prefer a moderate range of keratin containing substrates. Chicken feathers are the most preferred substrate, followed by feather meal, hair and wool (Williams *et al.*, 1990).

2.2.4.5 Trace elements

All microorganisms require certain mineral elements for growth and metabolism. In many media, magnesium, phosphorus, potassium, sulphur, calcium and chlorine are essential components, and because of the concentrations required, they must be added as distinct components. (Stanburry and Whittaker, 1997). For keratinase production, one of the most vital trace elements required to be added is Magnesium. Magnesium should be present in the media composition either in the form of $MgCl_2$ or $MgSO_4$ (Kim *et al.*, 2001; El-Refai *et al.*, 2005; Yamamura *et al.*, 2002).

2.2.5 Biochemical properties of keratinases

Keratinases [EC 3.4.21/24/99.11] are extracellular or intracellular proteases generated by microorganisms, which catalyze the specific breakdown of recalcitrant proteins, keratins, which are resistant to other proteases and peptidases (Onifade *et al.*, 1998). keratinases have played the prime role in valorization of the sizeable amount of keratinous waste that is continuously generated (Onifade *et al.*, 1998; Gupta and Ramnani, 2006). The intracellular fraction in most of these reports mainly assists to disulfide reductases, sulfite or thiosulfate that synergistically assists the extracellular keratinases to degrade keratin by reducing the disulfide bonds of keratin (Gupta and Ramnani, 2006). To be more specific, Keratin degradation required two steps: reduction in the disulfide bonds or sulfitolysis, and proteolysis (Bockle and Muller, 1995; Ramnani *et al.*, 2005). That is, the presence of keratin activates the release of keratinase, keratin thus playing as an inducer (Kim *et al.*, 2001). Although most keratinases have been categorized as inducible, certain studies on constitutive keratinases have been reported (Gessesse *et al.*, 2003; Manczinger *et al.*, 2003).

Another reason for this is that production of keratinases have been either suppressed or inhibited by the presence of a simpler carbon source such as glucose, sucrose and lactose in addition to keratin, indicating catabolite repression (Ignatova *et al.*, 1999; Wang and Shih, 1999; Yamamura *et al.*, 2002; Bernal *et al.*, 2006; Suntornsuk and Suntornsuk, 2003; Thys *et al.*, 2004). By catalytic nature, most keratinases, being inhibited by phenyl methyl sulfonyl fluoride (PMSF) belong to the serine protease category (Lin *et al.*, 1992; Bockle *et al.*, 1995; Friedrich and Antranikian, 1996; Bressolier *et al.*, 1999; Suh and Lee, 2001; Nam *et al.*, 2002), while some are metalloproteases (Allpress *et al.*, 2002). A brief review on biochemical properties of reported keratinases has been presented below:

2.2.5.1 Molecular Weight

Keratinases have molecular weights ranging from 18 kDa to 200 kDa. The keratinase with the lowest molecular weight belongs to the actinomycete *S. albidoflavus* SK 1-02 (Chitte *et al.*, 1999). The highest of 200 kDa for *Kocuria rosea* and *F. islandicum* have been reported (Nam *et al.*, 2002; Bernal *et al.*, 2006).

2.2.5.2 pH and temperature optima

Keratinases from most organisms are best active within the pH optima of neutral to alkaline range i.e. pH 7.5 onwards (Friedrich and Antranikian, 1996; Letourneau *et al.*, 1998; Bressollier *et al.*, 1999; Ignatova *et al.*, 1999; Gradisar *et al.*, 2000; Sangali and Brandelli, 2000; Yamamura *et al.*, 2002; Riffel *et al.*, 2003; Thys *et al.*, 2004; Anbu *et al.*, 2008, Ionata *et al.*, 2008).

2.2.5.3 Effect of inhibitors, metal ions, and other chemicals

Most of the keratinases which have been characterized are found to be serine proteases (Lin *et al.*, 1992; Bockle *et al.*, 1995; Friedrich and Antranikian, 1996; Bressolier *et al.*, 1999; Suh and Lee 2001; Nam *et al.*, 2002; Bernal *et al.*, 2006). A few keratinases from Gram negative isolates have been reported to be metalloproteases (Allpress *et al.*, 2002; Thys *et al.*, 2004). From the overall review of effect of metal ions on keratinase activity, it can be noted that most keratinases are activated in the presence of metal ions- Ca^{2+}, Mn^{2+}, Mg^{2+}, (Nam *et al.*, 2002; Macedo *et al.*, 2005). Most keratinases are inhibited by Cu^{2+} (Nam *et al.*, 2002; Riffel *et al.*, 2003; Thys *et al.*, 2004), Zn^{2+} and Hg^{2+} (Riffel *et al.*, 2003; Thys *et al.*, 2004).

2.2.6 Applications of keratinases

Some applications of keratinase enzyme are given below:

2.2.6.1 Production of amino acids

During the keratinase degradation of hair, wool and feather release some different types of amino acids such as alanine, valine, lysine, cysteine and it also release the essential amino acids lysine. So it has a role in the production of amino acids at a low cost (Taha *et al.*, 1998).

2.2.6.2 Human hair degradation

Human hair often causes clogging in the bath tub drains with bad odors at home and in hotels. Human hair composed of keratin having a lot of S-S bonds responsible for rigidity are hydrolyzed by keratinase from *Trichophyton mentagrophytes* reported by Takami *et al.*, (1992).

2.2.6.3 Poultry feed production by Poultry waste treatment

Feathers, which account for 5-7% of the total dry weight of mature chickens, are produced in huge quantities as a by-product at commercial poultry processing plants. Considering millions of tons of feather produced annually worldwide and that they made up primarily of keratin (90% or more), the by product (Regina *et al.*, 1996). Recently feeding experiments with chicken demonstrated a significant growth response when feather lysate produced by bacterial fermentation was added to the diet than adding untreated feather or commercially feather meal (Lin *et al*., 1995).

2.2.6.4 Pre-tanning processes

Keratinase enzymes could be promising in leather industries where they can be used to remove hairs in preference to traditional drastic chemical methods (Mukhopadhyay and Chandra, 1993). Keratinase reduces the use of lime and sulfide up to 50% required in non-enzymatic process (Kalisz *et a1*., 1988)

2.2.6.5 Bio- hydrogen production

There is increasing interest in utilization of renewable sources to satisfy the exponentially growing energy needs of mankind. Research on biological hydrogen production is propelled by the possible use of bio-hydrogen as the cleanest energy carrier and raw material (Benemann, 1996). Bio-hydrogen is a part of a broader concept of developing zero emission technologies employing production of H_2 from biomass in photo-biological or heterotrophic fermentation routes (Cammack *et al*., 2001).

2.2.6.6 Recycling of keratinous wastes

Keratinous wastes are a major by-product of poultry, slaughter house, leather- and fur-processing industries, and are abundantly generated in various forms such as feather, hair, horn, hoof, nails, claws, wool, and bristles (Kornillowicz, 2011; Karthikeyan *et al.*, 2007). A keratinous waste on hydrolysis is converted to keratin hydrolysate which has high nitrogen content, and is rich in hydrophobic amino acids (Gupta and Ramnani, 2006).

2.2.6.7 Medical applications

Keratinases are able to attack nail and skin keratin, and hence, find application as an additive to increase the efficacy of topical drugs.

They are also being utilized for various skin conditions such as acne (Spyros, 2003), corn, and callus. Keratinase used in nail treatment (Kumar and Raju, 2013). Keratinases act on both the intercellular matrix that holds the cells of the nail plate together as well as the dorsal nail corn eocytes by corroding their surface (Rajendra *et al.*, 2012). Keratinase was shown to partially disrupt the nail plates and increase the drug permeability (Mohorcic *et al.*, 2007).

2.2.6.8 Cosmetics/personal care products

Keratinases have been employed in cosmetic formulation for skin and hair. For skin, keratinases have been added in compositions for skin whitening, freckle dispelling, and bleaching (Yang, 2012). Keratinases can also be used for exfoliation and removal of stratum corneum as described by Ding and Sun, 2009 and Yong *et al.*, (2009).

2.3 Advantages of heterologous expression

Historically, *E. coli* was the first recombinant expression host for any cloned gene. The advantages of using *E. coli* for heterologous gene expression include the ease of growth and manipulation of this organism using simple laboratory equipment, the availability of dozens of vectors and host strains that have been developed for maximizing expression, a wealth of knowledge about the genetics and physiology of *E. coli*, and the influence of specific genetic and environmental factors on expression of heterologous proteins. Moreover, expression can often be achieved quite rapidly: it is possible to express a protein in *E. coli* and purify it in milligram quantities in less than two weeks. Shake flask cultures can produce many tens of milligrams of a heterologous protein per liter of culture. In fermenters, where much higher cell densities can be achieved, it is possible to produce more than a gram per liter of a heterologous protein. Recombinant proteins can be accumulated at levels up to 50% of total cell protein (Higgins and Hames, 1999).

2.4 An overview on Gene cloning

Gene cloning is the insertion of a fragment of DNA that carries a gene into a cloning vector, and thus generating recombinant DNA or chimera and its subsequent propagation in host organism (Brown, 2006). Gene cloning is a logical extension of the ability to manipulate DNA molecules with restriction endonucleases and ligases.

Cloning is a generic term meaning the duplication of genetic material. Recombinant cloning, also called DNA cloning, is the replication of specific genes of a DNA molecule. Recombinant cloning techniques have been in use since the 1970s and are now a common practice in research laboratories around the world. In 1973, a group of scientists produced the first organisms with recombinant DNA molecules. They called the hybrid DNA molecules *chimeras*. Recombinant DNA technology is a set of methods used to locate, analyze, alter, study and recombine DNA sequences.

Many recombinant DNA methods require numerous copies of a specific DNA fragment. One way to obtain these copies is to place the fragment in a bacterial cell and allow the cell to replicate the DNA. This procedure is termed gene cloning, because identical copies (clones) of the original piece of DNA are produced. The genome of an organism contains thousands of different genes. Cloning is frequently used to amplify DNA fragments containing whole genes, but it can also be used to amplify any DNA sequence such as promoters, non-coding sequences and randomly fragmented DNA. It is used in a wide array of biological experiments and practical applications ranging from genetic fingerprinting to large scale protein production.

According to Brown (2006), basic steps (Fig. 2.3) in a gene cloning experiment are as follows:

1. A fragment of DNA, containing the gene to be cloned, is inserted into circular DNA molecule called a vector, to produce a chimera or recombinant DNA molecule.

2. The vector acts as a vehicle that transports the gene into a host cell, which is usually a bacterium, although other types of living cell can be used.

3. Within the host cell the vector multiplies, producing numerous identical copies not only of itself but also of the gene that it carries.

4. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place.

5. After a large number of cell divisions, a colony, or clone, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule; the gene carried by the recombinant molecule is now said to be cloned.

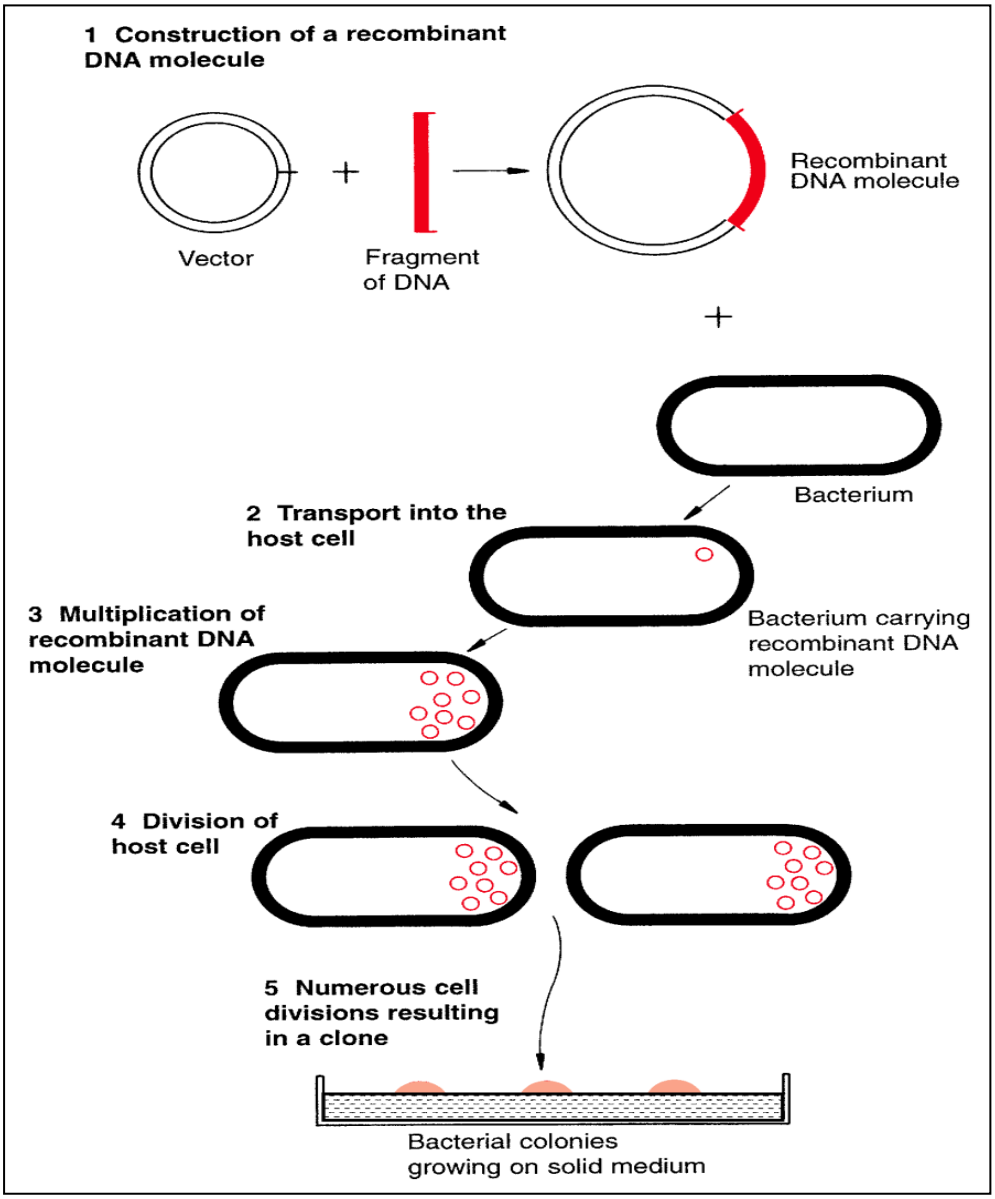

Figure 2.3: The basic steps in gene cloning.

2.4.1 Primer designing:

A primer is a short synthetic oligonucleotide which is used in many molecular techniques from PCR to DNA sequencing. These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which the primers are intended to anneal. When designing primers for PCR, sequencing or mutagenesis, it is often necessary to make predictions about these primers, for example melting temperature (T_m) and propensity to form dimers with it or other primers in the reaction.

So some thoughts should be given on primers designing. These include -

1. Primers should be 17-28 bases in length. This length is long enough for adequate specificity, and short enough for primers to bind easily to the template at the annealing temperature.

2. Base composition should be 50-60% of (G+C).

3. T_m values between 55-80^oC are preferred. Primer Melting Temperature (T_m) by definition is the temperature at which one half of the DNA duplex will dissociate to become single stranded. It actually indicates the duplex stability. Primers with melting temperatures in the range of 52-58^oC generally produce the best results. Primers with melting temperatures above 65^oC have a tendency for secondary annealing.

4. 3'-ends of primers should not be complementary (i.e. base pair), as otherwise primer dimers will be synthesized preferentially to any other product.

5. The presence of G or C bases within the last five bases from the 3'-end of primers (GC clamp) helps promote specific binding at the 3'-end due to the stronger bonding of G and C bases.

6. Primers with long runs of a single base should generally be avoided as they can misprime. For example, AGCGGGGGATGGGG has runs of base 'G' of value 5 and 4. A maximum number of runs accepted are 4bp.

2.4.2 Cloning vectors

A cloning vector is a stable, replicating DNA molecule to which a foreign DNA fragment can be inserted/ attached to introduce into a cell. The insertion of the fragment into the cloning vector is carried out by treating the vehicle and the foreign DNA with the same restriction enzyme, then ligating the fragments together. The simplest cloning vectors and the ones in most widespread use in gene cloning are those based on small bacterial plasmids. A large number of different plasmid vectors are available for use with *E. coli*, many obtainable from commercial suppliers (Brown, 2002). They combine ease of purification with desirable markers for transformants and recombinants (Brown, 2001).

2.4.2.1 Common features of the vector pGEX-6p-2

The pGEX-6p-2 vector is the commercial vector with GST tag (Glutathione-S-transferase) on N-terminal end. This vector was chosen for its following unique characteristics:

- A tac promoter for chemically inducible, high-level expression.
- An internal lac I^q gene for use in any *E. coli* host.
- Very mild elution conditions for release of fusion proteins from the affinity matrix, thus minimizing effects on antigenicity and functional activity.
- PreScission™ for cleaving the desired protein from the fusion product, its PreScissionion cleavage site allows cleavage at low temperature.

Properties of vector pGEX-6P-2 (Smith and Johnson, 1998) is given in Table 2.2

Table 2.2: Properties of vector pGEX-6P-2.

Vector Type	Promoter	Expression level	Size	Tag	Bacteria Resistance
Bacterial, Expression vector	Tac	High (activate with IPTG)	4985 bp	GST (N-term) glutathione S-transferase	Ampicillin

2.4.2.2 Common features of the vector pET-30a(+)

The pET vectors were developed at Novagen to designed with enhanced features to permit easier subcloning, detection, and purification of target proteins. This vector was chosen for its following unique characteristics:

- A tac promoter for chemically inducible, high-level expression.
- An internal lac I^q gene for use in any *E. coli* host.
- Very mild elution conditions for release of fusion proteins from the affinity matrix, thus minimizing effects on antigenicity and functional activity.

- Many strategies can be used for subcloning a protein-coding region of DNA into a pET vector for expression. pET-30a(+) vector is provide convenient method that does not require restriction digestion is the ligation-independent cloning (LIC) method. Ligation-independent cloning was developed for the directional cloning of PCR products without restriction enzyme digestion or ligation reactions.

The properties of vector pET-30a(+) is given in Table 2.3.

Table 2.3: Properties of vector pET-30a(+)

Vector Type	Promoter	Expression level	Size	Tag	Bacteria Resistance
Bacterial, Expression vector	Tac	High (activate with lactose)	5200 bp	His (N-term and C-term)	Ampicillin

2.4.3 Restriction endonucleases

A restriction enzyme (or restriction endonuclease) is an enzyme that binds to a DNA molecule at a specific sequence and makes a double-stranded cut at or near that sequence (Brown, 2002). These enzymes are produced naturally by bacteria, where they are used in defense mechanism against invading viruses. Inside a bacterial host, the restriction enzymes selectively cut up foreign DNA in a process called restriction.

***Bam*HI** (from *Bacillus amyloliquefaciens*) is a type II restriction endonuclease, having the capacity for recognizing short sequences 6 bp of DNA and specifically cleaving them at a target site. *Bam*HI binds at the recognition sequence 5'-GGATCC-3', and cleaves these sequences just after the 5'-guanine on each strand. This cleavage results in sticky ends which are 4 bp long. In its unbound form, *Bam*HI displays a central b sheet, which resides in between a helices. *Bam*HI is an extraordinarily unique molecule in that it undergoes a series of unconventional conformational changes upon DNA recognition. This allows the DNA to maintain its normal B-DNA conformation without distorting to facilitate enzyme binding.

***Xho*I** is a type II restriction enzyme that recognise the double-stranded DNA sequence CTCGAG and cleaves after C-1 (Theriault, 1985). Type II restriction endonucleases (EC) are components of prokaryotic DNA restriction-modification mechanisms that protect the organism against invading foreign DNA. These site-specific deoxyribonucleases catalyse the endonucleolytic cleavage of DNA to give specific double-stranded fragments with terminal 5'-phosphates.

2.5 Protein expression

Protein expression refers to the way in which proteins are synthesized, modified and regulated in living organisms. In protein research, the term can apply to either the object of study or the laboratory techniques required to manufacture proteins. Proteins are synthesized and regulated depending upon the functional need in the cell. The message coded by an mRNA is then translated into a protein. Transcription is the transfer of information from DNA to mRNA, and translation is the synthesis of protein based on a sequence specified by mRNA.

2.5.1 Expression of GST-fusion protein

Some vector like pGEX have GST (Glutathione S-transferase) gene under the control of strong tac promoter which is induced by IPTG (Isopropyl β-D-1-thiogalactopyranoside) an analogue of lactose. There is a multiple cloning site downstream to the GST gene. When any gene of interest is inserted into the multiple cloning sites, the expressed protein translated as GST fused expressed protein.

2.5.2 Expression of His-tag protein

The DNA sequence specifying a string of six to nine histidine residues is frequently used in vectors for production of recombinant proteins. The result is expression of a recombinant protein with a 6xHis or poly-His tag fused to its N- or C-terminus. Expressed His-tagged proteins can be purified and detected easily because the string of histidine residues binds to several types of immobilized metal ions, including nickel, cobalt and copper, under specific buffer conditions. In addition, anti-His-tag antibodies are commercially available for use in assay methods involving His-tagged proteins.

2.6 Purification of recombinant protein

2.6.1 Purification of GST-fusion protein

Protein purification with affinity tags such as Glutathione S-transferase (GST), histidine (His), and other affinity tags, enables purification of proteins with both known and unknown biochemical properties. Therefore, this methodology has become a widely used research tool for determining the biological function of uncharacterized proteins. GST is a 211 amino acid protein (26 kDa) whose DNA sequence is frequently integrated into expression vectors for production of recombinant proteins. The result of expression from this vector is a GST-tagged fusion protein in which the functional GST protein (26 kDa) is fused to the N-terminus of the recombinant protein. Because GST folds rapidly into a stable and highly soluble protein upon translation, inclusion of the GST tag often promotes greater expression and solubility of recombinant proteins than expression without the tag.

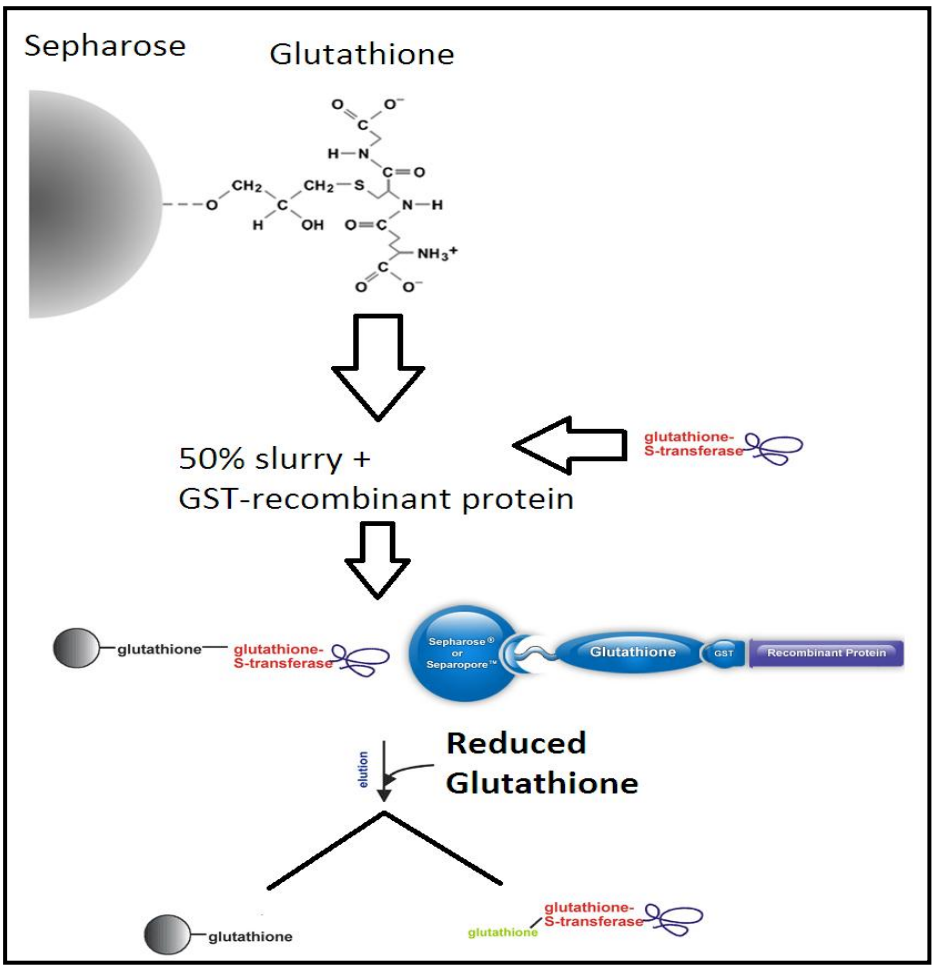

Figure 2.4: Purification steps of GST-fusion protein

In addition, GST-tagged fusion proteins can be purified or detected based on the ability of GST (an enzyme) to bind its substrate, glutathione (GSH). Glutathione is a tripeptide (Glu-Cys-Gly) that is the specific substrate for Glutathione S-transferase (GST). When reduced glutathione is immobilized through its sulfhydryl group to a solid support, such as cross-linked beaded agarose, it can be used to capture pure GST or GST-tagged proteins via the enzyme-substrate binding reaction (Fig. 2.4).

2.6.2 Purification of His-tag protein

A polyhistidine-tag is an amino acid motif in proteins that consists of at least six histidine (His) residues, often at the N- or C-terminus of the protein. It is also known as hexa histidine-tag, 6xHis-tag, His6 tag and by the trademarked name His-tag (Hochuli *et al.*, 1988). Polyhistidine-tags are often used for affinity purification of polyhistidine-tagged recombinant proteins expressed in *Escherichia coli* (Hengen Paul, 1995) and other prokaryotic expression systems. The DNA sequence specifying a string of six to nine histidine residues is frequently used in vectors for production of recombinant proteins. The result is expression of a recombinant protein with a 6xHis or poly-His-tag fused to its N- or C-terminus.

Expressed His-tagged proteins can be purified and detected easily because the string of histidine residues binds to several types of immobilized metal ions, including nickel, cobalt and copper, under specific buffer conditions. In addition, anti-His-tag antibodies are commercially available for use in assay methods involving His-tagged proteins. Depending on downstream applications, including the purification of protein complexes to study protein interactions, purification from higher organisms such as yeasts or other eukaryotes may require a tandem affinity purification (Gavin *et al.*, 2002) using two tags to yield higher purity.

Immobilized metal ion affinity chromatography (IMAC) is a widely-used method for rapidly purifying polyhistidine affinity-tagged proteins, resulting in 100-fold enrichments in a single purification step. The chelators most commonly used as ligands for IMAC are nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA).

2.7 Whole genome sequencing

Whole genome sequencing (also known as WGS, full genome sequencing, complete genome sequencing, or entire genome sequencing) is the process of determining the complete DNA sequence of an organism's genome at a single time. Whole-genome sequencing is the most comprehensive method for analyzing the genome. Whole genome sequencing has largely been used as a research tool, but is currently being introduced to clinics (Gilissen, 2014; Van *et al.*, 2013; Nones *et al.*, 2014).

In the future of personalized medicine, whole genome sequence data will be an important tool to guide therapeutic intervention (Mooney, 2014).Whole genome analysis has yielded basic scientific information about the relationship between genotype and phenotype, biotechnological advances in enzyme discovery, bioprospecting of genes for medicine, agriculture etc. There are several genome sequencing platforms like ABI SOLiD, Helicos Heliscope, IonTorrent PGM, Roche 454, Pacific Biosciences RS1, and Illumina. Of these, data generated by the Illumina instrument is the most prevalent data type at the moment.

2.8 Researches done worldwide on keratinase

At first Molyneux *et al.*, (1959) attempted to isolate some bacteria that are able to degrade keratin. He isolated organism from the contents of experimentally induced dermoid cysts from mid lateral region of sheep. Examination of wool sample showed degraded wool with numerous corticle and cyticular cells. He showed that the organisms belong to genus *Bacillus* and the organism was capable of attacking native wool protein. The same year published another article on enzymatic decomposition of native keratin by *Streptomyces fradiae* (Noval *et al.*, 1959). They showed extracellular enzyme secreted by this bacteria capable of degrading the human hair in its native state. Keratinolytic protein from keratinophilic fungi were reported by Yu *et al.*, (1968), Asahi *et al.*, (1985), Willams *et al.*, (1989) and Mukhopadhay *et al.*, (1990) reported keratinase production by *Streptomyces* spp. He isolated an inducible extracellular homogenous enzyme, which shows 7.5 fold increases in its activity after DEAE cellulose column chromatography. The enzyme-activity was inhibited by reduced glutathione, PMSF and 2-Mercaptaethanol.

Researchers are trying to recycle bird feathers by turning them into nutritious food for livestock. As mentioned, *Bacillus licheniformis* is commonly found on bird feathers; by fermentation with *B. licheniformis*, the large amounts of non-digestible proteins found in the feathers can turn into a feather meal for livestock. This is desired because it is cheap and nutritious. *B. licheniformis* can also give more information about the evolution of molting and patterns of color in birds due to its feather degrading capability. Ecologists are looking for signs of association between the plumage feathers and *B. licheniformis* activity.

B. licheniformis is also an important ingredient in laundry detergent. Since it can grow in alkaline conditions, it produces a protease that can survive at high pH levels. The protease has an optimum pH at around 9 and 10, which is desirable since it can remove protein-comprised dirt in clothes. Researchers culture and isolate this protease to add it into detergents. This protease prevents shrinkage and fading colors since it allows lower temperatures to be used, which in turn lowers energy use as well.

B. licheniformis is used to make the antibiotic Bacitracin. Bacitracin is composed of a mixture of the cyclic polypeptides that *B. licheniformis* produces; ironically the purpose of Bacitracin is to inhibit the growth of *B. licheniformis*. Bacitracin lyses the proplasts of *B. licheniformis* in the presence of cadmium or zinc ions. There is current research on *B. licheniformis* (strain SB3086) and its effects as a microbial fungicide. Novozymes Biofungicide Green Releaf contains *B. licheniformis* strain SB3086 as an active main ingredient. This fungicide can be used on lawns, conifers, tree seedlings, ornamental turf and ornamental plants in outdoor, greenhouse, and nursery sites.

The Research/Technology Invention Award 2006 was given to members of the Biotech Geno Mik project on *B. licheniformis;* they invented a system for controlling industrial fermentation, which they named Bio chip. This system uses DNA-based diagnostic tool to monitor fermentation processes such as the production of enzymes for Henkel laundry detergents. Currently there are many electrical techniques for food processing, one such example is Ohmic heating. Ohmic heating has potential uses such as blanching, evaporation and pasteurization of food; it is a high temperature, short time, and a purely bulk heating method. There are increased concerns regarding microbial contaminations, from such bacteria as *E. coli* and *B. licheniformis,* when it comes to food processing.

2.9 Aims and Objectives of the study

Processed leather is one of the major export items in Bangladesh, and in a survey it was found that about 200 tanneries in Bangladesh mostly employ conventional method of lime sulphide process in bating step and other harsh chemicals in soaking and dehairing steps of hide processing, which is both economically and environmentally unfriendly. Alternatively, the usage of microbial enzymes can overcome many of the problems as associated with conventional processing (Hossain *et al.*, 2006). As the requirement of biotech products including enzymes are met through import, about 30% of enzyme activity are lost during storage and transportation. As a result, the cost of the source gets higher. In this regard, strain of *Bacillus licheniformis* MZK05 was isolated from tannery effluents and identified by 16S rRNA gene sequence analysis (Hoq *et al.*, 2005). The strain was tested for keratinase production in shake and bioreactor cultures using feather meal medium (Hossain *et al.*, 2006). The enzyme preparations were tested for their technical applications in hide processing (Azad *et al*., 2002), feather solubilization (Hossain *et al.*, 2006) and as cleansing agent in detergents (Uddin *et al.*, 2006). Although the keratinase enzyme was found to be suitable in the above mentioned technical applications, the enzyme concentration produced by the wild strain MZK05 of *B. licheniformis* was not sufficient for the development of commercial bioprocess. For this, the *B. licheniformis* strain has to be improved by gene cloning. So, the present study is aimed to clone the gene *ker*A encoding keratinase from the most potential strain of *B. licheniformis* MZK05 in suitable hosts such as *E. coli*. This will follow the investigation into expression and purification of the enzymes, their characterization, production and some technical applications.

The main objectives of this study were:

1. Assessment for best keratinolytic strains of *Bacillus licheniformis*.
2. Amplification of the gene *ker*A from the potential strains by Polymerase Chain Reaction (PCR).
3. Cloning of the PCR amplified products into suitable plasmid vectors pGEX-6p-2 and pET-30a(+).
4. Expression of cloned *ker*A in *Escherichia coli* BL21.

5. Purification of the respective enzymes from the successful recombinant clone.
6. Characterization of the purified enzymes.
7. Whole genome sequencing of *Bacillus licheniformis* MZK05.
8. Optimization of fermentation condition for keratinase production by the recombinants.
9. Use of purified enzymes for some technical applications.

CHAPTER 3

Cloning, expression, purification and structure simulation of keratinase from *Bacillus licheniformis* MZK05 in pGEX-6P-2 system

Cloning, expression, purification and structure simulation of keratinase from *Bacillus licheniformis* MZK05 in pGEX-6P-2 system

3.1 Introduction

Keratinases are a group of proteases that exert keratinolytic activity on insoluble keratin which are fibrous and insoluble structural proteins, extensively cross-linked with disulphide, hydrogen and hydrophobic bonds that result in mechanical stability and resistance to common proteolytic enzymes such as pepsin, trypsin and papain. Keratinase belongs to the subtilisin family of serine proteases and has a high homology with subtilisin Carlsberg from *Bacillus subtilis* (Evans *et al.*, 2000). Keratinases use in leathers in leather industry, feed and bio fertilizers production from feather and treatment of keratin-rich wastes from poultry etc.

Keratinolytic enzymes have important uses in biotechnological process for the development of non-polluting processes and for the treatment of keratin-containing wastes from poultry and leather industry (Sangali and Brandelli, 2000). Keratinase treatment process replaces the use of sodium sulfide in the dehairing process in leather industry (Macedo *et al.*, 2005). Due to the growing needs of keratinase in the industrial application, the focus is now on improving the level of keratinase production through gene cloning for development of recombinant keratinase (Rao *et al.*, 1998). Although many bacteria, actinomycetes and fungi are known to produce keratinase (Bockle *et al.*, 1995; El-Naghy *et al.*, 1998; Thys *et al.*, 2004), only few keratinase encoding genes from the genus *Bacillus* have been cloned and expressed in heterologous systems (Lin *et al.*, 1997; Porres *et al.*, 2002). Similar to the problem associated with the expression of several other recombinant proteins, cloned protease accumulated within *Escherichia coli* (Jacobs *et al.*, 1985; Pan *et al.*, 2004; Tang *et al.*, 2004).

However, the keratinase cloned and expressed in *Bacillus subtilis* resulted in extracellular production of keratinase but at lower level when compared with the wild type strain (Wang and Shih, 1999; Pan *et al.*, 2004).

Keratinase enzymes reduced the use of lime and sulfide up to 50% required in non-enzymatic process (Kalisz *et al.*, 1988). The chemicals causing pollution in pre-tanning processes are lime, sodium sulphide and caustic soda. In fact, one third of the pollution caused by the leather industries results from the wastes generated during dehairing operations. *Bacillus licheniformis* MZK03 and MZK05 strains, previously isolated, exhibited promising keratinase activity in dehairing of hide and feather hydrolysis (Hoq *et al.*, 2005). Crude protease and keratinase enzymes alone (without chemicals) were able to dehair the goat skin completely with increased area yield (98%), less wrinkled grain and smooth surface but no swelling of the skin (Hossain *et al.*, 2008). Combined treatment of the enzymes with 50% chemicals was much better as it de-haired the skin from the hair root, retained desired fibrous structure, and rendered moderate area yield (94%). Hence, the use of keratinase from indigenous *B. licheniformis* strains can be a useful alternative in eco-friendly de-hairing step of hide processing over conventional method.

Since the production of keratinase by wild type strain is limited to a certain level, the industrial need could be met by improving the production level through gene cloning and expression of recombinant keratinase. In this connection, the cloning of *ker*A gene from *Bacillus licheniformis* MZK05 into pGEX-6p-2 vector, its expression in *Escherichia coli* BL21 host, purification by PreScission protease and prediction of 3D model of the expressed protein were performed in this chapter.

3.2 Materials and Methods

3.2.1. Materials

Chemicals and Reagents: Chemicals and Reagents used in this study were listed in Appendix A.

Media: Media were listed in Appendix B.

Buffers and Solutions: Buffers and solutions were presented in Appendix C.

Equipments: Appendix D.

3.2.2 Methods

3.2.2.1 Bacterial strains and plasmid (pGEX-6P-2)

Bacillus licheniformis MZK05 was collected from the laboratory of the Department of Microbiology, University of Dhaka. This strain was previously isolated from feather-decomposed soil and identified by 16S rRNA typing by the International Center for Biotechnology, Osaka University, Japan (Hoq *et al.*, 2005). The organism was then preserved as stock culture in cryogenic tubes containing 20% Trypticase Soya broth and 80% glycerol at -70°C. Organisms were collected at preserved condition from laboratory of the Department of Microbiology, University of Dhaka and revived carefully onto nutrient agar medium. *E. coli* DH5α was used as a propagation host of the plasmids (Blattner *et al*., 1997) which was collected commercially. pGEX-6P-2 vector was used to clone the *ker*A gene which was also collected commercially. This vector has a tac promoter possessing chemically inducible (with 1-5 mM IPTG**)**, high-level expression and an internal lac I^q gene for use in any *E. coli* host. The vectors offer very mild elution conditions for release of fusion proteins from the affinity matrix, thus minimizing effects on antigenicity and functional activity. pGEX-6P-1, pGEX-6P-2, and pGEX-6P-3 each encode the recognition sequence for site-specific cleavage by PreScission Protease, between the GST domain and the multiple cloning site. Low temperature (5°C) digestion minimizes the degradation of the protein of interest. Because PreScission Protease has been engineered with a GST-tag; it can also be removed from the cleavage mixture simultaneously with the GST portion of the fusion protein.

The pGEX-6P series provides all three translational reading frames linked between the GST coding region and the multiple cloning sites. Map of these vector is mentioned in **Appendix 5.**

3.2.2.2 Cloning of *ker*A gene in pGEX-6p-2

3.2.2.2.1 Primers designing

The keratinase protein (sequenced) was purified and characterized afterwards (Lin *et al.*, 1995) and was reported in the gene bank with the Accession No s78160.1. The sequence revels that the actual size of the gene is 1156 bp. The primers were redesigned previously (Shishir, 2007) to avoid spurious PCR products. The *ker*A gene sequence of *Bacillus licheniformis*, PWD-1 (ATCC 53757) with Accession No s781601 was used as a query sequence and blast search was executed on NCBI server (http://www.ncbi.nlm.nih.gov/blast/). From the search result, the *ker*A gene sequences of *B. licheniformis* with maximum identity above 95% were used for multiple sequence alignment analysis in EBI server (http://www.ebi.ac.uk/Tools/clustalw/index.html). The result of multiple sequence alignment was then analyzed to select the consensus sequences of *ker*A gene adjacent to start and stop codons. The selected consensus sequences were joined with the *Bam*HI and *Xho*I recognition sequences to design the primers.

The primers were designed from the alignment of the sequences obtained for *ker*A genes from different *Bacillus licheniformis* strains. Six sequences that exhibited maximum 100% and minimum 95% similarity were aligned and 18 bp consensus sequences at 5'-3' initiated with start codon (Fig. 3.2.1A) and at 3'-5' including stop codon (Fig. 3.2.1B) were chosen for forward and reverse primers respectively. Restriction sites for *Bam*HI and *Xho*I were then patched before the 5'-end with two additional nucleotides so that the amplicon could be ligated at right reading frame of the cloning vector after restriction digestion.

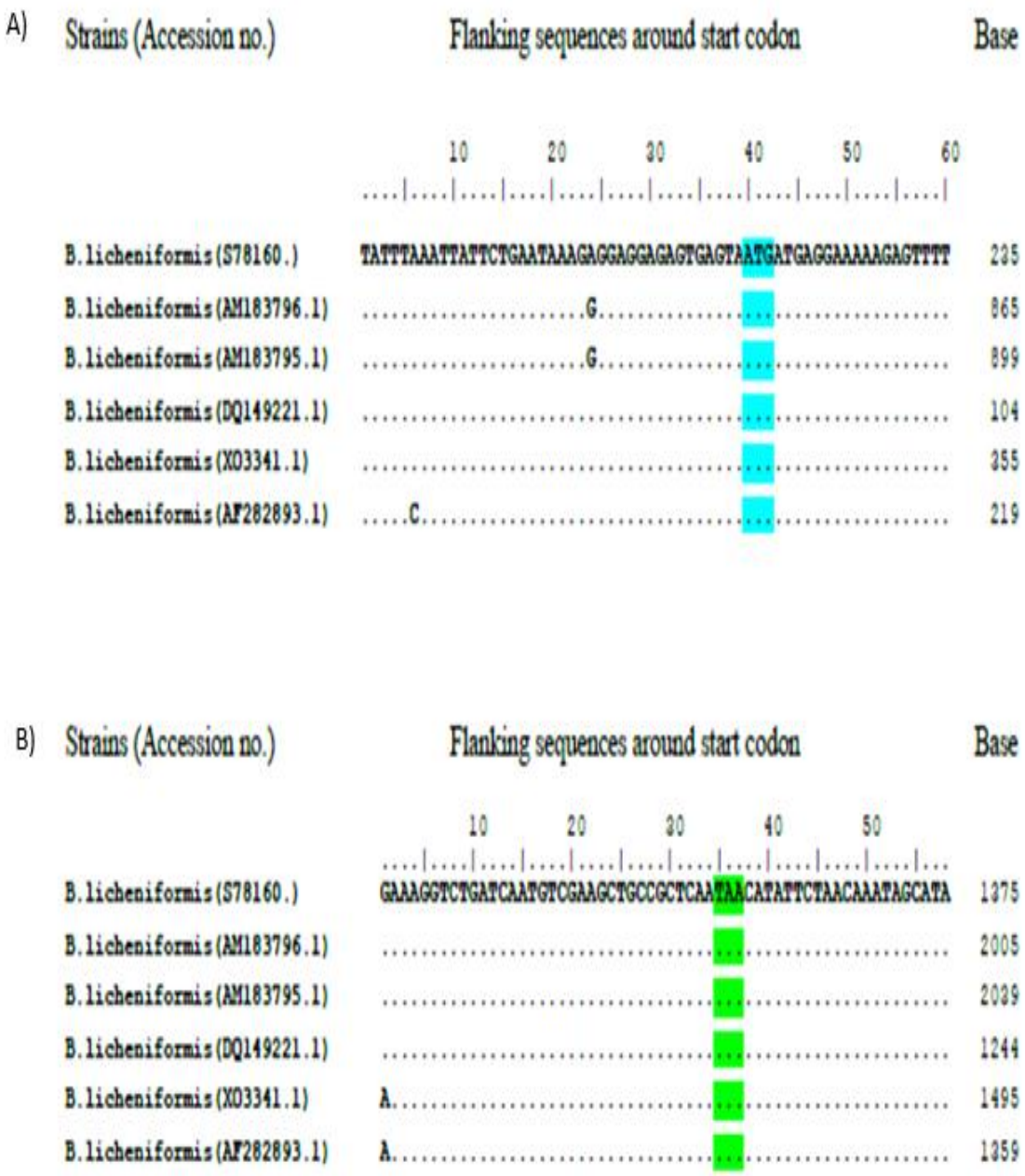

Figure 3.2.1: Primer designing to amplify *ker*A gene from *Bacillus licheniformis* MZK05 A) 18 bases started from start codon-ATG were chosen as part of the forward primer B) 18 bases ended with stop codon-TAA was selected as part of the reverse primer.

3.2.2.2.2 Polymerase chain reaction to amplify *ker*A gene

The colony direct PCR was done. A single colony was picked up by the autoclaved toothpick. Then the colony was placed on the PCR tube. The composition of colony PCR carried out in a 30 µl volume is detailed in Table 3.2.1.

Table 3.2.1: The composition of colony PCR carried out in a 30 µl volume.

Ingredients	Volume added
10X PCR buffer (with 15mM $MgCl_2$)	3 µl
Forward primer, 10 µM (10 pmol/µl)	3 µl
Reverse primer, 10 µM (10 pmol/µl)	3 µl
dNTP mix (2.5mM)	2.5 µl
Commercial Taq polymerase (5 U/µl)	0.3 µl
PCR grade water	To make final volume 30 µl

The PCR reactions were performed in a thermal cycler (MJ Mini™ BIO RAD, USA). The PCR program initiated with initial denaturation of template DNA at 94°C for 4 minute, (Sharkey *et al.,* 1994). Followed by 30 cycles of the following steps:

PCR condition	Temperature (°C)	Time
Denaturation	94	1min
Annealing	56	1min 30s
Extension	72	1min

A single final extension was done at 72°C for 10 minute (Chien *et al*., 1976). After PCR reaction, amplification was checked by horizontal electrophoresis in 1.0% agarose slab gel in Tris-borate EDTA (TBE) buffer.

3.2.2.2.3 Agarose gel electrophoresis

The DNA samples from the sources like PCR products, plasmids etc were separated in this whole study by Agarose gel electrophoresis (Sambrook *et al.,* 1989). The concentration of agarose was varied depending of the samples such as 1.5% in 1× TBE buffer for the analysis of PCR products and 0.75% in 1× TBE buffer for Plasmids. Agarose gel was prepared by dissolving agarose powder completely in 1× TBE buffer by heating and then pouring onto a mold with combs placed when cooled down at temperature 50^oC.

When the molten agarose solidified and became ready for use, the comb was removed carefully to keep the wells unbroken. The gel was then submerged in the electrophoresis tank with 1× TBE buffer. The DNA samples were then mixed with 0.2 volume of 6× DNA loading buffer by micropipette and loaded into the wells of the submerged gel. The electrophoresis was carried out at 60 volt and a DNA size marker was run alongside the samples. The gel was then visualized against UV trans-illumination in a gel documentation system (Alpha imager mini, USA) following staining in Ethidium Bromide (EtBr) solution (0.5 µg/ml) and de-staining in distilled water.

3.2.2.2.4 Purification of PCR product

To obtain the desired purified product, purification kit was used (ATP Gel/PCR Extraction Kit, ATP biotech Inc Ltd). The steps of the purification are given below:

Up to 100 µl reaction products were transferred into a microcentrifuge tube. 5 volumes of DF buffer was added to 1 volume of the sample and mixed by vortexing. A DF Column was placed in a 2 ml collection tube. The sample mixture was applied from previous step 2 into the DF column and centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded and the DF column was placed back in the collection tube. 600 µl of Wash buffer (ethanol added) was added into the DF column and centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded and placed the DF column back in the collection tube and centrifuged again for 3 minutes at 13,000 rpm to dry the column matrix. Dried column was transferred in a new microfuge tube. 15-50 µl of Elution buffer was added or water onto the center of the column matrix. The columm was stand for 2 minutes until Elution buffer or water is absorbed by the matrix and centrifuged at full speed for 2 minutes to elute purified DNA.

3.2.2.2.5 Restriction digestion of pGEX-6p-2 vector

Purified 1156 bp PCR product and pGEX-6p-2 vector (4985bp) were digested separately with both of the selected restriction enzymes, *Bam*HI and *Xho*I to produce molecules with single strand overhangs. Double digestion was carried out using enzymes and respective buffers of TAKARA, JAPAN.

Restriction digestion was done subsequently by the both enzymes with their own buffer (for *Bam*HI, buffer K, and for *Xho*I, buffer H) in the recommended incubation temperature (for *Bam*HI: 30°C and for *Xho*I: 37°C) overnight. Composition of the reaction mixtures given in Table 3.2.2.

Table 3.2.2: Composition for digestion of vector and PCR product by *Bam* HI

Ingredients	Amount (µl)	Ingredients	Amount (µl)
pGEX 6p-2 vector (55 ng/µl)	20 µl	PCR product (300 ng/µl)	10 µl
10× K buffer	3 µl	10× K buffer	2µl
*Bam*HI (1U/µl)	2µl	*Bam*HI (1U/µl)	4 µl
ddH_20	5 µl	ddH_20	4 µl
Total volume	30 µl	Total volume	20 µl

Digestion reaction was initially carried out with *Bam*H1 at 30^{o}C with overnight incubation. Then the second restriction enzyme, *Xho*1 was added and incubated at 37^{o}C for overnight. Composition of the reaction mixtures were given in Table 3.2.3.

Table 3.2.3: Composition for digestion of vector and PCR product by *Xho*1

Ingredients	Amount (µl)	Ingredients	Amount (µl)
pGEX 6p-2 vector (55 ng/µl)	20 µl	PCR product (300 ng/µl)	10 µl
10× K buffer	3 µl	10× K buffer	2µl
*Bam*HI (1U/µl) *Xho*1 (1U/µl)	2µl	*Xho1* (1U/µl)	4 µl
ddH_20	5 µl	ddH_20	4 µl
Total volume	30 µl	Total volume	20 µl

After completing the reaction, restriction digestion was confirmed by the demonstration of the digested band on the agarose gel.

3.2.2.2.6 Ligation reaction

The single stand overhangs of the PCR product are complementary to those of the pGEX-6p-2 vector. For this sticky ligation, 3X more insert were used than the number of vector molecules.

The following formula was used to calculate the molar ratio.

$$x = \frac{y \text{ bp of insert} \times \text{amount of vector}}{\text{Size in bp of vector}}$$

Where x ng is the amount of insert of y base pairs to be ligated for a 1:1 (vector: insert) ratio. In order to achieve a 1:3 ratio, x should be multiplied by 3 and for 1:5 ratios; x should be multiplied by 5 to get the amount of insert needed for ligation. The size of insert (y) is 1156 bp and the size of vector is 4962 bp. For 40 ng digested vector, the value of x was found 9.4 ng according to the above formula. The composition of the reaction mixture was given in Table 3.2.4.

Table 3.2.4: Ligation mixture compositions

Ingredients	1:1(vector: insert)	1:3(vector: insert)	1:0 (vector: insert)
10X ligation buffer	2µl	2µl	2µl
Digested vector (40 ng / µl)	5µl	5µl	5µl
DNA inserts (150ng/ µl)	0.5µl	0.75µl	0
T4 DNA ligase (5U/ µl)	1µl	1µl	1µl
Double deionized water	11.5µl	11.25µl	12µl
Total reaction volume	20µl	20µl	20µl

The reaction tube was vortexed and then spins down in a microcentrifuge for 3-5 seconds. A control reaction was carried out (1:0: vector: insert) in which insert DNA was replaced by ddH_2O. The reaction mixture was then incubated at 4^oC for overnight. After incubation, the reaction mixture was used for transformation.

3.2.2.2.7 Preparation of competent cells

50 ml of LB medium was inoculated with a colony of *E .coli* DH5α in a 250 ml flask and incubated in a 37°C orbital shaker at 150 rpm. Some of the overnight culture was poured into 50 ml of fresh LB broth so that the final absorbance should be 0.1. It was then incubated in a 37°C orbital shaker at 150 rpm. Until the absorbance at 600nm reached 0.4-0.6. Then, 45 ml culture was transferred to a sterile sorbal tube and chilled on ice for 10-15 min. It was centrifuged at 8,000 rpm for 5 minutes at 4°C Centrifuge (TOMY, MX-305, JAPAN). The supernatant was discarded and the cells were suspended in 22 ml (0.5 volumes) ice cold, sterile 0.1 M $CaCl_2$. It was kept on ice for 15-20 minutes and again centrifuged at 8,000 rpm for 5 minutes at 4°C Centrifuge (Tomy, MX-305, high speed Refrigerated micro centrifuge, Japan). The supernatant was discarded and the pellet was resuspended in 11 ml ice cold, sterile 0.1 M $CaCl_2$. It was kept on ice for at least 2 hours. (Transformation should be done immediately or the competent cells should be stored at-70°C by using 15% glycerol broth).

Transformation

50 µl competent cells were mixed with 1 µl (about 100 ng) of purified pGEX-6p-2 plasmid in a fresh eppendorf tube. Negative control was used with competent cells but had no plasmid. These were kept on ice for 30 minutes. Then they were kept in 42°C water bath for 1 minute and immediately transferred to ice for additional 2 minutes. 450 µl of fresh LB broth was added to each tube and incubated at 37°C for 45 minutes for cell recovery. From each tube 50 µl was spreaded on containing LB agar media containing ampicillin (100µg/ml).

3.2.2.2.8 Isolation of pGEX- 6p-2 plasmid from transformed *E. coli* DH5α

In the isolation of plasmid, The PureLink™ Quick Plasmid Miniprep Kit, invitrogen, U.S.A, plasmid preparation kit was used. 1-3 ml of well-grown bacterial culture was transfered to a centrifuge tube. The tube was centrifuged at 11,000 X g for 1 minute to pellet the cells and the supernatant was completely discarded. 200 µl of FAPD1 buffer (RNase A added) was added to the cell pellet and the cells were resuspended. Completely by pipetting. 200 µl of FAPD2 buffer was added and gently inverted the tube 5-10 times. The sample mixture was incubated at room temperature for 2-5 minutes to lysed the cells.

300 µl of FAPD3 buffer was added and the tube was inverted 5-10 times immediately to neutralize the lysate. Then centrifuged at full speed (18,000 X g) for 5 min to clarify the lysate. During centrifugation, FAPD column was placed in a collection Tube. The supernatant was transferred carefully to the FAPD column and centrifuged at 11,000 X g for 30 seconds. The flow-through was discarded and the column placed back to the collection Tube. 400 µl of W1 buffer was added to the FAPD Column and centrifuged at 11,000 X g for 30 seconds. The flow-through discarded and the column was placed back to the collection Tube. 700 µl Wash buffer was added to the FAPD Column and centrifuged at 11,000 X g for 30 seconds. The flow-through was discarded and the column was placed back to the collection tube and centrifuged at full speed (18,000 X g) for an additional 3 minutes to dry the FAPD column. The FAPD column was placed to a new 1.5 ml micro centrifuge tube. 50 µl ~ 100 µl of Elution buffer was added or ddH_2O to the membrane center of the FAPD Column. The column was stand for 1 minute and centrifuged at full speed (18,000 X g) for 1 minute to elute plasmid DNA and stored the DNA at -20°C.

3.2.2.2.9 Transformation of *E. coli* DH5α with ligation mixture

The competent cells were thawed gently. 5 µl of ligation mixture containing recombinant plasmids was added to 50 µl aliquot of competent cells. In another tube, 5µl of the reaction mixture without insert was added to 50 µl of competent cells. A positive control with commercial pGEX-6p-2 plasmid solution and a negative control without any plasmid were used to check the efficiency of the competent cells for transformation. The reaction tubes were incubated on ice for 30 min and then heat pulsed for 45 seconds at 42°C without shaking. The reaction tubes were again replaced on ice for 2 min. Then 0.5 ml of LB (preheated to 37°C) was added to each tube and incubated at 37°C for 1 hour with shaking at 225–250 rpm. After incubation, centrifugation was done at 5000 rpm for 5 min on a tabletop microfuge. Supernatant was discarded leaving ~100 µl to resuspend the pellet. The resuspended solution was then plated on LB agar containing ampicillin. The transformation plates were incubated at 37°C for >16 hours.

3.2.2.2.10. Confirmation of the recombinant vector

Growth on the plate containing ligation mixture was carried out under the further screening procedure to confirm the clone containing recombinant vector DNA. It was done by following PCR reaction. PCR reaction was done with the randomly selected colonies from the plate containing ligation mixture. For the recombinants, PCR products were found which were compared with the positive control. Same band like the insert was the indication to the positive result and of recombinant vector DNA molecules. For more confirmation, miniprep analysis was done. The agarose gel document of the single digestion of the normal and assumed recombinant one revealed the size difference between two plasmids is another proof. Further double digestion of the miniprep preparation would confirm the presence of *ker*A gene and of recombinant vector DNA molecule.

3.2.2.3 Heterologous expression of the recombinant protein in *E. coli*

3.2.2.3.1 Transformation of *E. coli* BL21 with recombinant pGEX-6p-2–kerA plasmid

2 µl of each of the ligation mixtures were added to 50 µl aliquot of competent cell separately in 1.5 ml fresh microfuge tubes. A positive control with commercial pGEX-6p-2 plasmid solution and a negative control without any plasmid were used to check the efficiency of the competent cells for transformation. The procedure for transformation of the vector into the bacterial cell has been described in section 3.2.2.2.9.

3.2.2.3.2 Expression of recombinant protein

The recombinant vector, pGEX-6P-2-kerA, was transformed into the chemically developed competent *Escherichia coli* BL21 cells for the expression of GST-KerA fusion protein. *Escherichia coli* BL21 carrying pGEX-6P-2-kerA was cultured overnight at 37°C in LB medium containing ampicillin (100 µg/mL) and was used as inoculum to start IPTG induced protein expression. Fresh LB ampicillin medium was inoculated with the overnight culture to start with an OD_{600nm}= 0.1 and incubated at 37°C with shaking at 200 rpm.

IPTG was added aseptically in the culture when the OD_{600nm} reached 0.5 and three different concentrations were applied (i.e. 0.1, 0.3 and 0.5 mM IPTG in 3 separate flasks) for optimization. Simultaneously to optimize the induction time, 1 mL of culture from each flask was collected aseptically at 1 h interval up to 5 h. Then the cells were harvested by centrifugation at 10,000×g for 10 min at 4°C Centrifuge (Tomy, MX-305, high speed Refrigerated micro centrifuge, Japan) and resuspended in 100 µL of 1× Laemmli buffer to denature the protein incubating at 95°C for 5 min. Equal amount of protein from each IPTG concentration and each time interval was then analyzed by SDS-PAGE in a 10% separating gel. Gel was then stained in staining solution (0.02% CBB-G250 in 2% (w/v) phosphoric acid, 5% aluminum sulfate and 10% ethanol) for 2 h.

3.2.2.3.3 SDS-PAGE analysis of proteins

3.2.2.3.3.1 Gel preparation

Clean and dry glass plates with spacer were assembled and placed on the gel casting stand (Bio- Rad instrument) 10% separating gel was prepared by gently mixing distilled water, lower gel buffer, 30% acrylamide and bisacrylamide solution (29:1), 10% APS and TEMED. Freshly prepared solution was carefully poured into the glass plate chamber, without generating bubble using a 1 ml micropipette. The edge of the separating gel was below 5 cm of the glass plate. It was kept undisturbed for 45-60 minutes for polymerization. 4% stacking gel was prepared using upper gel buffer and rest of the components in different ratio and the solution was poured using a 1 ml micropipette ensuring no generation of bubble.

A comb was placed carefully in the cleavage of two glass plate and allowed to rest for 45-60 minutes for polymerization and creation of well to load protein samples. After removing the comb the amount of each sample to be poured was determined. Prior to loading onto the gel, the samples were denatured by diluting them in 2X sample loading buffer and then heating at 95°C for 5 min. 2µl sample 0.1% bromophenol blue and sample was loaded into the well using a micro pipette. Electrophoresis buffer specific for SDS-PAGE was used for gel run at 100 volts until the tracking dye reached the bottom line of the gel. After completion of the electrophoresis the glass plates were immersed in distilled water with the plate separator gel was released from the glass plates.

SDS-PAGE gel composition was given in Table 3.2.5

Table 3.2.5: SDS-PAGE gel composition.

Ingredients	Separating gel 10%	Stacking gel 4%
dH20	1.9ml	1.5ml
Lower gel buffer	1.3ml	-
Upper gel buffer	-	0.625ml
30 % polyacrylamide	1.7ml	0.325ml
10% APS	50µl	12.5µl
TEMED	2.0µl	2.0µl

3.2.2.3.3.2 Staining and destaining of the gel

The glass plates were immersed in distilled water and by using the plate separator, the gel was released from the glass plates. Then the gel was placed into staining solution (0.02% Coomassie Brilliant Blue, G-250 in 2% (w/v) phosphoric acid, 5% aluminum sulfate and 10% ethanol) for a period of 2 hours on a rotary shaker. The gel was then transferred to a container containing distilled water and was rinsed with distilled water repeatedly to visualize the protein bands. The gel was visualized at 302 nm UV light. Gels were recorded in a gel documentation system (Gel Doc, Alpha imager mini, USA, Bio-science Corporation).

3.2.2.4 Purification of GST-fusion protein

The GST-KerA fusion protein was expressed as described after optimizing the IPTG concentration and time. The liquid culture (50 mL) was then transferred into falcon tube and the cells were harvested by centrifugation at 7700×g for 10 min at 4°C Centrifuge (Tomy, MX-305, Japan) and resuspended in 2.5 mL of 1× PBS **(**140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.3) from which 10 µL was preserved for SDS-PAGE. The suspended cells were then lysed using microtip sonicator in short bursts keeping in ice. Triton X-100 was then added for a final concentration of 1% and mixed gently for 30 min for the solubilization of the fusion protein.

Then, 1.0 mL of 50% slurry of Glutathione Sepharose 4B was added into the bacterial sonicate and kept at room temperature for 30 min with gentle mixing. The supernatant was then separated carefully by decanting following centrifugation at 500×g for 5 min. The sediment was then washed thrice with 5 mL of 1× PBS and once with 5 mL PreScission cleavage buffer (50 mM Tris-HCl; pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The pellet was then resuspended by gentle mixing in 500 µL of PreScission protease mixture [40 µL (80 units) of PreScission Protease added into 960 µL of PreScission Cleavage Buffer] and incubated at 5^{o}C for 4 h. The supernatant containing KerA protein was finally collected very carefully following centrifugation at 500×g for 5 min and was analyzed by SDS-PAGE as described in section 3.2.2.3.3.

3.2.2.5 Protease and keratinase assay

Protease activity of the purified KerA was determined by a modified method described by Kreger and Lockwood (1981) using azo-casein (Sigma, USA) as the substrate. Briefly, 400 µL of appropriate dilution of KerA was added to 400 µL of 1% azo-casein solution (suspended in 0.05 M Tris- HCI; pH 8.5) and the mixture was incubated at 37°C for 1 h. The reaction was terminated by adding 135 µL of 35% trichloroacetic acid (TCA) and left for 15 min on ice followed by centrifugation at 13,000 rpm, at 4°C for 10 min. Then, 750 µL of the supernatant was neutralized with equal volume of freshly prepared 1.0 N NaOH by gentle mixing. Absorbance (OD_{440nm}) of the mixture was then measured in a UV spectrophotometer (Thermo spectronic Genesys, USA) keeping the solution from a parallel reaction as blank where TCA was added before the enzyme. One unit of protease activity was defined as the amount of enzyme required to yield an increase in absorbance (OD_{440nm}) of 0.01 in 1 h at 37°C.

Keratinolytic activity was determined by the method of Bressollier *et al.*, (1999). The enzyme was incubated with 5 mg of keratin-azure (Sigma, USA) in 1 mL of 50 mM/l Na-phosphate (pH 7.5) at 37°C for 1 h with constant agitation (900 rpm). One unit of keratinase activity was defined as the amount of enzyme required to result in an increase in absorbance (OD_{595}) of 0.01 in 1 h.

3.2.2.6 Nucleotide Sequence and deduced amino acid sequence

The sequence of the *ker*A amplicon was determined by the dideoxy cycle sequencing method (Sanger *et al.,* 1992) on a model ABI 373 automated DNA sequencer (DNA Sequencing Laboratory, CARS, University of Dhaka, Bangladesh) and deposited in GenBank under the accession no. KJ661542. The similarity for the sequence was searched at the GenBank (National Centre for Biotechnology Information, NIH, USA) using BLAST and FASTA programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequence of the *ker*A ORF was deduced with the aid of the software MEGA 5.2.

3.2.2.7 3D Model Building for KerA Protein

Three dimensional model of KerA protein was built with ProMod Version 3.70 from a fully automated server, SWISS-MODEL (http://swissmodel.expasy.org/). Homology modeling typically comprises the following steps: (i) template identification, (ii) template selection (iii) model building and (iv) model quality estimation (Sali and Blundell, 1993; Schwede *et al.,* 2008). The deduced amino acid sequences were used in this process to build the models based on the target-template alignment. Coordinates which are conserved between the target and the template were copied from the template to the model. Insertions and deletions were remodeled using a fragment library. Side chains were then rebuilt. Finally, the geometry of the resulting model was regularized by using a force field. In case loop modeling with ProMod (Guex *et al.,* 1997) does not give satisfactory results, an alternative model was built with MODELLER (Sali and Blundell, 1993). The global and per-residue model quality has been assessed using the QMEAN scoring function (Benkert *et al.,* 2011).

3.3 Results

3.3.1 Amplification of *ker*A gene

Colony direct PCR reactions were carried out at annealing temperature of 56°C in the PCR machine of MJ Mini™ BIO RAD, USA. The annealing temperature was 56°C for 1 min 30 second with initial denaturation at 94°C for 4 min; denaturation at 98°C for 1 min; extension at 72°C for 1 min and final extension at 72°C for 10 min. 1156 bp amplicon was found (Fig. 3.3.1) For polymerization reaction, commercially derived Taq polymerase (5 U/µl) was used. After PCR reaction, amplification was checked by horizontal electrophoresis in 1.0% agarose slab gel in Tris-borate EDTA (TBE) buffer.

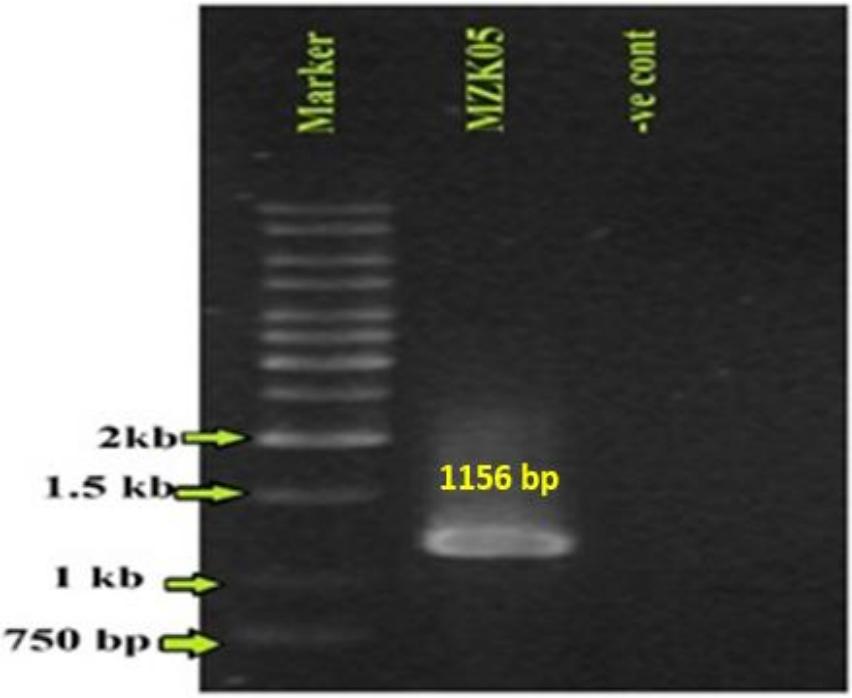

Figure 3.3.1: Agarose gel electrophoresis of PCR product (amplified *ker*A gene) of expected 1156 bp. Lane 1: Marker, lane 2: positive PCR products and lane 3: control.

3.3.2 Cloning of *kerA* gene from *Bacillus licheniformis* MZK05

3.3.2.1 Restriction digestion of pGEX-6p-2 vector and purified PCR product

For cloning of the *ker*A gene into pGEX-6p-2, both pGEX-6p-2 and the *ker*A amplicon were subjected to restriction digestion with the enzymes *Bam*HI and *Xho*I. Digested vectors were subjected to agarose gel electrophoresis in 1.0% gel with undigested vectors as control. Undigested vector in supercoiled form, moves faster than the digested vector that is in linear form. So, undigested vector appeared to be about 3.5 kb which upon digestion appeared to be about 5 kb (Fig. 3.3.2).

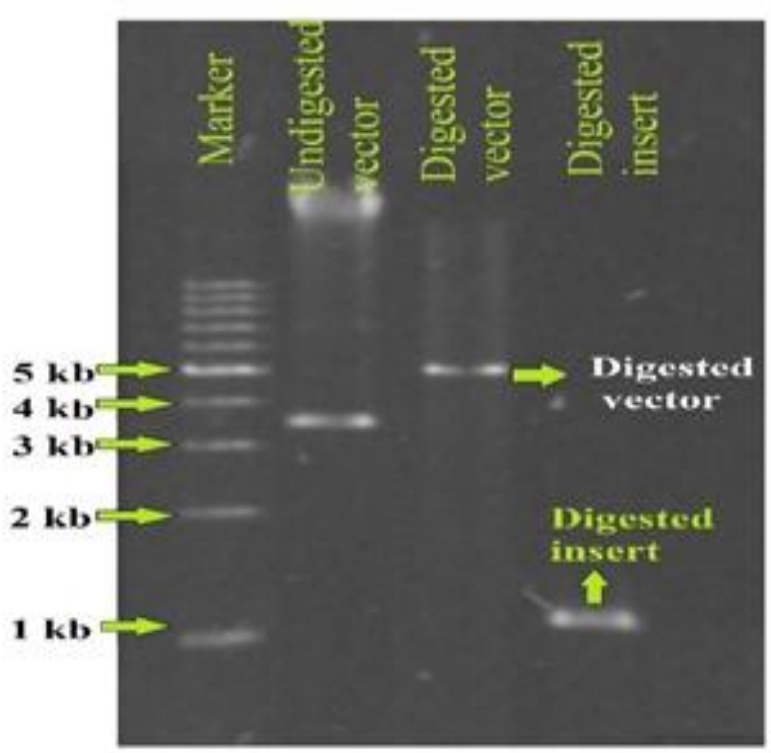

Figure 3.3.2: Agarose gel electrophoresis of restriction digestion of pGEX-6p-2 vector and purified PCR product

3.3.2.2 Transformation of *E. coli* DH5α with ligation mixture

After overnight incubation of the transformed cells in ampicillin LB agar plate the following observations were found. No colony was found in the 1:0 ligation mixture plates (Fig. 3.3.3 A) which indicate successful double digestion of the vector pGEX-6p-2.

In case of 1:1 ligation mixture plate (Fig. 3.3.3 B) and a number of 7 colonies were found and 1:3 ligation mixture plate (Fig. 3.3.3 C) contained the maximum number of colonies. These colonies from both plates were subjected to further analysis.

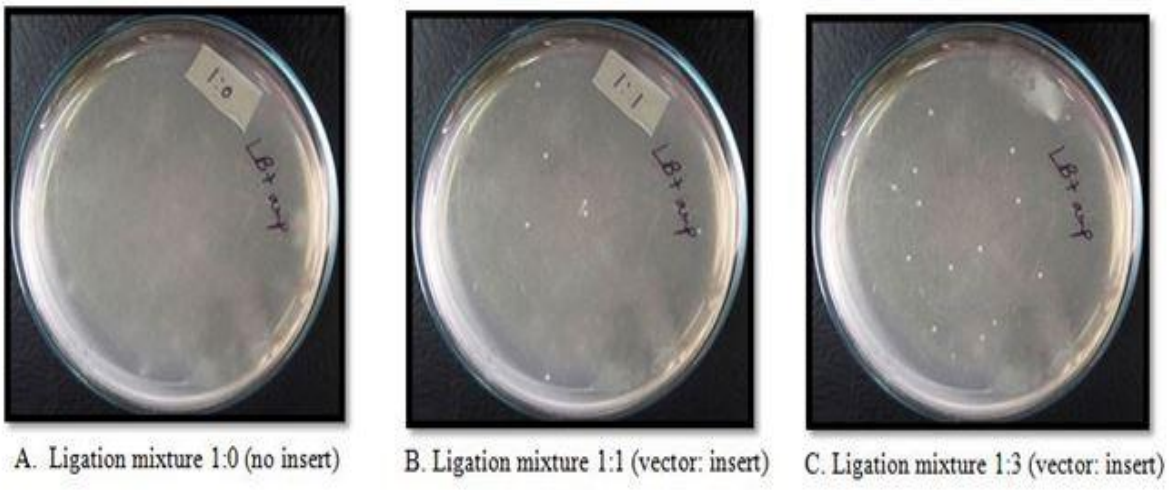

Figure 3.3.3: Ampicillin LB plates showing transformants. A: No colony on the 1:0 ligation mixture plate, B: 7 colonies on 1:1 ligation mixture, C: 15 colonies on 1:3 ligation mixture plate.

3.3.2.3 Confirmation and restriction digestion of the recombinant pGEX-6p-2-kerA

PCR was done with randomly selected colonies from the plates containing transformants. For the recombinants, PCR products were found which were compared with the positive control. Same band size like the insert (1156 bp) is the indication of positive result (Fig. 3.3.4 a). To confirm the insertion of the keratinase gene, the recombinant plasmid pGEX-6p-2-kerA was extracted and digested by *Bam*HI and *Xho*I. The molecular mass of *Bam*HI and *Xho*I double-digested DNA fragment in the positive clones was the same as the PCR product (Fig. 3.3.4 b), indicating the main keratinase gene was successfully cloned into plasmid pGEX-6p-2-kerA.

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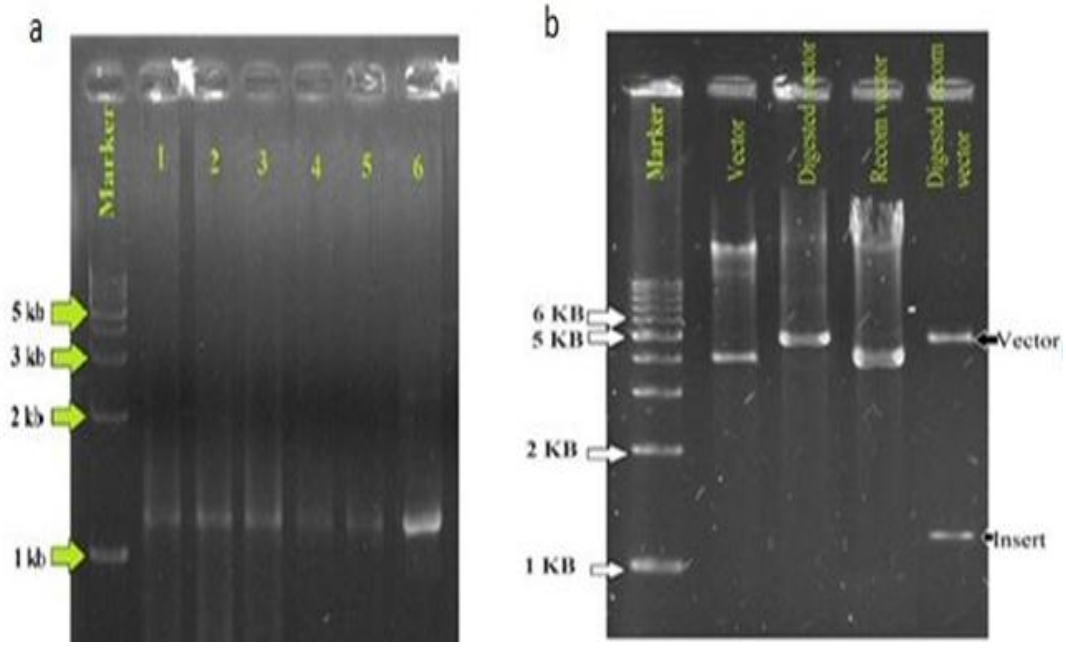

Figure 3.3.4: Confirmation and restriction digestion of the recombinant pGEX-6p-2-kerA. a. Confirmation of the clones harboring pGEX-6p-2-kerA recombinant vector by PCR amplification for *ker*A gene. Lanes other than DNA marker are labeled as per the clone number (1-6). b. Double digestion of both pGEX-6p-2 and pGEX-6p-2-kerA vector produced vector in linear form and vector as well as insert respectively.

3.3.3 Expression of *kerA* gene in *Escherichia coli* BL21

The pGEX-6p-2-kerA was transformed into *E. coli* BL21 and expression of GST-KerA fusion protein was observed within 1 h of induction at 0.1 mM IPTG concentration (Fig. 3.3.5). The maximum expression was 4 hr after the induction (Fig. 3.3.5). Different IPTG concentrations were tested to find out the maximum induction done. A 58 kDa GST-kerA fusion protein took place when induced with IPTG at a concentration of 0.1. Expression up to 3 hr was continued with different IPTG concentration starting from 0.1 mM to 0.5 mM and it was observed that the optimum IPTG concentration was 0.3 mM as the expression level remained unchanged from 0.3 Mm (Fig. 3.3.6)

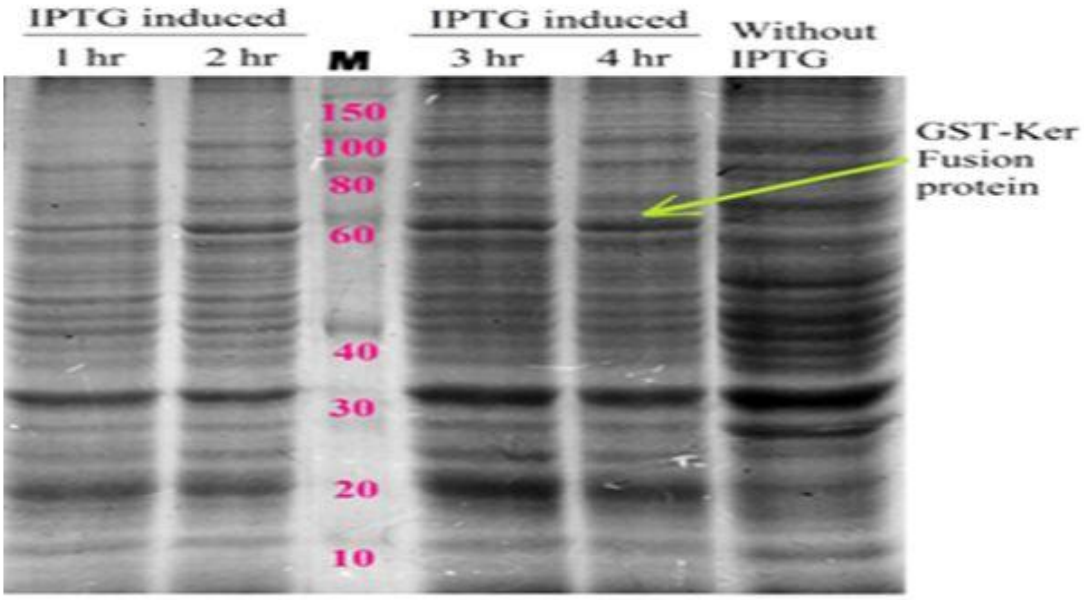

Figure 3.3.5: Time course study of GST-KerA fusion protein. Lane 1,2,3,5,6: IPTG induced protein, lane 7: uninduced, lane M: marker.

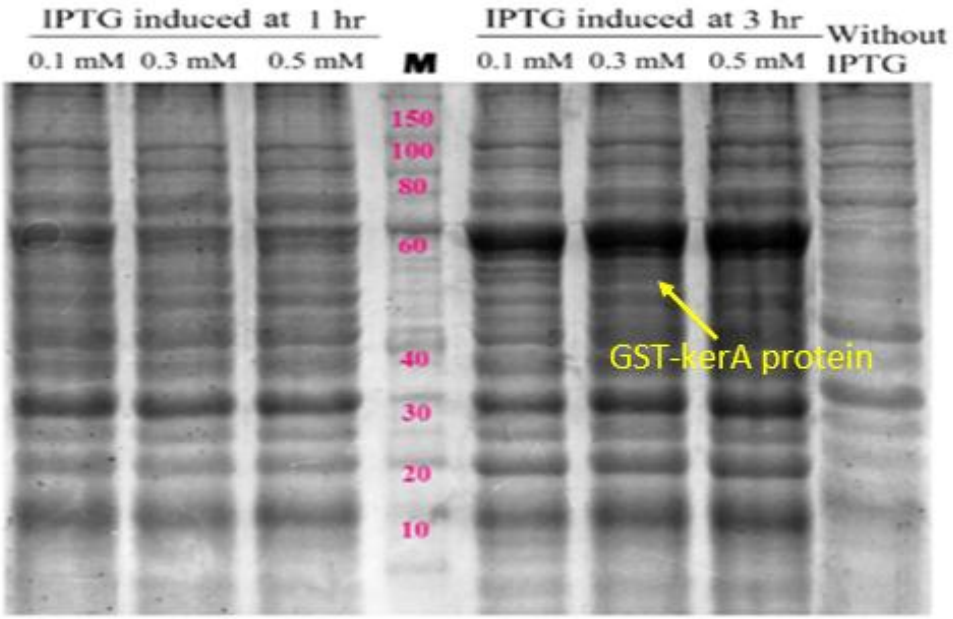

Figure 3.3.6: The optimum time and IPTG concentration for maximum expression of GST-KerA fusion protein. Lane 1, 2 and 3: different con. of IPTG induced at 1 hr, Lane 5, 6 and 7: different con. of IPTG induced at 3 hr, Lane 8: uninduced and lane M: marker.

3.3.4 Purification of recombinant protein

Purification of recombinant protein was done by the procedure described in the experimental section. Glutathione Sepharose 4B batch purification followed by PreScission protease cleavage produced the purified protein of about 39 kDa. From SDS-PAGE analysis, the molecular weight of the GST-KerA fusion protein was determined to be 58 kDa (Fig. 3.3.7). Glutathione Sepharose 4B batch purification followed by PreScission protease cleavage produced the KerA protein of about 39 kDa (Fig. 3.3.7).

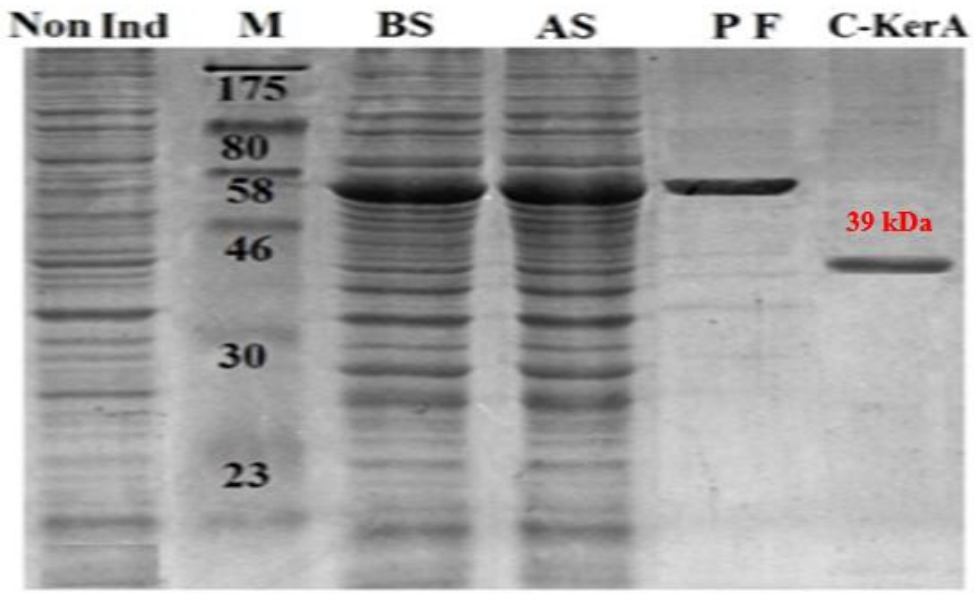

Figure 3.3.7: Purification of recombinant protein. Non Ind: Non induced. BS: Before sonication, AS: After sonication, PF: Purified fusion protein, C-KerA: KerA protein after cleavage by PreScission protease free from GST tag. (M: Pre-stained protein Marker, NEB, USA)

3.3.5 Protease and keratinase assay

Protease and keratinase activity of the recombinant purified protein was determined and was found 312 U/ml and 196 U/ml, respectively. The yield of purified protein was approximately 119 mg/L. The specific protease and keratinase activity was also determined and those were found to be 2621.84 U/mg and 1647 U/mg of protein respectively (Fig. 3.3.8).

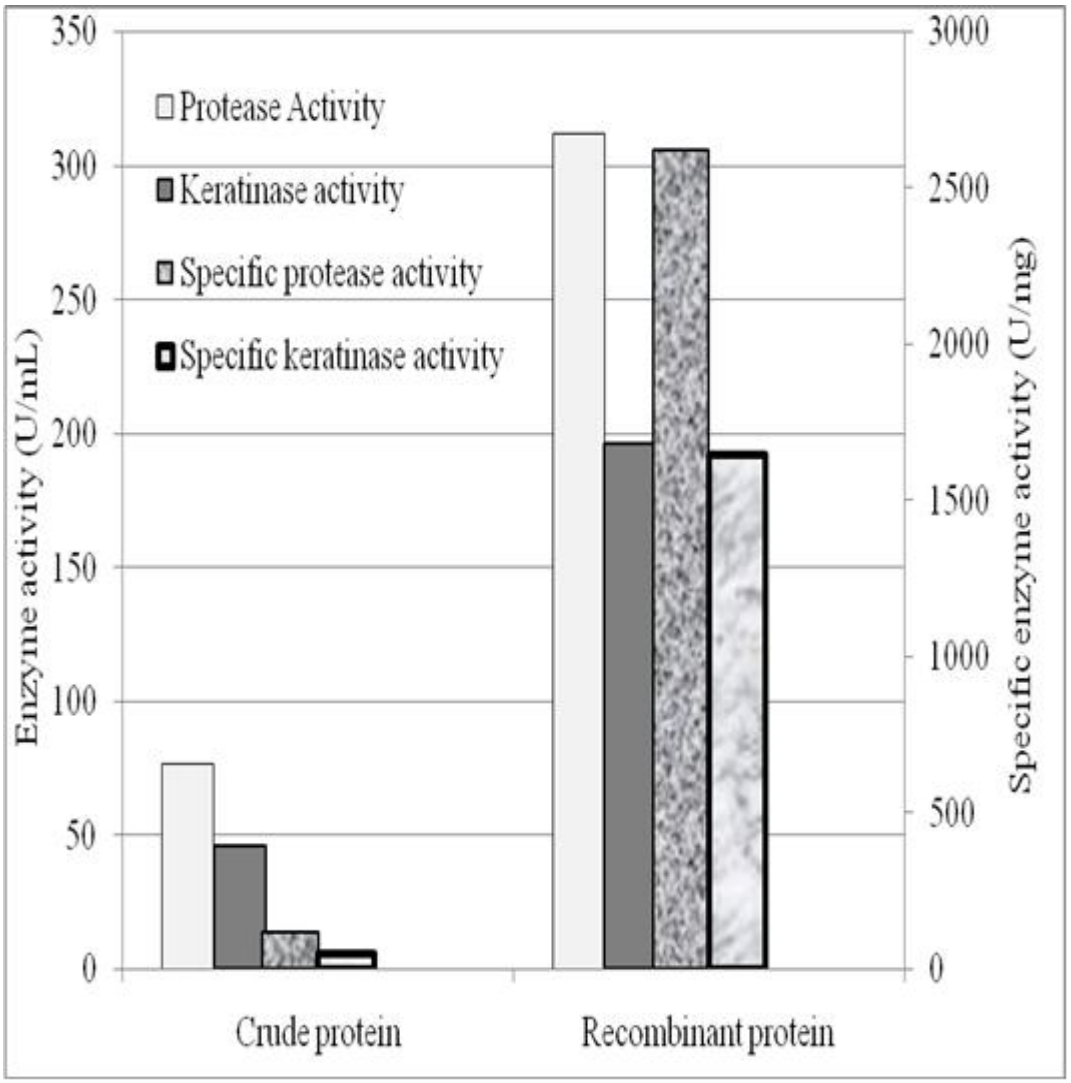

Figure 3.3.8: Comparison of yield in enzyme activity between crude and recombinant protein.

3.3.6 Sequence analysis

Sequence analysis of *ker*A indicated that the insert was in proper orientation and correct junction to maintain the reading frame. The deduced amino acid sequences of *ker*A gene of *B. licheniformis* MZK05 showed 100% identity with thermostable keratinase from *Bacillus licheniformis* DS23, KerA of *Bacillus licheniformis* OWU 1411T and 98.4% identity with kerA of *B. licheniformis* PWD-1 (*B. licheniformis* ATCC 53757).

The insert contained an ORF of 1137 nucleotides from which amino acid sequences were deduced and 379 amino acids were revealed with an N-terminal signal peptide consisting of 29 residues (Fig. 3.3.9), a pro-peptide of 76 residues followed by the matured protein comprising of 274 residues and thus the prokeratinase of 350 residues.

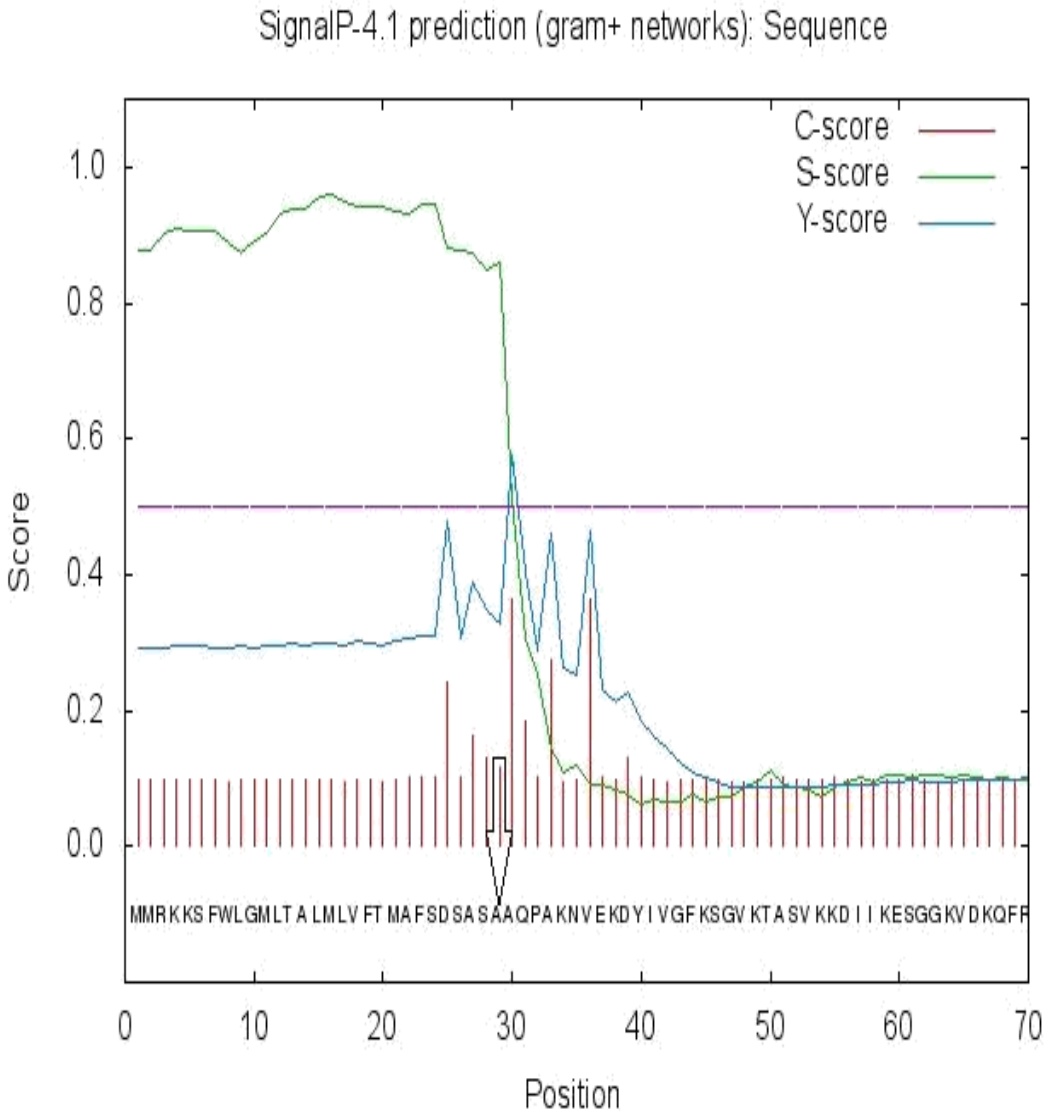

Figure 3.3.9: Amino acid sequence analysis of *ker*A gene of *B. licheniformis* MZK05.

The estimated molecular mass of matured keratinase deduced from the amino acid sequence was 27330 Da. Sequence alignment of the amino acids from matured KerA protein revealed that Tyr26 instead of Phe26, Asn86 for Ser86 and Ser211 for Asn211 are present (Fig. 3.3.10).

In the KerA from *B. licheniformis* MZK-05, discrepancies with other sequences, i.e. Tyr26 instead of Phe26, Asn86 and Ser211 instead of Ser86 and Asn211 respectively were observed (Fig. 3.3.10).

```
                        80        90       100       110       120       130       140
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MZK05 KerA      IINAAKAKLDKEALEEVKNDPDVAYVEEDHVAHALAQTVPYGIPLIKADKVQAQGYKGANVKVAVLDTGI
4gi3.1.A KerA   ------------------------------------.....................F..............
1c3l.1.A SUBTIL ------------------------------------.....................F..............
1yu6.1.A Subtil ----------------------------------M.....................F..............

                       150       160       170       180       190       200       210
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MZK05 KerA      QASHPDLNVVGGASFVAGEAYNTDGNGHGTHVAGTVAALDNTTGVLGVAPNVSLYAVKVLNSSGSGSYSG
4gi3.1.A KerA   ................................................S...................
1c3l.1.A SUBTIL ................................................S...................
1yu6.1.A Subtil ................................................S...................

                       220       230       240       250       260       270       280
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MZK05 KerA      IVSGIEWATTNGMDVINMSLGGPSGSTAMKQAVDNAYARGVVVVAAAGNSGSSGNTNTIGYPAKYDSVIA
4gi3.1.A KerA   ......................A.........................................
1c3l.1.A SUBTIL ......................A.............................S..............
1yu6.1.A Subtil ......................A............................N..S..............

                       290       300       310       320       330       340       350
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MZK05 KerA      VGAVDSNSNRASFSSVGAELEVMAPGAGVYSTYPTSTYATLNGTSMASPHVAGAAALILSKHPNLSASQV
4gi3.1.A KerA   ..................................N.................................
1c3l.1.A SUBTIL ..................................N.................................
1yu6.1.A Subtil ..................................N.................................

                       360       370
                ....|....|....|....|....|....
MZK05 KerA      RNRLSSTATYLGSSFYYGKGLINVEAAAQ
4gi3.1.A KerA   ..............................
1c3l.1.A SUBTIL ..............................
1yu6.1.A Subtil ..............................
```

Figure 3.3.10: Alignment of mature protein sequence (274 aa) of *B. licheniformis* strains MZK05 with the highly similar KerA and Subtilisin Carlsberg proteins of other strains.

3.3.7 Protein structure simulation

From the homology modeling results, a total of 317 templates were found to match the target sequence at different similarity index. This list was filtered by a heuristic down to 52 from which 3 models were built based on the maximum sequence coverage, similarity and identity. As to determine the 3D structure from the above mentioned 3 models, the correct one was considered to be similar to Subtilisin Carlsberg (PDB- 1c3l.1.A).

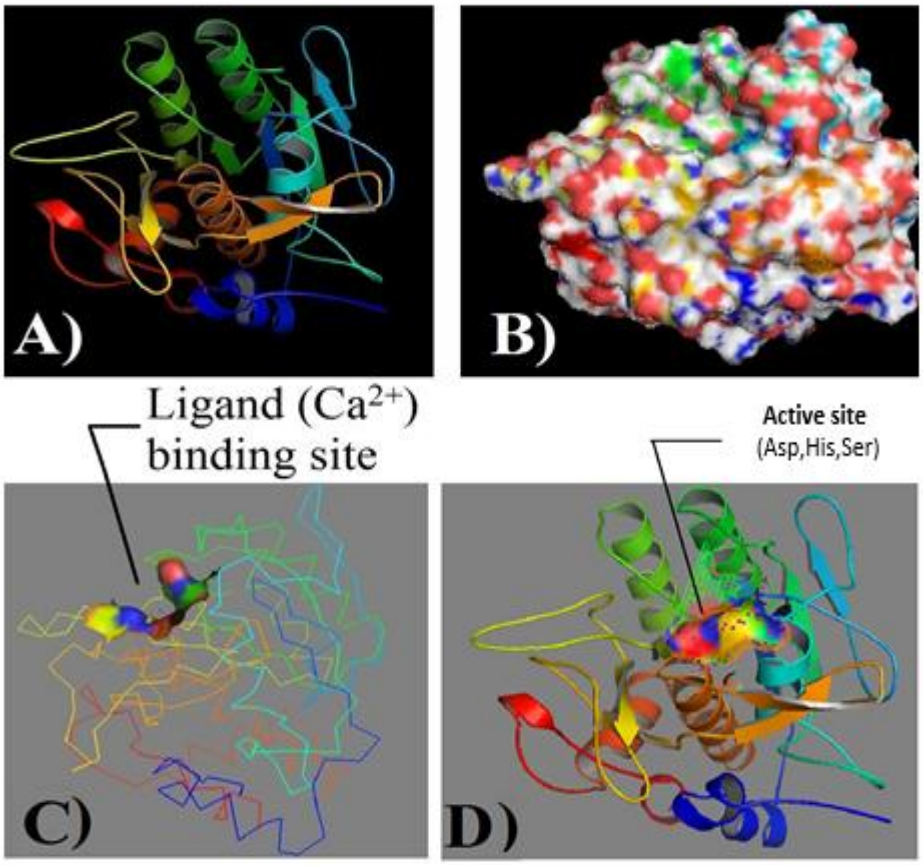

Figure 3.3.11: 3D modeling of KerA protein from *Bacillus licheniformis* MZK-05. A) Overall view of the mature Keratinase enzyme where N-terminal to C-terminal is indicated by rainbow colour (Blue to red). B) Surface of the modeled protein when H-ions are present. C) Ligand (Ca^{2+}) binding site of the protein. D) Active site of the protein (Asp, His, Ser).

There is no disulfide bond in the protein, as presence of Cysteine was not observed in the sequence (Ceroni *et al.*, 2006). There were 6 N- Glycosylation sites in the matured protein sequence which are 76NTTG, 86NVSL, 96NSSG, 122NMSL, 217NGTS and 249NLSA. The structure model and the hydrogen added surface of the protein were shown in Figure 3.3.11 A and Figure 3.3.11 B, respectively. This structure was chosen for its maximum sequence identity and Ca^{2+} binding site (Fig. 3.3.11C) with Subtilisin Carlsberg. Like all subtilisins, three residues at the active site (Fig. 3.3.11D) forming the catalytic triad (Asp-32, His-63 and Ser-220), are also conserved in the keratinase of *B. licheniformis* MZK05.

CHAPTER 4

Cloning, expression and purification of keratinase from *Bacillus licheniformis* MZK05 in pET-30a(+) system: High level expression of keratinase

Cloning, expression and purification of keratinase from *Bacillus licheniformis* MZK05 in pET-30a(+) system: High level expression of keratinase

4.1 Introduction

Microbial keratinases are a group of proteolytic enzymes with the capability to degrade recalcitrant proteins (Brandelli, 2008). Their unique property to attack tough insoluble structural proteins has made them attractive biocatalysts in areas dealing with such substrates e.g. dehairing of hides, textiles and keratin waste management (Gupta and Ramnani, 2006). However, keratinases have gained major impetus with the discovery of prion decontamination by keratinase *Ker*A of *Bacillus licheniformis* PWD-1 by Shih's group in 2003 (Langveld *et al.*, 2003). Since then, the use of keratinases has also been advocated in skin and nail medications (Brandelli *et al.*, 2010; Selvam and Vishnupriya, 2012). Thus, keratinases are increasingly being looked up as a potential solution to address several challenging issues dealing with hard to degrade proteins encountered both in environmental and pharmaceutical sectors.

Keratinases are broadly classified as serine and metalloproteases or serine-metallo-proteases based on their catalytic mechanism (Brandelli, 2008). They have the tendency to adsorb onto insoluble macromolecules and subsequently, cleave highly hydrophobic proteins (Bressollier *et al.*, 1999). By virtue of these exclusive characteristics, keratinases find applications in various biotechnological sectors, especially the ones that require selective cleavage of insoluble proteins over soluble proteins such as feather recycling, feed, pharmaceuticals and prion decontamination (Brandelli *et al.*, 2010). Further, since keratinases are proteases, thus are well suited to take over the hitherto protease-dominant sectors e.g. detergent, leather and textiles. Overall, keratinases have broadened the horizons of protease market especially in sectors where previously proteases had limited applicability. The biotechnological significance of keratinases over traditional proteolytic enzymes has been extensively reviewed several times during the last decade with major emphasis on their diversity, characterization and applications.

The earliest review by Onifade *et al.*, (1998) emphasized on biotechnological potential of keratinases with respect to feather meal production. Gupta and Ramnani (2006) provided a comprehensive over view about the microbial sources, physiology and fermentation mechanism of keratinolysis, biochemical and molecular characterization and prospective industrial applications of keratinases. This was followed by three reviews (Brandelli, 2008; Brandelli *et al.,* 2010; Gupta *et al.*, 2013) that focused on the diversity and properties of keratinases. All these reviews touched upon the potential applications of keratinases while highlighting feather recycling as the major application. Feathers are the major of by-product in poultry production and processing industry, amounting to several millions of tons every year globally (Porres *et al.*, 2002). Although feather meal is a cheap protein source, its use in the animal nutrition and feed industry is still not commercially feasible since keratin, the major component of feather meal, is poorly degraded by most proteinases e.g. trypsin, papain and pepsin (Son *et al.*, 2008). However, the keratin waste can be treated by applying keratinase, one of the important proteolytic enzymes used for hydrolyzing keratins. Keratinases are currently used in leather-processing industry (Lin *et al.*, 1995). The use of keratinase from indigenous *B. licheniformis* strains can be a useful alternative in eco-friendly de-hairing step of hide processing over conventional method (Nahar *et al.*, 2015).

The pET system is the most powerful system for the expression of proteins in *Escherichia coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. The target gene is expressed under the control of strong bacteriophage T7 transcription and translation signals on vector pET-30a(+).

In view of these, the keratinase gene from *B. licheniformis* MZK05 was cloned, expressed in *E. coli* using pET-30a(+) vector and purified by Immobilized metal affinity chromatography (IMAC) process in this chapter.

4.2 Methods

4.2.1 Bacterial strains and growth conditions

Bacillus licheniformis was from our laboratory stock and cultivated at 37°C for 24 h in Luria bertani medium. *Escherichia coli* DH5α and *E. coli* BL21 were grown in Luria-Bertani (LB) medium at 37°C. Whenever needed, ampicillin (50 mg/ml) was added to the medium.

4.2.2 Plasmids

TA vector (pCR2.1, Thermofisher scientific) was employed for cloning PCR products amplified using Taq DNA polymerase which adds adenine (A) to the 3′ end of its PCR products. This improves the sequencing of the gene. A second plasmid called pET-30a(+) was used for the cloning and expression of the gene and this vector was also collected commercially.

4.2.2.1 TA Vector (pCR2.1)

The TA Cloning vector (pCR2.1) provides a quick, one-step cloning strategy for the direct insertion of a PCR product into the vector. The diagram below shows the concept behind the TA Cloning method (Fig. 4.2.1). Map of these vector is mentioned in **Appendix 5.**

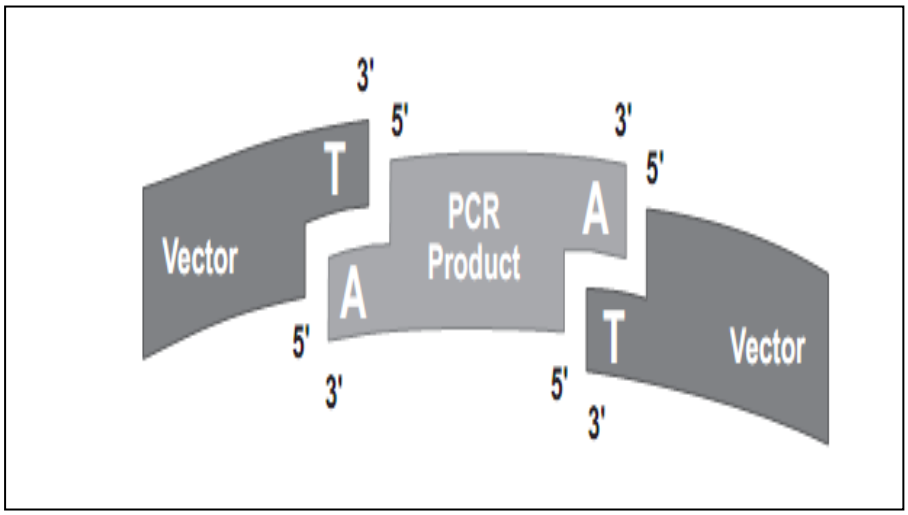

Figure 4.2.1: TA Cloning method

4.2.2.2 pET-30a(+) vector

The expression vector, pET-30a(+) was purchased from Novagen (Germany). The pET system is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. Once established in a non-expression host, plasmids are then transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control and expression is induced by the addition of lactose. This vector was chosen for its unique characteristics: A tac promoter for chemically inducible, high-level expression. An internal lac I^q gene for use in any *E. coli* host. Very mild elution conditions for release of fusion proteins from the affinity matrix, thus minimizing effects on antigenicity and functional activity. Map of these vector is mentioned in **Appendix 5.**

4.2.3 Cloning of *ker*A gene

4.2.3.1 Primers designing

Described in chapter 3, section 3.2.2.2.1.

4.2.3.2 Polymerase chain reaction to amplify *ker*A gene

Described in chapter 3, section 3.2.2.2.2.

4.2.3.3 Agarose gel electrophoresis

Described in chapter 3, section 3.2.2.2.3.

4.2.3.4 Purification of PCR product

Described in chapter 3, section 3.2.2.2.4.

4.2.3.5 Construction of recombinant TA vector

4.2.3.5.1 Preparation of competent cells

50 ml of LB medium was inoculated with a colony of *E. coli* DH5α in a 250 ml flask and incubated in a 37°C orbital shaker at 150 rpm. Some of the overnight culture was poured into 50 ml of fresh LB broth so that the final absorbance should be 0.1. It was then incubated in a 37°C orbital shaker at 150 rpm until the absorbance at 600 nm reached 0.4-0.6. Then, 45 ml culture was transferred to a sterile falcon tube and chilled on ice for 10-15 min. It was centrifuged at 8,000 rpm for 5 minutes at 4°C centrifuge. The supernatant was discarded and the cells were suspended in 22 ml (0.5 volumes) ice cold, sterile 0.1M $CaCl_2$. It was kept on ice for 15-20 minutes and again centrifuged at 8,000 rpm for 5 minutes at 4°C centrifuge. The supernatant was discarded and the pellet was resuspended in 11 ml ice cold, sterile 0.1M $CaCl_2$. It was kept on ice for at least 2 hours. (Transformation should be done immediately or the competent cells should be stored at -70°C by using 15% glycerol broth).

4.2.3.5.2 Ligation reaction

For sticky ligation, the following formula was used to calculate the molar ratio.

$$x = \frac{y \text{ bp of insert} \times \text{amount of vector}}{\text{Size in bp of vector}}$$

Where x ng is the amount of insert of y base pairs to be ligated for a 1:1 (vector: insert) ratio. Fresh PCR products (less than 1 day old) which contain single 3′ A-overhang were used to ligate with the linearized TA vector which contains 3′ T-overhang. Ligation reaction was done at a ratio of 1:1 (vector: insert) as this ratio gives the best efficiency of ligation. Self-ligation reaction was performed at a ratio 1:0 (vector: insert) as a control reaction. The size of insert (y) is 828 bp and the size of vector is ~3900 bp. For 50 ng of the vector, the value of x was found 10.6 ng according to the above formula. The composition of the reaction mixture was given in Table 4.2.1.

Table 4.2.1: Ligation mixture composition for cloning into TA vector

Ingredients	**1:1(vector:insert)**	**1:0(vector:insert)**
10X ligation buffer	1µl	1µl
pCR2.1 vector (25 ng/µl)	2µl	2µl
Fresh PCR product (19 ng/µl)	0.6µl	-
T4 DNA ligase (10U/ µl)	1µl	1µl
Double deionized water	5.4µl	6µl
Total reaction volume	10µl	10µl

The reaction tube was vortexed and then spins down in a microcentrifuge for 3-5 seconds. The reaction mixtures were then incubated overnight at 14°C. After incubation, the reaction mixture was used for transformation.

4.2.3.5.3 Transformation of competent cells with ligation mixture

The competent cells were thawed gently. 2 µl of each of the ligation mixtures were added to 50 µl aliquot of competent cell separately in 1.5 ml fresh microfuge tubes. A positive control with commercial pET-30a(+) plasmid solution and a negative control without any plasmid were used to check the efficiency of the competent cells for transformation. The reaction tubes were incubated on ice for 30 min and then heat pulsed for 45 seconds at 42°C without shaking. The reaction tubes were again replaced on ice for 2 min. Then 250 ml of LB media (preheated to 37°C) was added to each tube and incubated at 37°C for 1 hour with shaking at 225 rpm. After incubation, centrifugation was done at 5000 rpm for 5 min on a tabletop microfuge. Supernatant was discarded leaving ~100 µl to resuspend the pellet. The resuspended solution was then plated on LB agar containing ampicillin. The plates were incubated overnight at 37°C.

4.2.3.5.4 Confirmation of the recombinant TA-kerA vector

Growth of transformants on ampicillin plate was indicative of the insertion of the plasmids into the cells. Colonies from the plate were randomly picked for further screening to confirm that the clone contains recombinant vector DNA with desired insert. It was done by subsequent PCR reaction and restriction digestion of the recombinant vector.

Confirmation by Colony PCR

Colony PCR was done as same method mentioned in in chapter 3, section 3.2.2.2.2. Analysis of PCR products by electrophoresis was done on 1.5% agarose gel according the method described in chapter 3, section 3.2.2.2.3.

Confirmation by restriction digestion

Colonies those gave positive PCR reaction were then grown overnight in 5 ml LB broth containing 100 µg/ml ampicillin. Plasmid extraction was done by using the PureLink™ Quick Plasmid Miniprep Kit, described in chapter 3, section 3.2.2.2.8 and restriction digestion of the recombinant TA vector was performed. Analysis of digested vector was done by electrophoresis on 1.0% agarose gel described in chapter 3, section 3.2.2.2.3.

4.2.3.6 Subcloning of *Ker*A gene into pET-30a(+) vector

4.2.3.6.1 Restriction digestion of pET-30a(+) vector and TA-*Ker*A

Recombinant TA-*ker*A and pET-30a(+) vector were digested separately with both of the selected restriction enzymes, *Bam*HI and *Xho*I to produce molecules with single strand overhangs. Double digestion was carried out using enzymes and respective buffers of TAKARA, JAPAN. Restriction digestion was done subsequently by the both enzymes with a common buffer buffer K in the recommended incubation temperature (for *Bam*HI: 30°C and for *Xho*I: 37°C) overnight. Composition of the reaction mixtures are given in Table 4.2.2.

Table 4.2.2: Composition for digestion of pET-30a(+)vector and recombinant TA vector by *Bam*HI and *Xho*I

Ingredients	**Amount (μl)**	**Ingredients**	**Amount (μl)**
pET-30a(+)vector (55 ng/μl)	20 μl	Recombinant TA vector (300 ng/μl)	10 μl
10× K buffer and 10× H buffer	3 μl	10× K buffer and 10× H buffer	2μl
*Bam*HI (1U/μl) and *Xho*I(1U/μl)	2μl	*Bam*HI (1U/μl) *Xho*I(1U/μl)	4 μl
ddH_20	5 μl	ddH_20	4 μl
Total volume	30 μl	Total volume	20 μl

After completing the reaction, restriction digestion was confirmed by the demonstration of the digested band on the agarose gel and digested insert was purified by PCR purification kit.

4.2.3.6.2 Ligation reaction

The single stand overhangs of the gel purified product are complementary to those of the pGEX-6p-2 vector. The formula of ligation is described in detail in the section 4.2.3.5.2. Ligation reaction was done both at ratio of 1:1 and 1:3 (vector: insert). Ligation reaction at a ratio 1:0 (vector: insert) was done as a negative control which demonstrates successful digestion of the vector pET-30a(+). The size of insert (y) was 1156 bp and the size of vector is ~5400 bp. For 80 ng of the vector, the value of x was found 13.5 ng according to the formula. The composition of the reaction mixture was given in Table 4.2.3.

Table 4.2.3: Ligation mixture composition for cloning into pET-30a(+)

Ingredients	**1:1 (vector: insert)**	**1:3 (vector: insert)**	**1:0(vector: insert)**
10X ligation buffer	2µl	2µl	2µl
Digested vector (40 ng / µl)	5µl	5µl	5µl
DNA inserts (24 ng/ µl)	0.5µl	1.5µl	0
T4 DNA ligase (5U/ µl)	1µl	1µl	1µl
Double deionized water	11.5µl	10.5µl	12µl
Total reaction volume	20µl	20µl	20µl

The reaction tube was vortexed and then spins down in a micro centrifuge for 3-5 seconds. The reaction mixtures were then incubated overnight at 14°C. After incubation, the reaction mixture was used for transformation.

4.2.3.6.3 Transformation

2 µl of each of the ligation mixtures were added to 50 µl aliquot of competent cell separately in fresh eppendorf tubes. A positive control with commercial pET-30a(+) plasmid solution and a negative control without any plasmid were used to check the efficiency of the competent cells for transformation.

4.2.3.6.4 Confirmation of the recombinant kerA vector

Described in 4.2.3.5.4

4.2.4 Heterologous expression of the recombinant protein in *E. coli*

4.2.4.1 Transformation of *E. coli* BL21 with recombinant pET-30a(+) plasmid

2 µl of each of the ligation mixtures were added to 50 µl aliquot of competent cell separately in 1.5 ml fresh microfuge tubes.

A positive control with commercial pET-30a(+) plasmid solution and a negative control without any plasmid were used to check the efficiency of the competent cells for transformation. The procedure for transformation of the vector into the bacterial cell has been described in section 3.2.2.2.7.

4.2.4.2 Expression of recombinant pET-30a(+)-kerA

Escherichia coli BL21 carrying pET-30a(+)-kerA was cultured overnight at 37°C in LB medium containing ampicillin (100 µg/ml) and was used as inoculum to start lactose induced protein expression. Fresh LB ampicillin medium was inoculated with the overnight culture to start with an OD_{600nm}= 0.1 and incubated at 37°C with shaking at 200 rpm. Lactose was added aseptically in the culture when the OD_{600nm} reached 0.5 at a concentration of 0.3 mM.

To optimize the induction time, 1 ml of culture from each flask was collected aseptically at 1 hour interval up to 5 h. Then the cells were harvested by centrifugation at 10,000 X g for 10 min at 4°C and resuspended in 100 µl of 1X Laemmli buffer to denature the protein incubating at 95°C for 5 min. Equal amount of protein from each lactose concentration and each time interval was then analyzed by SDS-PAGE in a 10% separating gel as described in chapter 3, section 3.2.2.3.3.

4.2.5 Enzyme-substrate reaction on SDS PAGE gel

After electrophoresis the gel was incubated in NaOH- glycine buffer (PH 8.5) containing 1% Triton X-100 with shaking for 30 min to allow renaturation of proteins (Lin *et al.*, 1997). The gel was then washed twice with 100 ml of NaOH- glycine buffer, immersed into the same buffer containing azo casing dye for 1 h and then it was analyzed by SDS-PAGE in a 10% separating gel as described in chapter 3, section 3.2.2.3.3.

4.2.6 Purification of recombinant keratinase

The liquid culture (50 ml) was transferred and harvested by centrifugation at 7700×g for 10 min at 4^{o}C and washed once with deionized water. Then it was resuspended in 40 ml lysis buffer by sonicating the suspension on ice for 8x 30 sec. The lysate was incubated overnight at room temperature rolling on a roller. In the following morning, the cell debris was separated by centrifugation at 10000 rpm for 30 min in a cold rotor.

The cleared supernatant was mixed with 1 ml Ni^{2+}-NTA resin at room temperature for 30 min on a rotating platform. Then it was transferred to a column, protein materials were collected as the flow-through material. Then it was washed twice with 10 ml of lysis buffer. Then proteins were renatured in 10 ml washes with wash buffer and centrifuged at 7700×g for 1 min. Then eluted from the column in 1 ml fraction of elution buffer. KerA protein was finally collected very carefully following centrifugation at 500×g for 5 min and was analyzed by SDS-PAGE. After purification, low molecular weight proteins were purified by 10 KD centricon tube. The sample reservoir was firmly placed into the filtrate receiver. 500 µl of the sample was pipette into the sample reservoir. Then the filtration device was place into a fixed-angle centrifuge rotor. The sample was centrifugated at 14,000 x g for 12 mins. Samples were analyzed by SDS-PAGE and western blotting.

Western blotting

After gel electrophoresis, the membrane (pore size 0.2 µm, Protran, Bioscience) of equal size of the gel was rinsed in transfer buffer together with 4 pieces of blotting paper (Whatman 3 mm), the gel and the sponge. Placing the black side of the cassette down, the followings were placed one after another: the sponge, two blotting papers, gel, the membrane, two blotting papers. Before putting the other sponge, a tube was gently rolled on top of the blotting papers to release any air, if trapped in between the gel and the membrane. The cassette was closed, placed vertically in the tank (black part behind, flat part top) along with an ice-bar. Pour transfer buffer into the tank and place the tank onto a magnetic stirrer. Transfer was achieved by 100 volt/60 min. The ice-bar was replaced after 30 min. The strip was destained with water. The blot was washed with 2X TBS at room temperature each for 5 min to equilibrate the blot with the buffer. The membrane was block with blocking solution for 1 hour at $4^{o}C$ with gentle shaking. The blocked membrane was placed in the primary antibody (6x-His Tag) solution. It was rocked at $4^{o}C$ for 2 hours. The blot was washed 3 times briefly in deionized water at room temperature. Then the secondary antibody (Goat Anti-Mouse IgG+ IgM) was applied. It was rock at $4^{o}C$ for 45 min. BCIP/NBT solution was added as substrate then gel was visualized.

4.2.7 Keratinase assay

Keratinase activity of the purified KerA was determined by a modified method described by Kreger and Lockwood (1981) using azo-casein (Sigma, USA) as the substrate. Briefly, 400 µL of appropriate dilution of KerA was added to 400 µL of 1% azo-casein solution (suspended in 0.05 M Tris- HCI; pH 8.5) and the mixture was incubated at 37°C for 1 h. The reaction was terminated by adding 135 µL of 35% trichloroacetic acid (TCA) and left for 15 min on ice followed by centrifugation at 13,000 rpm, at 4°C for 10 min. Then, 750 µL of the supernatant was neutralized with equal volume of freshly prepared 1.0 N NaOH by gentle mixing. Absorbance (OD_{440nm}) of the mixture was then measured in a UV spectrophotometer (Thermo spectronic Genesys, USA) keeping the solution from a parallel reaction as blank where TCA was added before the enzyme. One unit of protease activity was defined as the amount of enzyme required to yield an increase in absorbance (OD_{440nm}) of 0.01 in 1 h at 37°C.

4.3 Results

4.3.1 Cloning of the *ker*A gene into TA vector

A 1,156 bp keratinase gene was amplified from the *B. licheniformis* DNA (Fig. 4.3.1 a) with designed primers and cloned into TA cloning vector (Fig. 4.3.1 b). The TA Cloning vector (pCR2.1) provides a quick, one-step cloning strategy for the direct insertion of a PCR product into the plasmid vector. This improves the sequencing of the gene whereas sequencing from the purified product may result in incorrect sequencing due to presence of unwanted products.

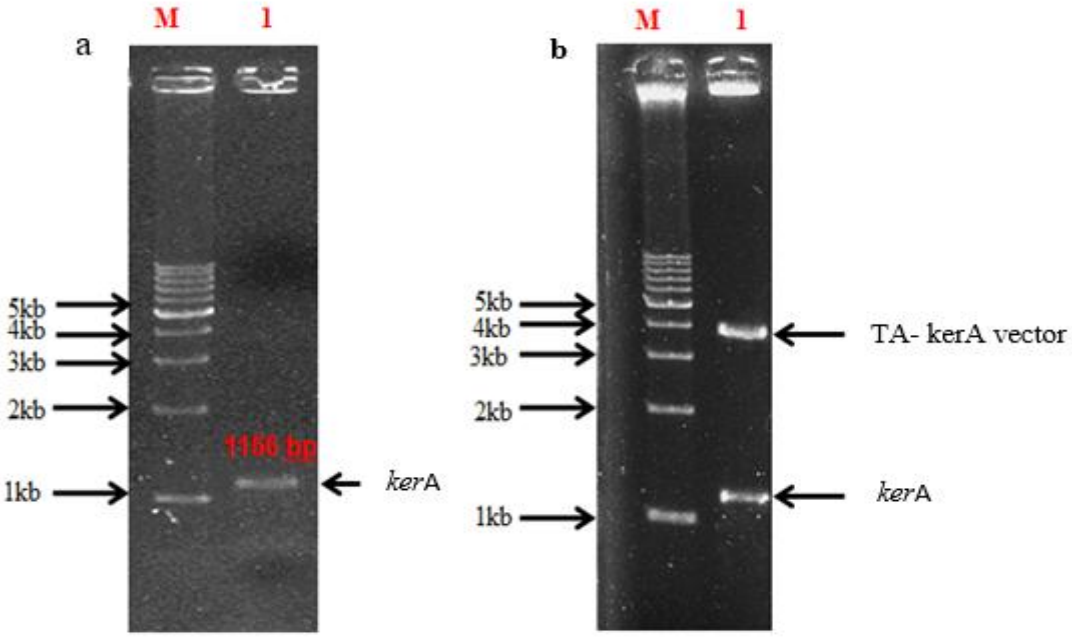

Figure 4.3.1: Agarose gel electrophoresis of cloning of the *ker*A gene into TA vector. (a) Amplification of DNA fragment encoding the keratinase gene. Lane M: DNA markers, lane 1: 1156 bp PCR product. (b) Restriction enzyme digestion map. Lane M: DNA markers, lane 1: TA-kerA vector.

4.3.2 Construction of Recombinant pET-30a(+)-kerA vector

Proteins difficult to purify can be subjected to heterologous expression by cloning the gene that encodes the protein into an expression vector. Heterologous expression improves the purification of the protein. The recombinant protein can then be investigated for a particular biological activity (enzymatic activity, signaling capacity).

4.3.2.1 Transformation

After overnight incubation of the transformed cells in ampicillin LB agar plate the following observations were found. No colony was found in the 1:0 ligation mixture plates (Fig. 4.3.2 A) which indicate successful double digestion of the vector pET-30a(+). In case of 1:1 ligation mixture plate (Fig. 4.3.2 B) and a number of 7 colonies were found and 1:3 ligation mixture plate (Fig. 4.3.2 C) contained the maximum number of colonies. These colonies from both plates were subjected to further analysis.

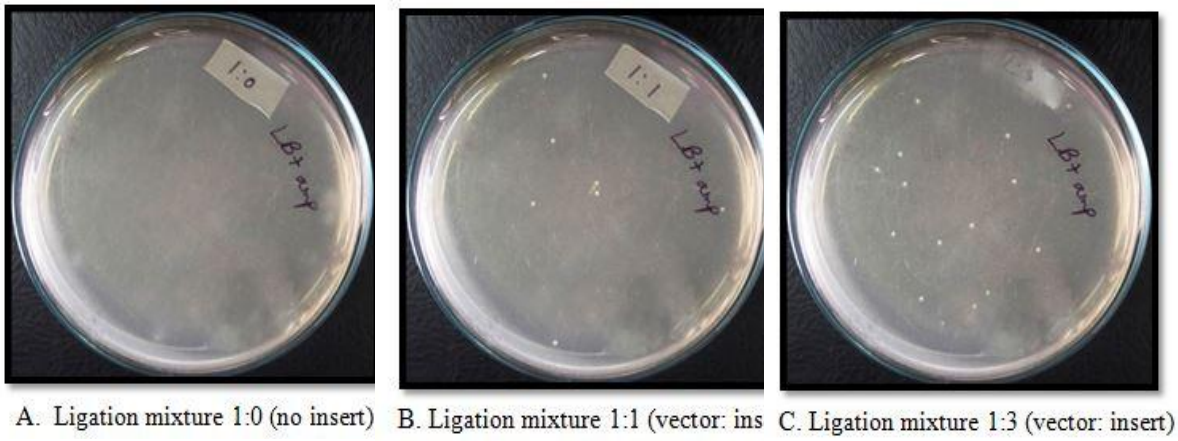

Figure 4.3.2: Ampicillin LB plates showing transformants. A: No colony on the 1:0 ligation mixture plate, B: 7 colonies on 1:1 ligation mixture, C: 15 colonies on 1:3 ligation mixture plate.

4.3.2.2 Confirmation and restriction digestion of the recombinant pET-30a(+)-kerA

PCR was done with randomly selected colonies from the plates containing transformants. For the recombinants, PCR products were found which were compared with the positive control. Same band size like the insert (1156 bp) is the indication of positive result (Fig. 4.3.3 a). To confirm the insertion of the keratinase gene, the recombinant plasmid pET-30a(+)-kerA was extracted and digested by *Bam*HI and *Xho*I. The molecular mass of *Bam*HI and *Xho*I double-digested DNA fragment in the positive clones was the same as the PCR product (Fig. 4.3.3 b), indicating the main keratinase gene was successfully cloned into plasmid pET-30a(+).

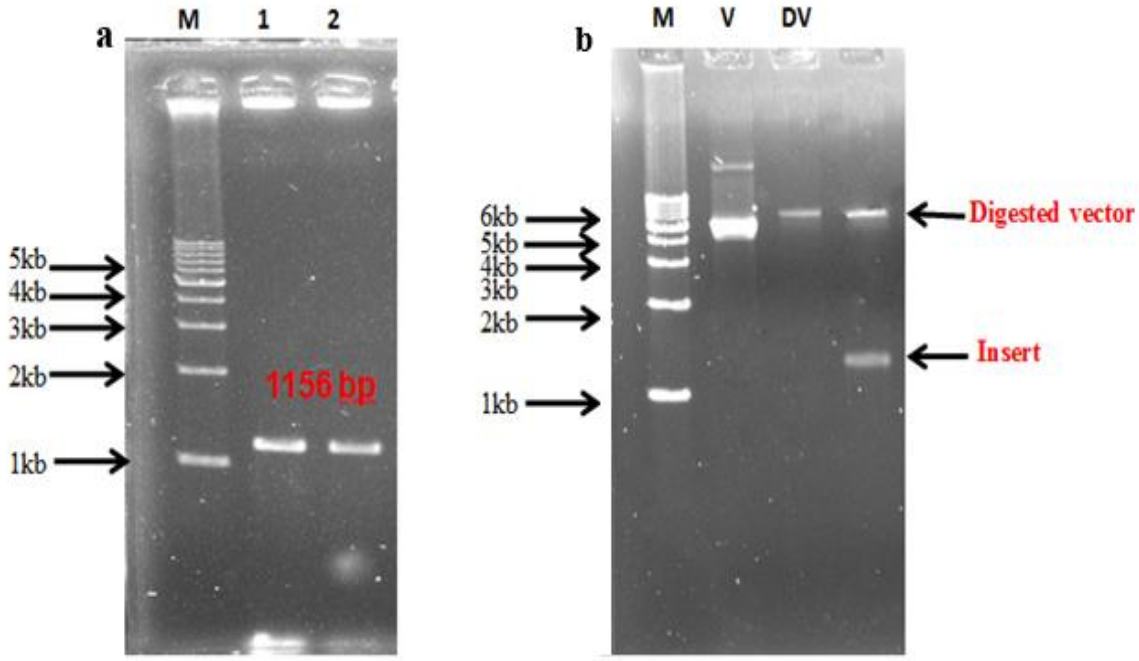

Figure 4.3.3: Confirmation and restriction digestion of the recombinant pET-30a(+)-kerA. a. Confirmation of the clones harboring pET-30a(+)-kerA recombinant vector by PCR amplification for *ker*A gene. b. Double digestion of both pET-30a(+) and pET-30a(+)-kerA vector produced vector in linear form and vector as well as insert respectively.

4.3.3 Expression of recombinant keratinase

The pET-30a(+)-kerA was transformed into *E. coli* BL21 and the expressed products analyzed by SDS-PAGE. The optimum time for maximum expression of the desired protein was found to be 3 h with 2 mM lactose concentration (Fig. 4.3.4) and produces a band with MW of 40 kDa respectively

4.3.4 Enzyme-substrate reaction on keratinolytic activity

The activity staining of keratinase produced by transformed *E. coli* BL21 is shown in figure 4.3.5. The 10% SDS PAGE gel was stained for keratinolytic activity (clear band) and subsequently with coomassie brilliant blue for proteins. Proteins having keratinolytic activity appear as clear bands on a dark background (Fig. 4.3.5).

Keratinolytic activity was found with or without lactose induction. Time required for maximum protein expression at 2 mM lactose.

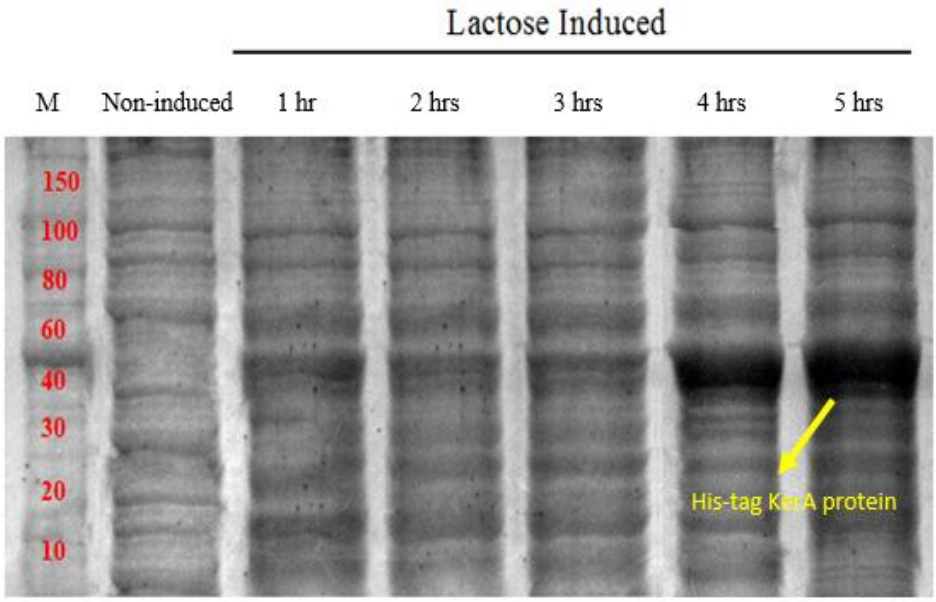

Figure 4.3.4: Time course study of His-tag KerA protein. Lane1: Marker, Lane 2: Non-induced, Lane 3-5: Different time interval (1-5hrs) for maximum protein expression.

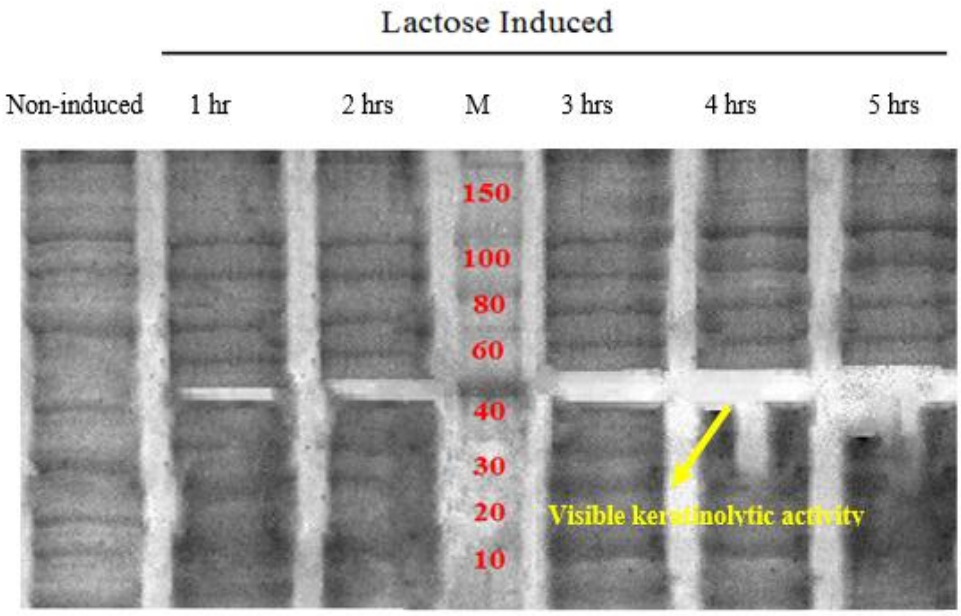

Figure 4.3.5: keratinolytic activity of kerA protein in enzyme-substrate reaction on SDS PAGE Lane 1: Non-induced, Lane 2,3,6-7: Different time interval (1-5hrs) for maximum protein expression and Lane 4: Marker.

4.3.5 Purification of recombinant keratinase

The crude recombinant protein was successfully purified using affinity chromatography on Ni^{2+}-NTA resin. But some low molecular weight protein bands were observed and it was purified by 10 KDa centricon tube. Low molecular weight proteins separated and only one clear band with a MW of approx. 39 kDa was observed on SDS-PAGE (Fig. 4.3.6.a) and for further confirmation, western blot was done. Same 39 KDa KerA protein was observed (Fig. 4.3.6.b).

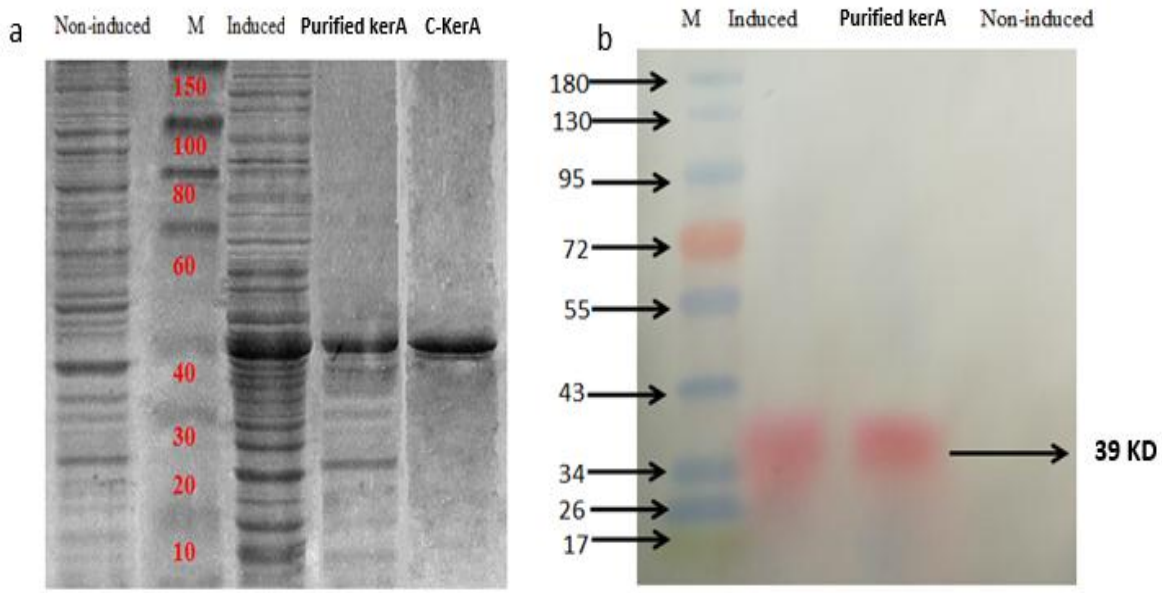

Figure 4.3.6: Purification of recombinant His-tag KerA protein. a.SDS PAGE analysis of recombinant KerA protein. Lane 1: Non induced, Lane 2: Marker, Lane 3: Induced, Lane 4: purified KerA protein; Lane 5: C- KerA: KerA protein after purified by Centricon (10-30 KDa) tube. b. Western blotting analysis of purified protein; Lane 1: Marker, Lane 2: Induced, Lane 3: Purified KerA protein and Lane 4: Non induced.

4.3.6 Keratinase assay

The keratinase activity was determined of the recombinant purified keratinase and wild MZK05 and the activity found to be 358 U/ml and 78 U/ml (Fig. 4.3.7). The recombinant keratinase showed 4.5 fold higher keratinase activity respectively than that of wild strain. The yield of purified protein was approximately 145 mg/L.

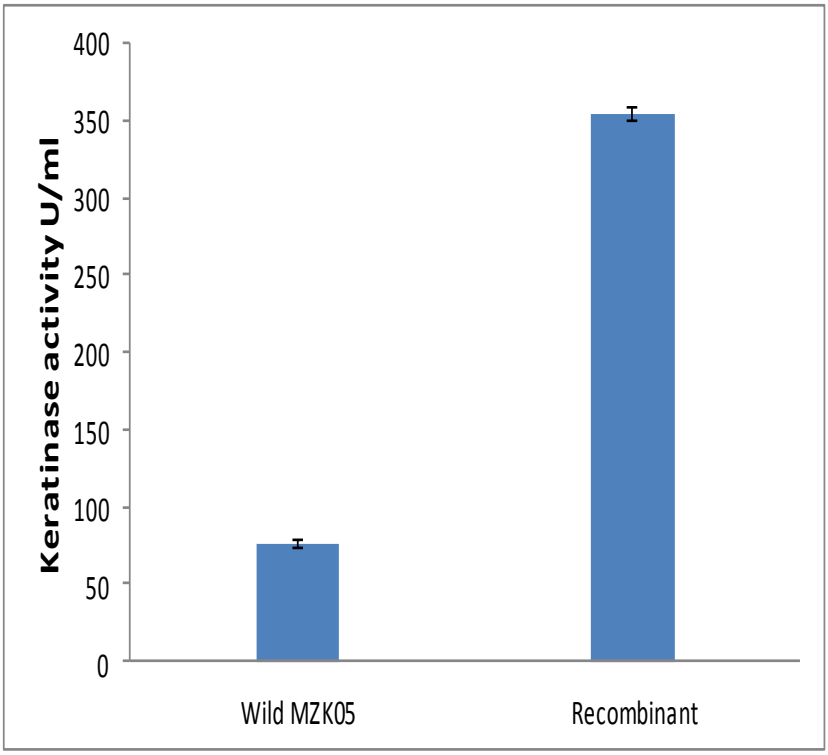

Figure 4.3.7: Comparison of yield in enzyme activity between crude and recombinant protein.

CHAPTER 5

Characterization of recombinant keratinases and whole genome sequencing of *Bacillus licheniformis* MZK05

Characterization of recombinant keratinases and whole genome sequencing of *Bacillus licheniformis* MZK05

5.1 Introduction

Keratinases are proteolytic enzymes in nature and have 97% sequence homology with alkaline protease and it is also inhibited by the same inhibitor that inhibits serine protease. Keratinases are produced only in the presence of keratin containing substrates. Microbial keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide "keratin". Commonly known proteolytic enzymes trypsin, pepsin and papain do not attack on the disulfide (-S-S-) bond of the keratin substrate. Keratinases from microorganisms have many applications in the feed, fertilizer, detergent, leather and pharmaceutical industries (Gupta and Ramnani, 2006). For example, the feather hydrolysates of *Bacillus licheniformis* PWD-1 and *Vibrio* spp. strain kr2 (Williams *et al.*, 1991; Grazziotin *et al.*, 2006) can be used as animal food. Indeed, addition of the crude keratinase from *B. licheniformis* PWD-1 improved the poultry growth (Odetallah *et al.*, 2003). Also, the keratinase can degrade the infectious form of prion, PrP^{sc}, in the presence of detergents and heat treatment (Langeveld *et al.*, 2003), which could be important for the utilization of animal meal as feed. As for leather industry, the keratinase from *Bacillus subtilis* S14 exhibits remarkable dehairing capabilities (Macedo *et al.*, 2005) without the degradation of collagen and this ecofriendly dehairing approach shows great utilization potential. Characterization and purification of an enzyme is highly important in its potential uses in industrial process as the aptness of an enzyme depends on its unique characteristics such as optimum temperature, pH, mode of action etc. (Zhang *et al.*, 2011).

B. licheniformis is extremely important for commercial processes for heterologous exoenzymes as they frequently exhibit higher enzyme yields than *B. subtilis* (Schallmey *et al.*, 2004). *B. licheniformis* belongs to the *B. subtilis* group (group II) of the genus *Bacillus* together with other well known species whose complete genome sequence has been determined.

These are *Bacillus anthracis* (Read *et al.*, 2003), *Bacillus cereus* (Ivanova *et al.*, 2003; Rasko *et al*., 2004), *Bacillus thuringiensis*, the alkaliphilic species *Bacillus halodurans* (Takami and Horikoshi, 2000; Takami *et al*., 2000) and *B. subtilis* (Kunst *et al*., 1997).

Whole genome sequencing of *Bacillus licheniformis* revealed several releasing genes both from inorganic and organic sources, which might be important to deal with both organic and inorganic rich environments. Presence of various transporter genes in both the genome would help in the transport of organic acids (for phosphorus solubilization) and phosphorus across cell walls.

Thus in this study the purified keratinase enzyme of recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA were characterized, which would allow for further improvements in the potential industrial applications of keratinase and the whole genome sequencing of *Bacillus licheniformis* MZK05 was done for molecular characterization.

5.2 Methods

5.2.1 Characterization of purified keratinase enzyme

5.2.1.1 Effect of temperatures on enzyme activity and stability

The optimum temperature for purified recombinants pGEX-6P-2-kerA or pET-30a(+)-kerA were measured by incubating the enzyme with 1% (w/v) azocasein as a substrate at various temperature ranging from 20^{o}C to 70^{o}C for 60 min.

The temperature stability of purified recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA were determined by pre-incubating the enzyme in various temperatures ranging from 20^{o}-70^{o}C for 60 min. After incubation, the enzyme was assayed by incubating with 1% azocasein as substrate at 37^{o}C and the relative keratinase activity was determined. The untreated enzyme was treated as control.

5.2.1.2 Effect of pH on keratinolytic activity and stability of the keratinase

The optimum pH of the purified recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA were determined with 1% (w/v) azocasein as substrate in different buffers (potassium phosphate, pH 6-7.5; Tris-HCl, pH 8.0-9.5 and Glycine, pH 10-11). The enzymes were incubated with substrate of different pH and incubated at 37^{o}C for 1 hr for the determination of enzyme activity.

The pH stability of purified recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA were determined by pre-incubating the enzyme in various buffers (potassium phosphate, pH 6-7.5; Tris-HCl, pH 8.0-9.5 and Glycine, pH 10-11) for 1 hr at room temperature. After incubation, the enzyme was assayed by incubating with 1% azocasein as substrate at 37^{o}C and the relative keratinase activity was determined. The untreated enzyme was treated as control.

5.2.1.3 Effect of various metal ions on keratinase activity

To detect the effect of various metal ions (Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}) on the keratinolytic activity on recombinants purified enzymes, metal salt solutions were prepared in a concentration of 5 mM and one ml of metal solution was mixed with 5 ml of crude enzyme. The solutions were incubated for 1 h. After pre-incubation the enzyme assay was carried out under standard assay condition.

5.2.1.4 Effects of various inhibitors on keratinase activity

The effect of various enzyme inhibitors on keratinase activity was studied using ethylenediaminetracetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT). The purified keratinase enzymes pGEX-6P-2-kerA and pET-30a(+)-kerA were incubated with each inhibitor (5 mM) for 60 min at room temperature and then the remaining enzyme activity was determined under standard conditions. The activity of the enzyme without any inhibitor was taken as control.

5.2.2 Whole genome sequencing of *Bacillus licheniformis* MZK05

5.2.2.1 Genomic DNA extraction

Bacillus licheniformis strain was grown in LB broth and plasmid extraction was done using GeneJET Genomic DNA Purification kit. The procedure is described here.

Bacterial cells were harvested up to $2x10^9$ in a 1.5 or 2 ml microcentrifuge tube by centrifugation for 10 min at 5000 x g. The supernatant was discarded. The pellet was resuspend in 180 µl of lysis buffer. Incubated for 30 min at 37°C. 200 µl of lysis solution and 20 µl of Proteinase K was added. Mixed thoroughly by vortexing or pipetting to obtain a uniform suspension. The sample was incubate at 56°C while vortexing occasionally or used a shaking water bath, rocking platform or thermo mixer until the cells are completely lysed (~30 min). 20 µl of RNase A solution was added, mixed by vortexing and the mixture was incubated for 10 min at room temperature. 400 µl of 50% ethanol was added and mixed by pipetting or vortexing. The prepared lysate was transfered to a GeneJET genomic DNA purification column inserted in a collection tube. The column was centrifuged for 1 min at 6000 x g. The collection tube was discarded containing the flow-through solution. The GeneJET genomic DNA purification column was placed into a new 2 ml collection tube (included). 500 µl of wash buffer I (with ethanol added) was added. Centrifuged for 1 min at 8000 x g. The flow-through was discarded and the purification column was placed back into the collection tube. 500 µl of wash buffer II (with ethanol added) was added to the GeneJET genomic DNA purification column and centrifuged for 3 min at maximum speed (≥12000 x g).The collection tube containing the flow-through solution was discarded and the GeneJET genomic DNA Purification column was transferred to a sterile 1.5 ml microcentrifuge tube.

200 µl of elution buffer was added to the center of the GeneJET genomic DNA purification column membrane to elute genomic DNA. Incubated for 2 min at room temperature and centrifuged for 1 min at 8000 x g.

5.2.2.2 Measurement of DNA concentration and purity

DNA concentration and purity was measured using NanoDrop 2000 spectrophotometer produced by Thermo SCIENTIFIC. 1 µl of nuclease free water or buffer in which DNA was eluted was used as blank. Another 1 µl of sample was loaded and DNA concentration was shown at ng/µl unit. The OD260/0D280 ratio was also shown with the software indicating the purity of the sample. Pure DNA preparations have OD260/OD280 values of 1.8 (Maniatis *et al.*, 1982). Concentration of double stranded DNA is calculated according to the following formula:

Concentration of ds DNA (µg/ml) = OD reading x dilution factor x 50 (an OD value 1 corresponds to approximately 50 µg/ml of purified ds DNA)

5.2.2.3 Electrophoretic analysis of plasmids

The plasmids were checked by horizontal electrophoresis in 0.7% agarose slab gel in Tris-borate EDTA (TBE) buffer. Agarose was dissolved in 1X Tris borate EDTA buffer to give a final concentration of 0.7% agarose and was heated to dissolve in a microwave oven for about 1 minute and then allowed to cool down to about 50°C. Then, 1µl of Ethidium bromide (EtBr) per 50 ml gel from 10 mg/ml stock was added. Then it was poured on the tray previously set with the comb and allowed to solidify. 5µl aliquot of the product was mixed 1µl of loading dye, (6X MBI fermentas) and was loaded into the individual well of the gel. 1kb marker (Invitrogen, USA) was used as molecular size standards. DNA bands were observed on a UV transilluminator (Gel Doc, Bio Rad, USA). Photographs were taken using Gel Doc (Bio Rad, USA) machine attached to a computer and bands were analyzed with 'Alpha Imager' software (Bio Rad, USA).

5.2.2.4 Sequencing and data analysis

Whole genome sequencing of *Bacillus licheniformis* MZK05 strain was performed using Illumina MiSeq technique (BGI; Shenzhen, China). Sequencing data of whole genome were processed and assembled by SPAdes-St. Petersburg genome assembler (SPAdes3.11). Annotation was done by rapid prokaryotic genome annotation tool Prokka (http://github.com/tseumann/prokka) (Seemann, 2014). Genomic similarities of *Bacillus licheniformis* MZK05 with other *Bacillus licheniformis* was predicted by NCBI blast. Circular genome map was designed by prokaryotic genome comparisons tool- Blast Ring Image Generator (BRTG) (Alikhan *et al.*, 2011). tRNA genes and rRNA operons were identified by the tRNAScan and RNAmmer software programs, respectively (Li *et al.*, 2009; Lowe *et al.*, 1997). Subsystem category distribution of *Bacillus licheniformis* MZK05 annotation was done by RAST server (Overbeek *et al.*, 2014).

5.3 Results

5.3.1 Characterization of the recombinant keratinase

5.3.1.1 Effect of temperature on activity of the keratinase

Both purified enzymes of pGEX-6P-2-kerA or pET-30a(+)-kerA were active in a large temperature range with an optimum at 50^{o}C. The relative activities of pGEX-6P-2-kerA and pET-30a(+)-kerA at 50^{o}C were about 83% and 74% respectively (Fig. 5.3.1).

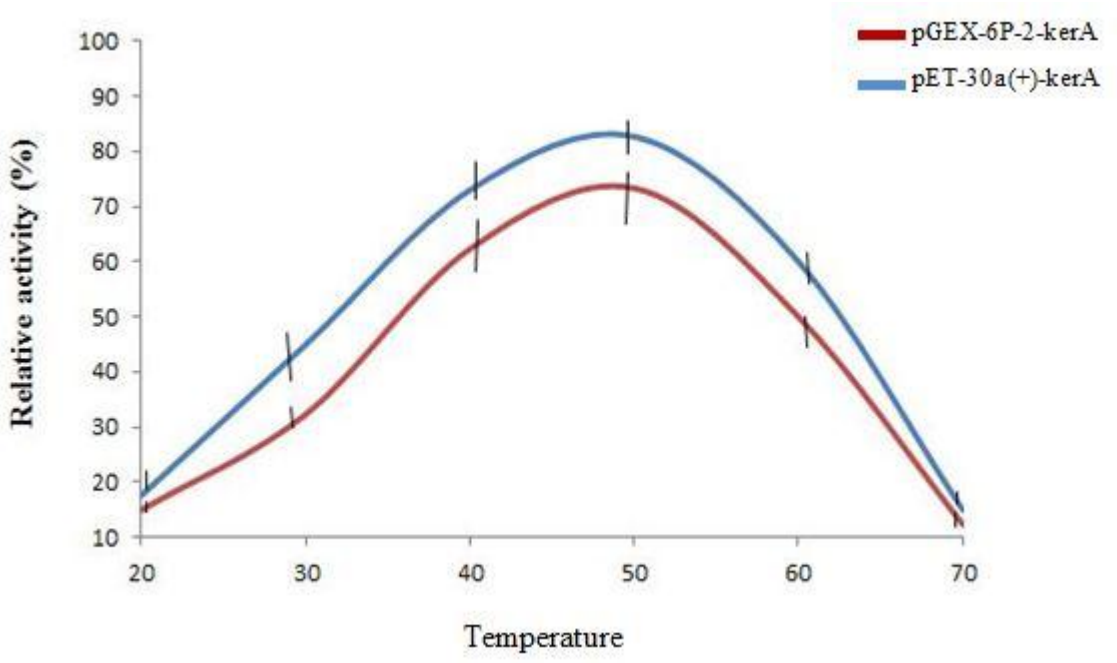

Figure 5.3.1: Temperature activity of the keratinase by the recombinants

5.3.1.2 Effect of temperatures on enzyme stability

Both purified enzyme of pGEX-6P-2 kerA and pET-30a(+)-kerA showed different temperature stability at various temperatures (20-70^{o}). Results indicate that the both enzymes were stable at 40^{o}C without loss of any activity. After 40^{o}C, it began to decrease (Fig. 5.3.2).

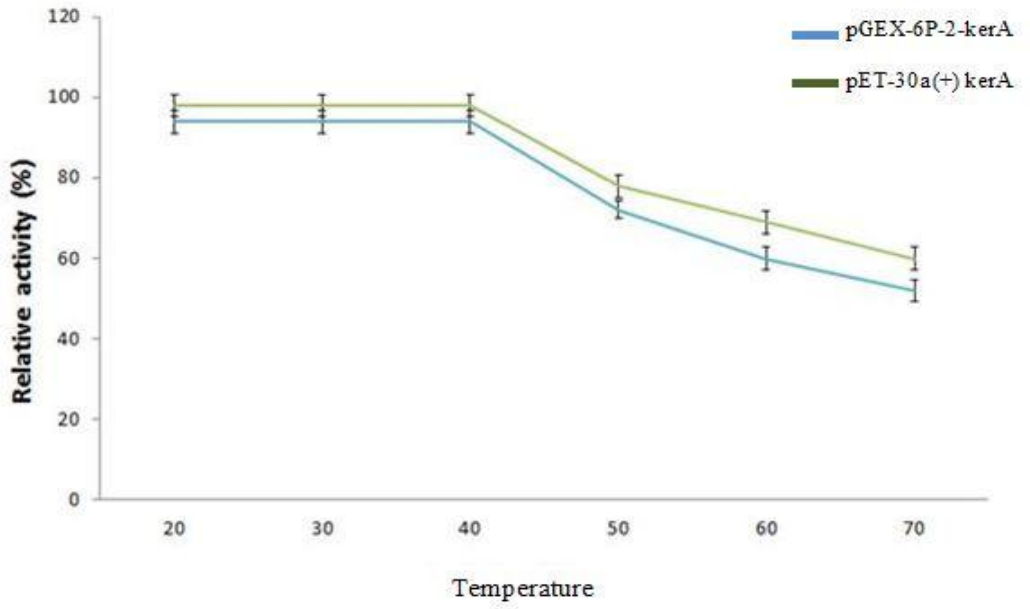

Figure 5.3.2: Temperature stability of keratinase by the recombinants

5.3.1.3 Effect of pH on activity of the keratinases

The purified enzymes pGEX-6P-2-kerA or pET-30a(+)-kerA were found highly active between pH 7.0 and 9.0 with an optimum pH 8.0, indicating its alkaline nature. The relative activities of pGEX-6P-2-kerA and pET-30a(+)-kerA at pH 8.0 were about 85% and 72% respectively (Fig. 5.3.3).

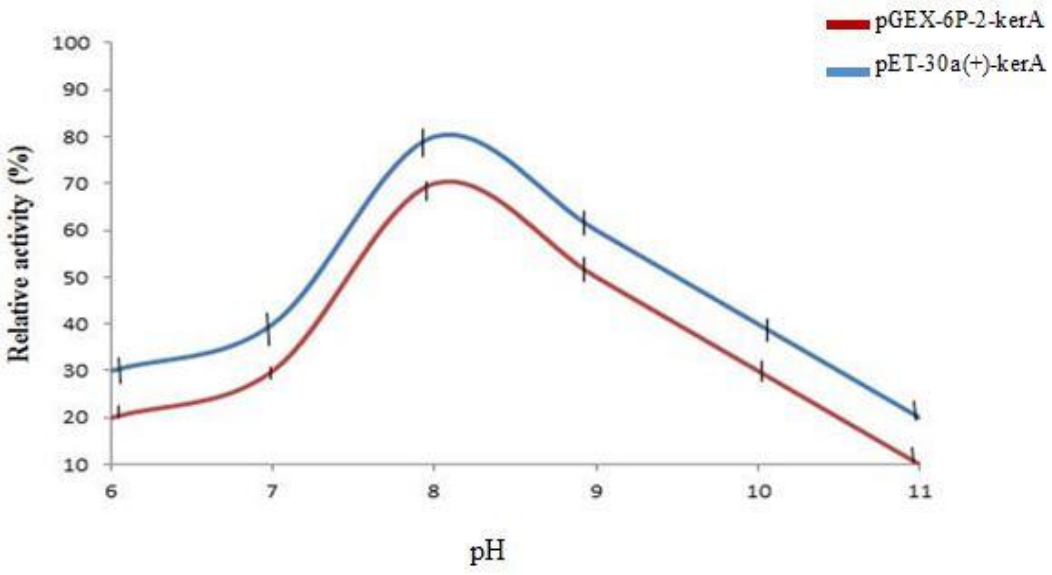

Figure 5.3.3: pH activity of the keratinase by recombinants

5.3.1.4 Effect of pH on stability of recombinant keratinase

The pH stability profile of keratinase showed that both the recombinants pGEX-6P-2-kerA or pET-30a(+)-kerA were stable between pH 6-8. After PH 8, it began to decrease (Fig. 5.3.4).

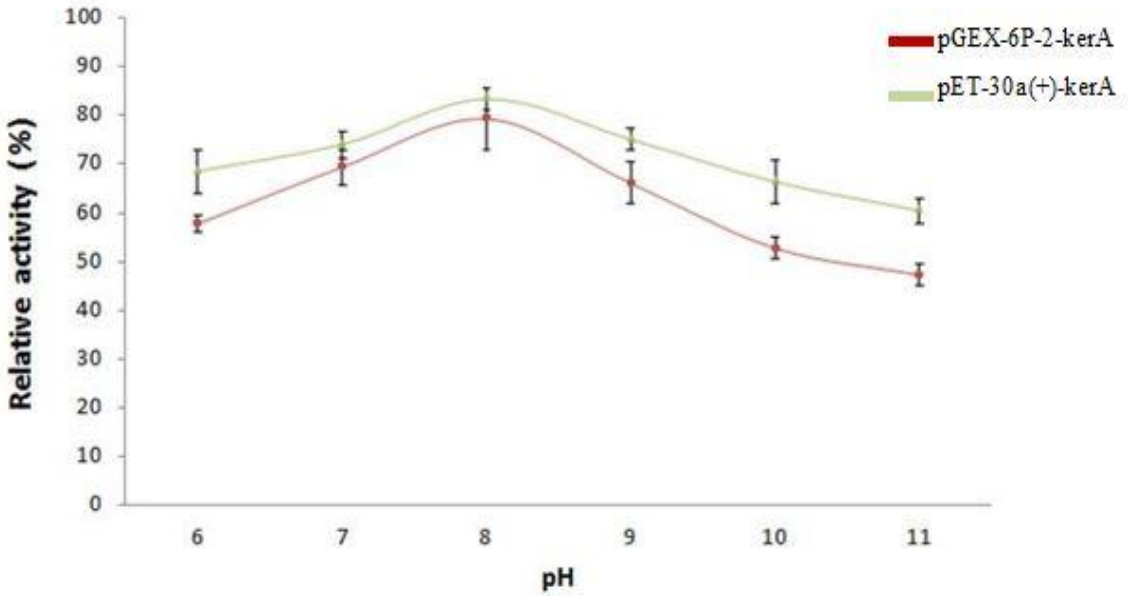

Figure 5.3.4: pH stability of recombinants keratinase enzyme

5.3.1.5 Effect of metal ions on recombinant keratinase

To detect the effect of various metal ions (Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}) on the keratinolytic activity of the enzymes, the same were pre-incubated in different metal salt solutions and then was assayed by a procedure described by azo-casein digest method. The results obtained that the both the enzymes showed comparable pattern with an increased activity in presence of Ca^{2+}, Mg^{2+} and Mn^{2+}. Whereas lower the activity was demonstrated in presence of Co^{2+}, Zn^{2+} and Cu^{2+}. Metal like Co^{2+} restored the enzyme activity of the recombinant strains as 66% and 61.50% while considering the control as having 100% activity. Whereas Zn^{2+} and Cu^{2+} showed an appreciable inhibitory effect on the enzyme with activity of 42.50% and 39.24% respectively (Fig. 5.3.5).

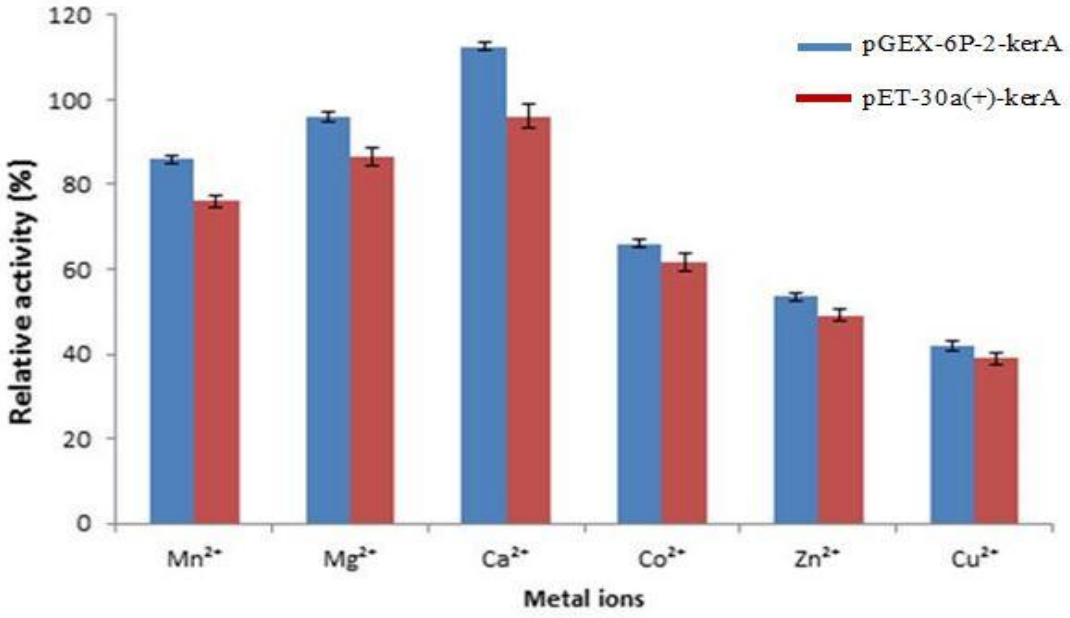

Figure 5.3.5: Metal ions effect of the recombinant keratinases

5.3.1.6 Effects of inhibitors on the keratinase activity

The specific influence of inhibitors (EDTA, PMSF, DTT) on the keratinase activity was determined by using inhibitors of a recommended concentrations (5 mM) and data obtained showed that enzymes were strongly inhibited 73% for pET-30a(+)-kerA and 62% for pGEX-6P-2-kerA by PMSF. On the other hand, EDTA and DDT showed only 26% and 45% with pET-30a(+)-kerA and that of 19% and 33% for pGEX-6P-2-kerA respectively (Fig. 5.3.6).

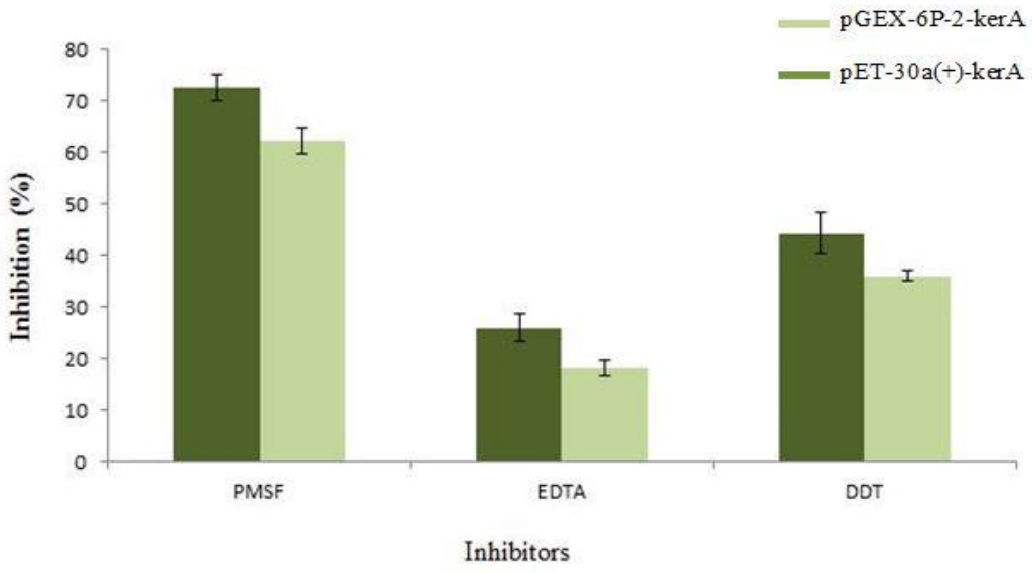

Figure 5.3.6: Effects of inhibitors on keratinase enzyme

5.3.2 Whole genome sequencing of *Bacillus licheniformis* MZK05

5.3.2.1 Analysis of gene map of *Bacillus licheniformis* MZK05

From NCBI genome data blast, the annotated whole genome sequence had 99.99% similarities with existing whole genome of *Bacillus licheniformis* (Accession no CP21507.1, CP000002). The genome of *Bacillus licheniformis* MZK05 is 4,145,737 bp long. There is high and low GC region present with a GC content of 43.05 % (Fig 5.3.7).

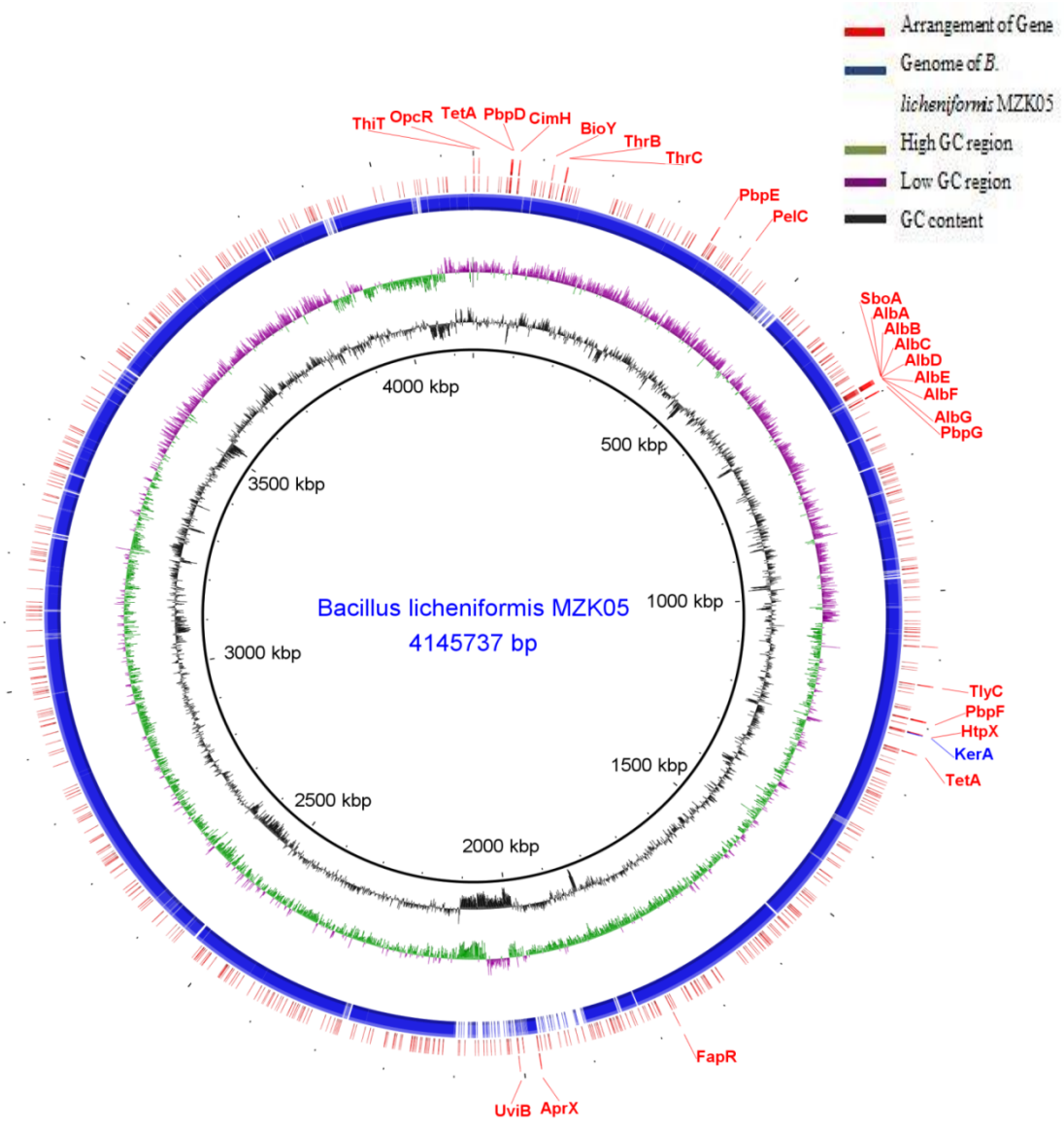

Figure 5.3.7: Circular genome map of *Bacillus licheniformis* MZK05.

The number of tRNA genes and rRNA operons are 85 and 6, respectively. Circular bar diagram indicating different type of genes (*Tly*C, *Pbp*F, *Htp*X, *Tct*A, *Opc*R, *Cim*H, *Thr*B, *Thr*C, *Alb*A, *Alb*B, *Alb*C, *Alb*D, *Alb*E, *Alb*F, *Alb*G, *Pbp*E, *Pbp*G and *Pel*C) where gene of interest that is *ker*A, is also present (Fig. 5.3.7). The position of *Ker*A gene in whole genome sequencing of *Bacillus licheniformis* MZK05 is (1,211,369-1,212,344) bp. The number of known protein coding gene is 2796 and hypothetical gene is 1282.

5.3.2.1 Subsystem statistics of *Bacillus licheniformis* MZK05

Annotations by RAST revealed a total of 419 subsystems with 48% subsystem coverage (Fig. 5.3.8). A subsystem represents a collection of functional roles that make up a metabolic pathway, a multi-subunit complex (e.g., the ribosome) or a specific class of proteins (e.g., signal transduction).

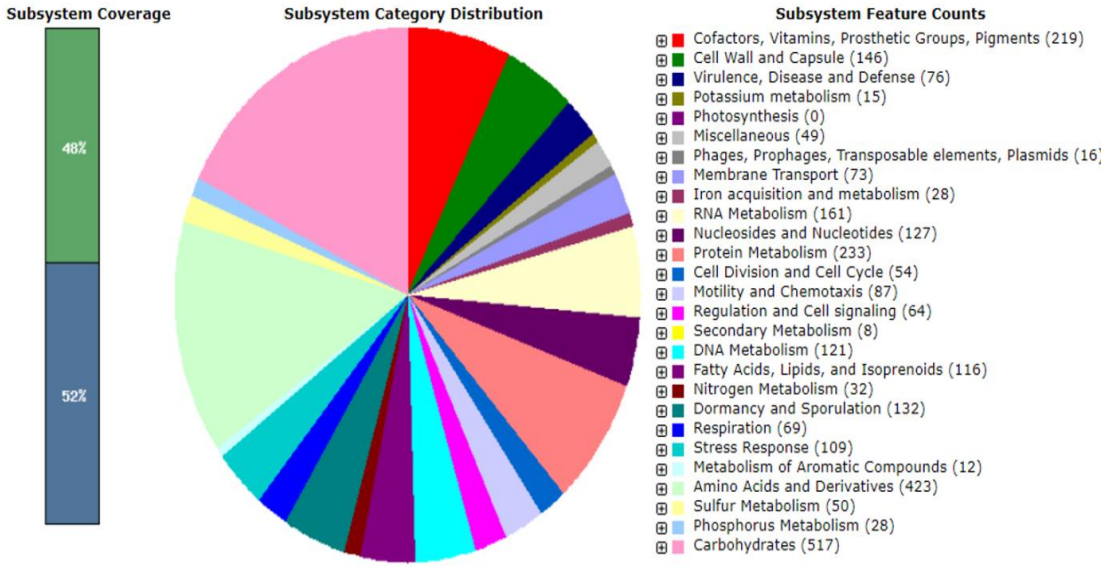

Figure 5.3.8: Subsystem distribution of *Bacillus licheniformis* MZK05 based on RAST annotation server. The green bar of the subsystem coverage indicates the percentage of the proteins included in the subsystems while the blue bar refers to the percentage of the proteins that are not included in the subsystems.

Meanwhile, subsystem coverage shows the percentage of the FIGfams (a set of proteins that are "glob-ally similar" and in which all members share a common function) that is covered by subsystems (Aziz *et al*., 2008).

Among the features of the subsystems, at least two third encode the basic core functions and metabolic pathways of the organism. The most abundant of the subsystems are related to amino acids and derivatives (n=423), followed by carbohydrates (n=517), cofactors, vitamins, prosthetic groups, pigments (n=219), protein metabolism (n=233), RNA metabolism (n=161), fatty acids, lipids and isoprenoids (n=116).

CHAPTER 6

Optimization of fermentation conditions for enhanced production of keratinase by recombinant of *Bacillus licheniformis* MZK05

Optimization of fermentation conditions for enhanced production of keratinase by recombinants of *Bacillus licheniformis* MZK05

6.1 Introduction

Bacillus strains are known to produce and secrete large quantities of extracellular enzymes and constitute a major group of industrial enzyme producers due to the robust nature of the organism. Several *Bacillus* isolates produce extracellular proteases e.g., *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus and B. pseudofirmus* (Priya *et al*., 2011) with various applications.

Cultivation conditions are essential in successful production of an enzyme and optimization of parameters such pH, temperature and media composition is important in developing the optimum fermentation conditions. Keratinase production is affected by various nitrogen sources and its concentration such as raw feather, feather meal, powdered chicken nails, bovine hair or wool (Riffel *et al*., 2002). Besides these, several other physical factors such as aeration, inoculum's density, pH, temperature and incubation time also affect the amount of keratinase production (Sangali *et al*., 2000).

Wild strain of microbes has limitations for industrial production due to not only as hypo-producer but also in the characteristic of thermal, pH stability and nutritional requirements. By using recombinant strain, proteins of interest can be produced in large quantities, greatly simplifying the task of protein purification in comparison to the wild strain. In this study, two recombinant strains were developed in the laboratory by cloning *ker*A gene from *Bacillus licheniformis* MZK05 using two vectors pGEX-6P-2 and pET-30a(+). The pGEX-6p-2 vector is the commercial vector with GST tag (Glutathione S-transferase) on N-terminal end and IPTG was used as an inducer. On the other hand, pET-30a(+) was chosen to reduce the cost of production where lactose used as a unlike IPTG inducer.

At the industrial level, biochemical and process engineers use several strategies to obtain high yields of protease in a fermenter. Fermentation condition is an important parameter to consider that can influence the cost and rate of enzyme production (Beg *et al*., 2002). Controlled batch and fed-batch fermentations using simultaneous control of glucose, ammonium ion concentration, oxygen, tension, pH and salt availability have successfully increased keratinase production by 10 fold (Wang *et al*., 1999).

In a recent study, the overall keratinase yield from *B. licheniformis* PWD-1 was improved up to 10 times those found during regular batch culture using multistage continuous and fed-batch operations (Raninger *et al.,* 2003).

Bacillus licheniformis MZK05 was a newly isolated and identified bacterium produce alkaline protease on APPB and soybean meal medium. The wild strain also tested for keratinase production on feather meal in liquid culture at 37^{o}C and exhibited keratinase activity on hairs or keratin substrates (Hoq *et al.,* 2005). The present study was carried out on the optimization of fermentation conditions for the expression of the keratinase production by the two recombinant systems pGEX-6P-2 and pET-30a(+) in *E. coli* BL21.

6.2 Methods

6.2.1 Bacterial strains

Two recombinant strains were developed in the laboratory by cloning *ker*A gene from *Bacillus licheniformis* MZK05 using two vectors pGEX-6P-2 and pET-30a(+). Both pGEX-6P-2-kerA or pET-30a(+)-kerA were expressed in *E. coli* BL21 and used for fermentation in shake flask and bioreactor cultivations.

6.2.2 Production of inoculum

The recombinant pGEX-6P-2-kerA and pET-30a(+)-kerA was streaked onto LB plates and incubated overnight at 37^{o}C. One loopful of the recombinant organism from these plate was transferred to 250 ml Erlenmeyer flask containing 50 ml LB and incubated for 12 hrs at 37^{o}C and 150 rpm. This was used as inoculum for shake culture and bioreactor cultivation.

6.2.3 Optimization of fermentation conditions for keratinase production by two recombinants *E.coli* BL21 systems in shake flask

Optimization of the fermentation conditions for keratinase production by recombinant pGEX-6P-2-kerA and pET-30a(+)-kerA were studied in LB medium at 37^{o}C in an orbital shaker. The experiments were carried out systematically in such a way that the parameter optimized in one experiment was maintained at its optimum level in the subsequent experiments. Various process parameters that enhance the yield of keratinase under submerged fermentation were investigated by one factor-at-a-time approach. All the experiments were conducted in triplicate and then the mean values were considered.

6.2.3.1 Effect of temperatures on keratinase production

To study the effect of temperatures on keratinase production by recombinant pGEX-6P-2-kerA and pET-30a(+)-kerA, the experiments were carried out in LB medium at various temperatures (30^{o}, 35^{o}, 40^{o} and 45^{o}). After autoclaving, the medium was cooled and inoculated with 5 ml of 12 hrs grown inoculum and incubated in orbital shaker at 150 rpm for 24 hrs.

6.2.3.2 Effect of pH on keratinase production

Effect of pH on the enzyme production by recombinant pGEX-6P-2-kerA and pET-30a(+)-kerA were carried out in 250 ml of Erlenmeyer flasks containing 100 ml of LB medium with various initial pH ranging from 6.5 to 9.0 with an interval of 0.5 adjusted by using 0.1 N NaOH and 0.1 N HCl. The volume of the medium was made up to desired level after adjusting the pH. After autoclaving, the medium was cooled and inoculated with 5 ml of 12 hrs grown inoculum and incubated in orbital shaker at 150 rpm for 24 hrs.

6.2.4 Optimization of fermentation conditions in bioreactor

6.2.4.1 Inoculum preparation for fermentation in bioreactor

Luria-Bertaini (LB) medium was used for inoculum preparation. After sterilization by autoclaving the inoculum medium was cooled and inoculated with the 16 hrs old culture and incubated for 16 hrs at 37^{o}C and 150 rpm in an orbital shaker.

6.2.4.2 Preparation of bioreactor

The vessel volume of the stirred tank bioreactor (model: BioFlo/CelliGen 115 Fermenter/ Bioreactor; company: New Brunswick Scientific) was 3 liters and the working volume was 1.5 liter. The bioreactor was equipped with instrumentation in order to measure and control the agitation, pH, temperature, foam, dissolve oxygen (dO_2) and exit gases. The medium was aerated by a pump (MTH) through a membrane filter. The agitator was equipped with six bladed impellers. These impellers homogenized air and the bio-controller displayed the dissolve oxygen concentration by sensing with an electrode. For temperature control, there was an outer jacket wrapping the vessel and a chiller was connected to supply cool water through a ring inside the vessel. The aeration was performed under cascade control air-inflow (1.0 vvm) and agitation rate (300 rpm).

6.2.4.3 Keratinase production in bioreactor

Recombinant *E. coli* BL21 culture was incubated at 37^{o}C, 24 hrs. Inoculum was inoculated into 1.5 L LB broth in 3 L bioreactor with optical density of 0.1 at 600nm. The broth was incubated at 37^{o}C for 3-5 hours to reach an optical density between 0.6-0.8. Then IPTG/lactose was added. Incubation was continued and samples were taken at different time interval.

Sampling was done at 1 hr of interval and enzyme activity was measured by standard assay method after separating the cell mass and protein concentration was measured by Bradford method (Bradford, 1976).

6.2.4.4 Keratinase assay

Keratinase activity was determined by a modified method described by Kreger and Lockwood (1981) using azo-casein (Sigma, USA) as the substrate. Briefly, 400 µL of appropriate dilution of KerA was added to 400 µL of 1% azo-casein solution (suspended in 0.05 M Tris- HCI; pH 8.5) and the mixture was incubated at 37°C for 1 h. The reaction was terminated by adding 135 µL of 35% trichloroacetic acid (TCA) and left for 15 min on ice followed by centrifugation at 13,000 rpm, at 4°C for 10 min. Then, 750 µL of the supernatant was neutralized with equal volume of freshly prepared 1.0 N NaOH by gentle mixing. Absorbance (OD 440nm) of the mixture was then measured in a UV spectrophotometer (U-2910, Hitachi) keeping the solution from a parallel reaction as blank where TCA was added before the enzyme. One unit of protease activity was defined as the amount of enzyme required to yield an increase in absorbance (OD 440nm) of 0.01 in 1 h at 37°C

6.3 Results

6.3.1 Effect of temperatures on enzyme production by recombinant strains

The LB medium was inoculated with 5% inoculum of recombinant pGEX-6P-2-kerA or pET-30a(+)-kerA at pH 7.5 and incubated at various temperatures ranging from 30-45^{o}C at 150 rpm for 24 hrs. Both recombinant strains, pGEX-6P-2-kerA or pET-30a(+)-kerA showed the maximum keratinase production as 120 U/ml was 210 U/ml respectively at 37^{o}C after 24 hrs. The results clearly indicate that the increase in the temperature from 30 to 37^{o}C, production was increased and it was decreased with further increase in temperature. Therefore, 37^{o}C was found optimum temperature for keratinase production by the both recombinants (Fig. 6.3.1).

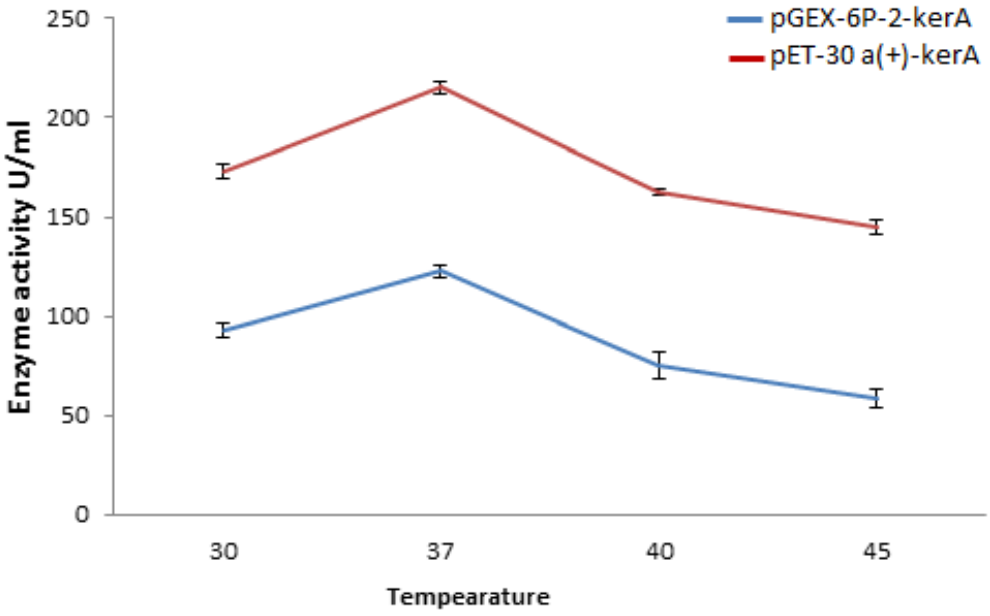

Figure 6.3.1: Effect of temperatures on keratinase production by recombinants

6.3.2 Effect of pH on keratinase production by recombinant strains

The results described in Figure 6.3.2 showed that the optimum pH for production of keratinase by recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA were pH 7.5 with highest enzyme activity 122 U/ml and 215 U/ml at 37^{o}C. The decrease in enzyme production was observed clearly by decrease and increase in pH beyond the optimum level.

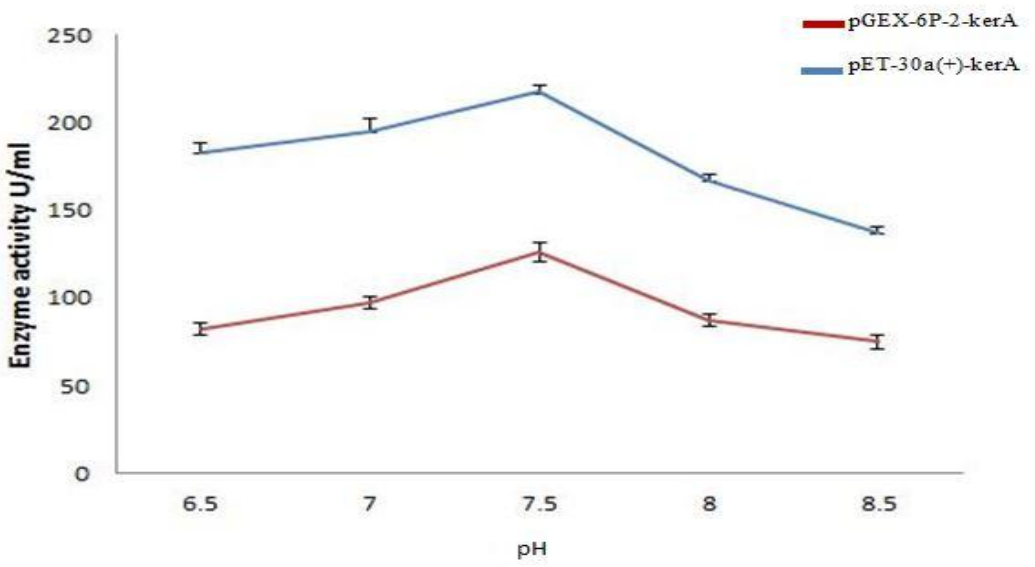

Figure 6.3.2: Effect of pH on keratinase production by recombinant strains

6.3.3 Production of keratinase in bioreactor

Keratinase production in 3L bioreactor was done using LB medium at 37^{o}C, 150 rpm. Over the 10 hours of fermentation, change in different fermentation parameters like pH and dO_2 concentration were observed. The result showed significant relationship of change of these parameters with cell number, protein concentration and enzyme activity over the time period (Fig. 6.3.3). The change of pH is notable with the increase of growth co relating metabolic changes during active fermentation period.

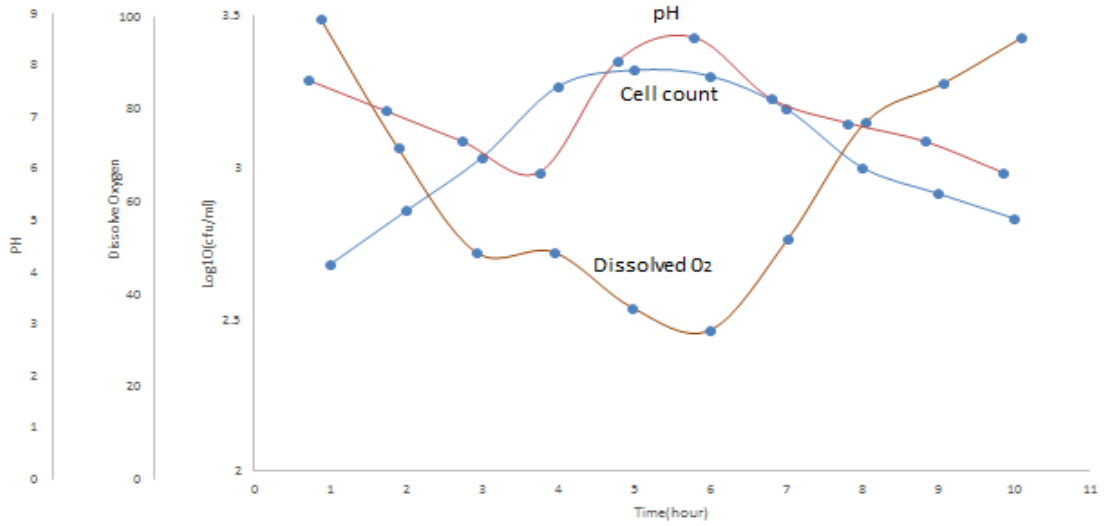

Figure 6.3.3: Kinetics of dissolve oxygen (dO_2) with bacterial growth and pH changes in bioreactor cultivation.

Both the recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA exhibited highest enzyme production after same time of 5 hrs. But with different level of enzyme production were 240 U/ml (Fig. 6.3.4) and 310 U/ml (Fig. 6.3.5) respectively by pGEX-6P-2-kerA and pET-30a(+)-kerA with IPTG and lactose induction.

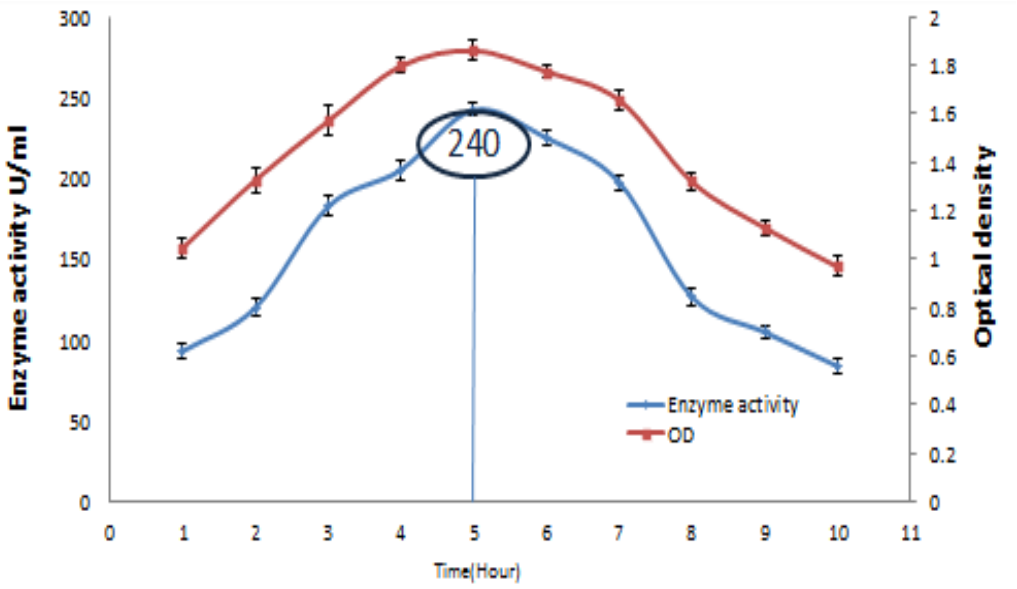

Figure 6.3.4: Kinetics of bacterial growth and productivity of pGEX-6p-2-kerA on

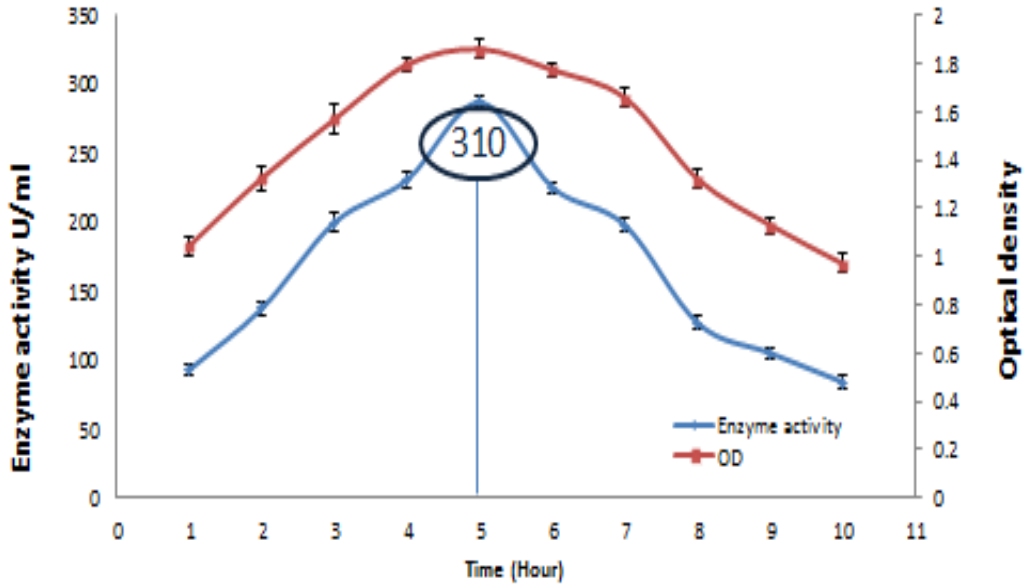

Figure 6.3.5: Kinetics of bacterial growth and productivity of pET-30a(+)-kerA on lactose

6.3.4 Comparison of enzyme productivity between shake flask and bioreactor

Keratinase productivity was compared between shake flask and bioreactor under the similar conditions in terms of media composition, inoculum volume and physico-chemical parameters. Productivity of keratinase in shake flask and bioreactor was measured by multiplying the highest enzyme activity with 1000 (for converting milliliter to liter) followed by divided by corresponding hour.

$$\text{Productivity of keratinase} = \frac{\text{Highest unit of keratinase} \times 1000}{\text{Corresponding hour of production}}$$

The result showed that keratinase productivity in bioreactor was pGEX-6p-2-kerA and pET-30a(+)-kerA were 1.50 and 1.90 times higher as compared to the shake flask (Table 6.3.1).

Table 6.3.1: Keratinase productivity comparison between shake flask and bioreactor of pGEX-6p-2-kerA and pET-30a(+)-kerA

Mode of Fermentation	**Productivity (Unit/Liter/Hour)**	**Productivity Increased (Fold)**
Shake flask	31250	1
pGEX-6p-2	48000	**1.5**
pET-30a(+)	62000	**1.9**

CHAPTER 7

Use of purified keratinase for some technical applications

Use of purified keratinase for some technical applications

7.1 Introduction

Keratinases have been employed in cosmetic formulation for skin and hair. For skin, keratinases have been added in compositions for skin whitening, freckle dispelling and bleaching (Yang 2012). Keratinases can also be used for exfoliation and removal of stratum corneum as described by Ding and Sun (2009) and Yong *et al.*, (2009). Chemical hair straighteners are highly alkaline. For this reason, changing the color of a chemically straightened hair, either with hydroxides or with thioglycolate, is a delicate procedure that may cause hair damage and breakage. Chemically treated hair is highly sensitive and prone to breakage. That's why keratinases have been added to hair compositions such as shampoo, hair gel and conditioner where they play a dual role in improving hair color, quality and luster with simultaneous cleaning and removal of impurities adhered on hair (Mo and Sook, 2010). Keratinase break down the disulfide bond of hair and as a result, curled hair becomes straight (Fig. 7.1). Here keratinase can play an excellent role to straighten curly hair in beauty parlour.

Keratinases encompasses a distinctive group of proteolytic enzymes, show their complete involvement in hydrolysis of insoluble keratin (feathers, hair, nails, horn and keratin-rich wastes) into simple polypeptides and amino acids (Gupta *et al*., 2013). At present, treatment of feather with microbial keratinase draws wide attention with several applications due to its substantiality as feasible source of dietary protein in food and feed supplements. Keratinases are likely to engender a prospective worldwide market similar to other proteases (Deivasigamani *et al*., 2008). As the feather has β-pleated sheet structure consists of 20 amino acids, mainly of cysteine and its structural configuration comprises of a central carbon linked to functional groups (amine, $–NH_2$ and carboxylic acid, –COOH), the hydrogen atoms and the group R further twisted and cross linked by disulfide bridges to form a cable-like structures (Belarmino *et al*., 2012). Disulphide reductase breaks the disulphide bridges which are responsible for the rigidity of keratin found in feather and keratinase cleaves the hydrogen bonds (hydrolysis) of β-pleated sheet and releases different amino acids.

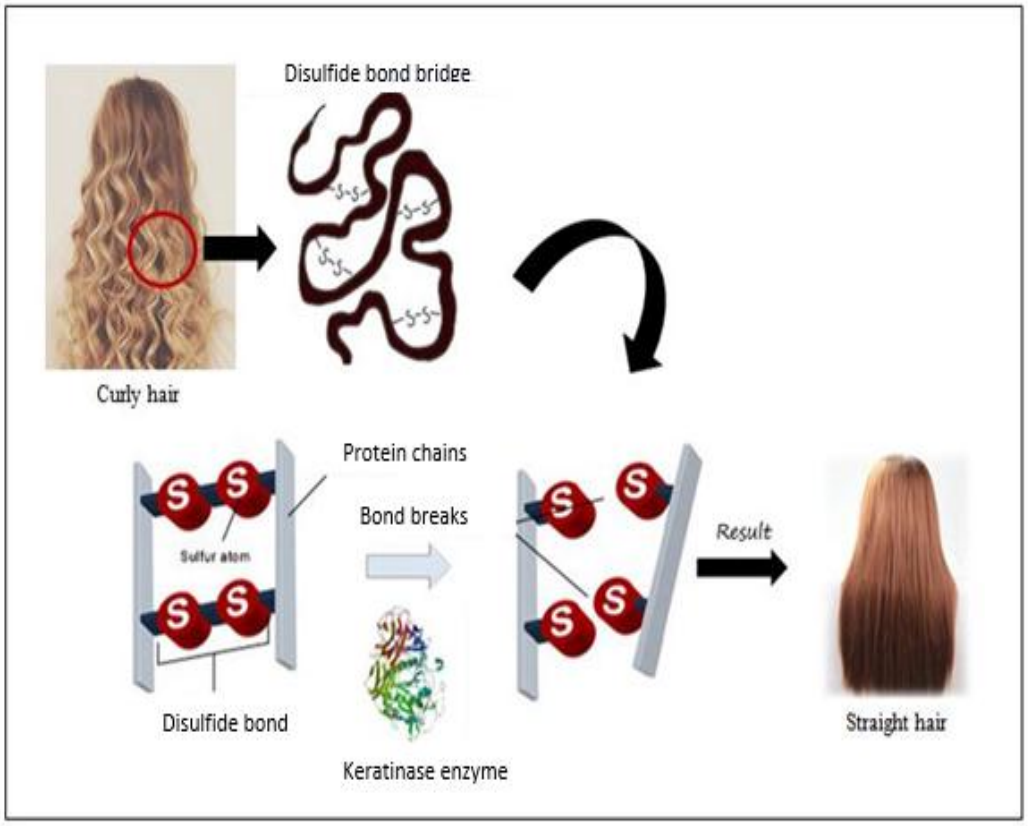

Figure 7.1: Straightening of curled hair by the keratinase through breaking of disulfide bond

Leather industries (Tanneries) offer various advantages in terms of manufacturing capacity and export potential (Luthra, 2006). There are about more than 200 tanneries in Bangladesh producing and exporting quality leathers with good international reputation and have been ranked fifth in the country's export earning sectors (Paul *et al.*, 2013). Animal skins/hides, byproduct of meat industries, are composed of proteins (90-95% of solid, 35% by weight), lipids, carbohydrates, mineral salts and water. Among several classes of proteins (collagen, elastin, keratin, glycoproteins, albumins and globulins), collagen as structural protein present in largest amount and is responsible for the formation of leather by combining with tanning agent (Kanagaraj *et al*., 2006).

The animal skins/hides have different processing steps like soaking, dehairing, deliming, bating, degreasing, pickling and tanning in beam house operations to prepare stabilized collagenous leather products (Zambare *et al*., 2013). For this purpose, all local tanneries still use traditional leather processing method utilizing mostly harsh chemicals.

The conventional chemical leather processing generates huge amount of environmental pollutions (Kanagaraj *et al.*, 2006; Thanikaivelan *et al*., 2004). Dehairing process, based on the use of lime and sodium sulfide, excretes highly alkaline effluent which contributes to 50–60% of total aquatic pollution load in terms of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (Marsal *et al*., 1999; Taylor *et al*., 1987).

The highly alkaline sodium sulfide having obnoxious odor is very toxic and can cause skin burn. Moreover, in oxidation-reduction reaction sodium sulfide produces hydrogen sulfide which is proven to be fatal even in concentrations as low as 200 ppm (Hannah and Roth, 1991; Roth *et al*., 1995). Hydrogen sulfide also reacts with water and produces sulfuric acid that causes health hazards to the tannery workers and damage buildings, bridge etc. (Puvanakrishnan and Dhar, 1988). The extensive use of hazardous sulfide not only leads to unfavorable consequences on the health and environment but also undermines the efficiency of the effluent treatment plants. The above mentioned situations necessitate the use of an alternative and eco-friendly process, which is most likely could be accomplished by a process involving enzymes, to reduce environmental pollution (Dettmer *et al*., 2011).

So, this study has been carried out for some technical applications like hair rebonding, feather hydrolysis and dehairing by recombinant keratinases.

7.2 Methods

7.2.1 Hair treatment of keratinase

Keratinase is a very good substitute for chemicals on hair treatment. Keratinase is used in different types of hair treatments and hair rebonding. For hair rebonding test, hair was washed with a mild shampoo. A flat iron was used to straighten hairs. Purified keratinase enzyme with moisturizer (1:5) was applied on a bunch of curly hair and another bunch of curly hair was treated with only rebonding cream. They were kept for 45 min and then the hair was rinsed with water and blow dried.

7.2.2 Whole feather hydrolysis by keratinase enzyme

For feather hydrolysis test, raw feathers from hens were obtained from the poultry farm. The feathers were washed in tap water and placed in a circulating air-drying oven at 60^{o}C for 24 h. Then, whole feather was immersed into 5 ml of purified keratinase enzyme of pGEX-6P-2-kerA or pET-30a(+)-kerA and incubated in shaking incubator for 24 to 48 hrs at 37°C. The control was raw feathers incubated with only 5 ml of distilled water for 24 h at 37^{o}C.

7.2.3 Dehairing test

Freshly flayed goat skin was obtained from the local slaughterhouse and washed properly with water and detergent to eliminate blood, dirt and other unwanted particles. After removing the adipose tissue layer with a knife the skin was cut into pieces (6cm x 6cm) and weighted. These pieces were used for dehairing tests-conventional (control), enzyme mediated and enzyme assisted methods. Both purified keratinase enzyme of pGEX-6P-2-kerA and pET-30a(+)-kerA has been used for dehairing test.

(a) Conventional method

In conventional method, the pieces of the goat skin was placed into a conical flasks (1000 ml) containing water (120 ml) to dip the skin pieces. Then 2% Na_2S and 5% CaO (of the skin weight) were used for dehairing of the goat skin. Flasks was transferred into orbital shaker rotating at 120 rpm for 26 hrs at 37^{o}C.

(b) Enzyme mediated (only enzyme) method

In enzyme mediated method, only 5% keratinase solution was used for dehairing of the goat skin. Then the flasks was transferred into orbital shaker rotating at 120 rpm for 26 hrs at 37^{o}C.

(c) Enzyme assisted (enzyme + chemical) method

For enzyme assisted method (enzyme + chemical) the piece of goat skin was soaked in 5% CaO (w/w of the skin) solution for 6 hrs at 37^{o}C. After CaO treatment, the skin piece was washed several times by water until pH of the skin dropped near 7.5. Then the pieces of the goat skin dipped in 120 ml of water in a 1000 ml conical flask and 5% keratinase solution was added. Then the flask was transferred into orbital shaker rotating at 120 rpm for 26 hrs at 37^{o}C.

The efficiency of the dehairing methods to remove the hair from skin was evaluated by determining the area of the dehaired portion and expressed as the percentage of the area of the treated skin.

7.3 Results

7.3.1 Hair rebonding test by the recombinant keratinase

For hair rebonding test, Purified keratinase enzyme with moisturizer (1:5) was applied on bunch of curly hair and another bunch of curly hair was treated with only rebonding cream. Purified keratinase with moisturizer applied hair showed better comparing to only rebonding cream used hair (Fig. 7.3.1)

Figure 7.3.1: Hair rebonding test by recombinant keratinase

7.3.2 Whole feather hydrolysis by keratinase enzymes

In feather hydrolysis test, after 48 hrs, pGEX-6P-2-kerA and pET-30a(+)-kerA both enzymes were completely hydrolyzed comparing to wild MZK05 (Fig. 7.3.2a). No hydrolysis occurred in control and partial hydrolysis observed in wild MZK05 (Fig. 7.3.2 b). Compared to pGEX-6P-2-kerA and pET-30a(+)-kerA, pET-30a(+)-kerA was demonstrated better hydrolysis compared to pGEX-6P-2-kerA.

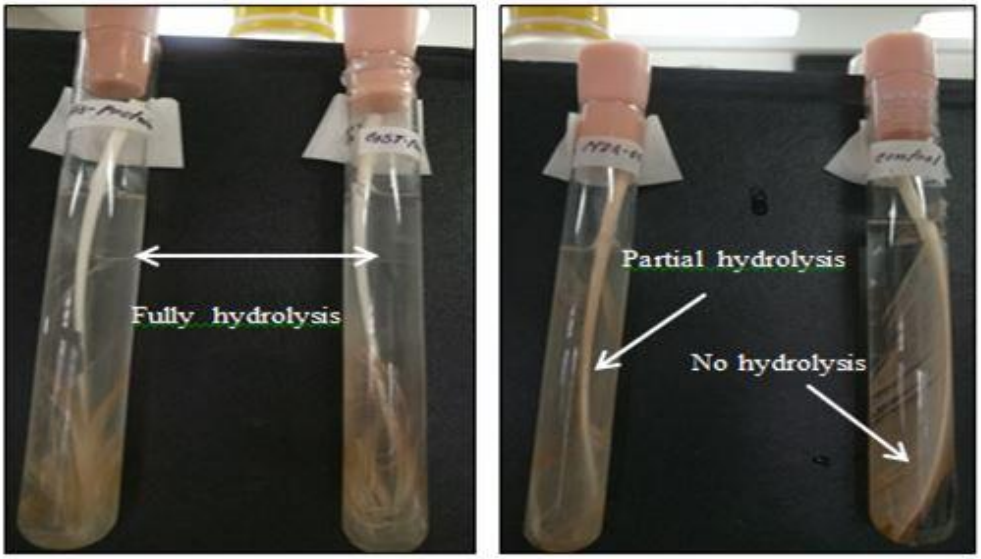

Figure 7.3.2: Physical observation of feather hydrolysis test. (a) After 48 hrs incubation purified keratinase were fully hydrolyzed. (b) Wild type was partially hydrolyzed.

7.3.3 Dehairing test

In dehairing test, conventional dehairing process using CaO and Na_2S needed 26 hrs for complete dehairing of the goat skin. Both enzyme assisted and enzyme mediated also needed 26 hrs (Table 7.3.1). From the results, it was found that the highest 85% and 95% dehaired area were resulted using enzyme assisted and enzyme mediated methods respectively (Fig. 7.3.3). Comparison between enzyme mediated and enzyme assisted method showed that complete dehairing was occurred by enzyme mediated method in small scale unhairing process (Fig. 7.3.4). But pET-30a(+)-kerA was demonstrated better dehairing process compared to pGEX-6P-2-kerA.

Table 7.3.1: Effect of the keratinase enzymes and chemical treatments on the dehaired area yield of the skin.

Types of method	Area of treated skin (cm^2)	Dehairing agents	Duration of treatment (hr)
Conventional	36	2% Na_2S and 5% Cao	26
Enzyme mediated	36	5% keratinase	26
Enzyme assisted	36	5% CaO and 5% keratinase	26

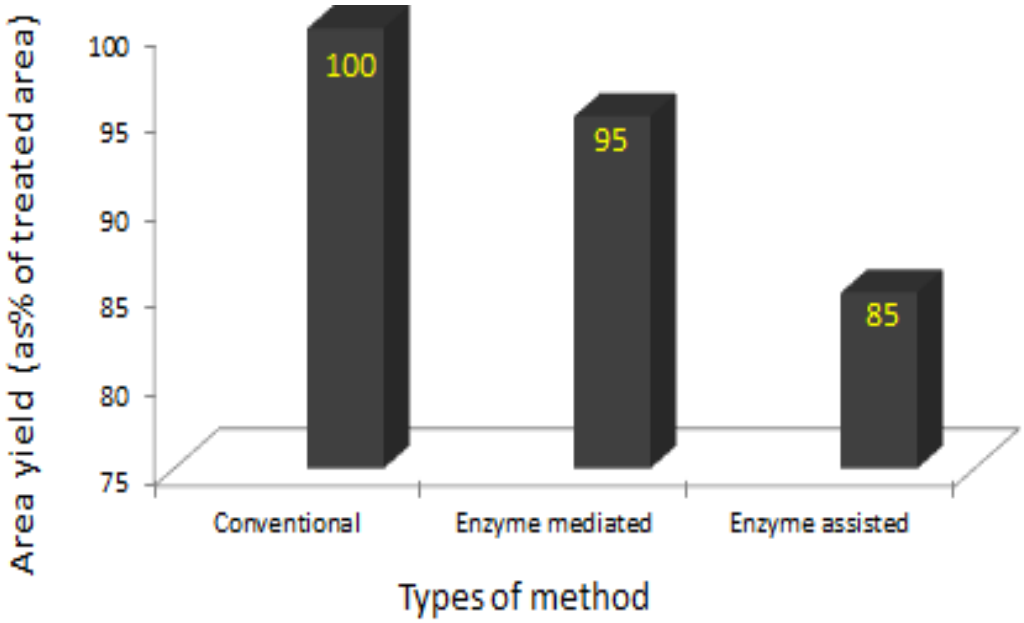

Figure 7.3.3: Comparative dehairing efficiency of conventional, enzyme mediated and enzyme assisted methods.

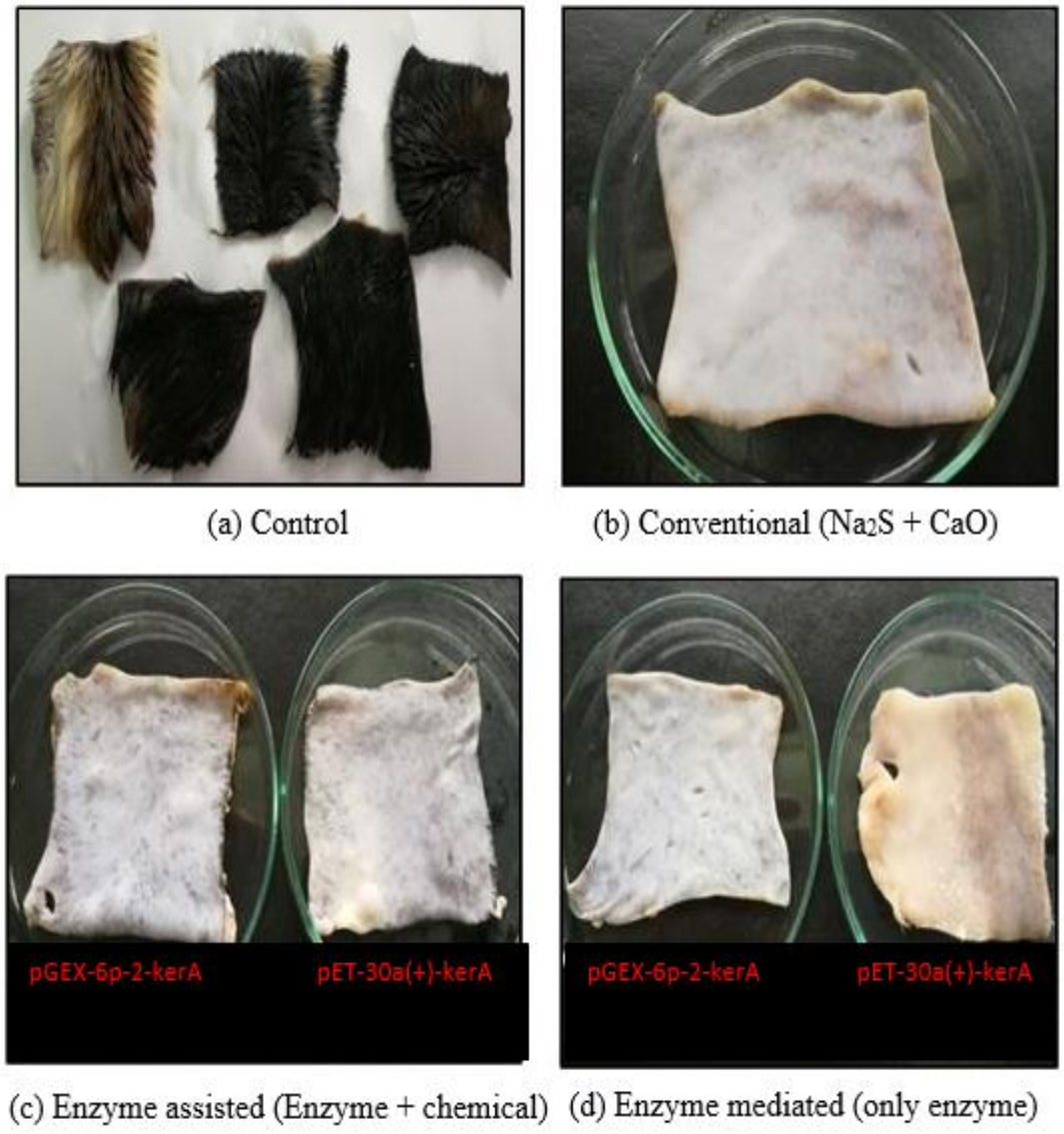

Figure 7.3.4: Evaluation of different treatment methods for dehairing of goat skin. (a) Control (b) Conventional (Na_2S + CaO) method (c) Enzyme assisted (enzyme+chemical) method (d) Enzyme mediated (only enzyme) method.

CHAPTER 8

Discussion and Conclusions

8.0 DISCUSSION AND CONCLUSIONS

8.1 DISCUSSION

Keratinase is a potential enzyme for removing hair and feather in the poultry industry (Takami *et al*., 1992) for nutritional upgrading of feather meal and conversion of feathers into a feed protein in feed industry (Williams *et al*., 1991) and for clearing obstructions in the sewage system during waste water treatment and eco-friendly de-hairing process in leather industry. However, much current research is centered on the potential use of keratinase of bacterial origin for the industrial treatment of keratin-containing compounds.

Different microorganisms have been employed for industrial enzyme production, varying from eukaryotic systems, such as yeast and fungi, to prokaryotic system involving both Gram positive and Gram negatives bacteria. Keratinolytic enzymes have been studied from a variety of fungi, but to a lesser extent in bacteria. *Bacillus* spp. is one of the very suitable microorganisms known to secrete industrial enzymes. In this regard, *Bacillus licheniformis* was newly isolated, identified and characterized (Hoq *et al.*, 2005). This bacterium was tested for its keratinolytic activity and its application was found suitable for further studies. Therefore the study was designed to develop high level expression of keratinase by the recombinants of *Bacillus licheniformis* MZK05 through cloning into two system, pGEX-6P-2 and pET-30a(+) in *E. coli* BL21. In addition, the keratinase enzyme was successfully purified, characterized and studied for some technical applications.

Cloning, expression and structure simulation of keratinase in pGEX-6P-2 system

The pGEX-6P-2 vector was chosen as the expression vector because of its tight and inducible high expression capacity, purification of the fusion protein by affinity chromatography in mild condition that minimizes the effects on the antigenicity and functional activity of the protein. The expression is under the control of *taq* promoter which is chemically inducible with lactose analog isopropyl β-D thiogalactoside (IPTG) and also engineered with an internal *lac*I^q gene, product of which acts as a repressor protein. It binds to the operator region of *tac* promoter that prevents expression until induction by IPTG. Thus a tight control over expression of insert is maintained.

The vector has multiple cloning sites (mcs) and a PreScission Protease cleavage site is present between the GST tag and the mcs. Thus it offers the most efficient method for cleavage and purification of GST fusion proteins. Site-specific cleavage is performed with simultaneous immobilization of protease as well as the GST tag on the column and thereby removed from the purified protein in the elute fractions.

Restriction sites, *Bam*HI and *Xho*I were chosen from the mcs of pGEX-6p-2 as these two sites are normally absent in the restriction mapping of previously reported *kerA* ORFs. To keep the insert in proper orientation and correct junction for maintaining the reading frame, *Bam*HI was used in forward primer and *Xho*I was used in reverse primer. Thus the desired amplicon reached 1156 bp in length (1140+ 2×8).

After the accomplishment of PCR amplification, double digestion of both vector and insert and ligation, construction of recombinant DNA was checked by transforming the ligation mixture into the competent *Escherichia coli* DH5α cells. Transformation with undigested vector and digested vector alone were also performed as positive and negative control of the experiment. A good number of transformants were found for the ligation mixture (vector: insert-1:3) and 15 clones were selected randomly to check for insert by PCR. PCR positive clones were then used for the propagation and extraction of pGEX-6p-2-kerA. Double digestion also revealed two DNA bands upon agarose gel electrophoresis at around 1 kb and 5 kb.

The recombinant pGEX-6p-2-kerA vector was used for sequencing with primers designed from the upstream and downstream region of PreScission Protease and *Xho*1 restriction site respectively to see whether the insert was in proper orientation and correct junction to maintain the reading frame. It was confirmed from the sequence analysis that Gln-Gly-Pro-Leu-Gly-Ser-Met was maintained i.e. the insert was in proper orientation. Thus the cloning of *kerA* gene was confirmed and the attempt was taken to express the GST-KerA fusion protein. The transformants of *E. coli* BL21 was prepared transforming the pGEX-6p-2-kerA vector and was induced with IPTG in LB-ampicillin broth.

Expression of a 58 kDa GST-KerA fusion protein took place when induced with IPTG at a concentration of 0.1 mM and maximum expression level was attained at 3 h. Hence, expression up to 3 h was continued with different IPTG concentrations starting from 0.1 mM to 0.5 mM and it was observed that the optimum IPTG concentration was 0.3 mM as the expression level remained unchanged from 0.3 mM onward.

Purification of fusion protein was performed by cleaving the kerA protein with PreScission Protease from its GST counterpart in Glutathione Sepharose purification method. Thus, the protein band for KerA (39 kDa) was observed after purification. As the heterologous expression of kerA was performed as GST-fusion protein, neither signal peptide nor pro-protein were processed after to generate matured protein. So, the molecular weight of the protein seemed to be unusual from other serine proteases which are around 30 kDa. It was also clarified when the molecular weight of the protein in pro-protein and matured form was determined from the deduced amino acid sequences to be 35.65 and 27.3 kDa respectively.

The concentration of purified protein was found to be 119 mg/L which is an appreciable yield. The protease and keratinase activity of the purified keratinase was found to be 312 U/ml and 196 U/ml as assayed on azocasein and keratin azure respectively. A 4-fold increase both in protease and keratinase activity have been obtained by heterologous expression of keratinase than the wild-type organism. The specific protease and keratinase activity was also determined and those were found to be 2621.84 U/mg and 1647 U/mg of protein respectively.

The sequences of all reported mature subtilisins of *B. licheniformis* strains have strong identity of amino acid sequences (Radha and Gunasekaran, 2007). It was not different in this study too. The blast result for the deduced amino acid sequences of kerA from *B. licheniformis* MZK05 revealed that 30 hits showed were with more than or equal to 98% identity. With thermostable keratinase from *Bacillus licheniformis* DS23 and kerA of *Bacillus licheniformis* OWU 1411T, 100% identity was observed and 98.4% identity was observed with kerA of *B. licheniformis* PWD-1 (*B. licheniformis* ATCC 53757). The signal sequence obtained (Petersen *et al.*, 2011) has similar properties with 29 residues to other signal sequences from Gram positive bacteria. This includes a basic N-terminal segment followed by a stretch of uncharged residues. It was also reported that the Gram positive signal peptides are unusual both in length and charge compared with other prokaryotic and eukaryotic organisms and subtilisin Carlsberg signal peptide was typical for Gram positive bacteria with 29 residues (Jacob *et al.*, 1985). The N-terminal signal peptide is followed by a pro-peptide of 76 residues followed by the mature protein comprising of 274 residues and thus the prokeratinase of 350 residues.

The amino acid residues at positions 150 and 153 are important for determining the specificity of Carlsberg protease (Takagi *et al.*, 1998) and for *B. licheniformis* MZK05, amino acids at these positions are same as others, i.e. Ala-Ala-Ala-Gly. Amino acids sequence alignment of the matured kerA from MZK05 and PWD-1 revealed that differences are present in the following positions, i.e. Tyr26 instead of Phe26, Asn86/Ser86, Pro128/Ala128, Ser211/Asn211 and Ala222/Val222. This divergence could be due to strain variation. The residue involved in catalysis is Ser220 and Val222 is one of the two residues near to the active site. It was reported that the larger side chain of Val222 in kerA in contrast to Ala222 may enhance keratin hydrolysis with the consequence of impaired kinetics on the charged amino acids at P1 (Evans *et al.*, 2000).

The three dimensional models of kerA_MZK05 were built with ProMod Version 3.70 simulating the 3D models of PDB templates 4gi3.1.A, 1yu6.1.A, 1c3l.1.A whose sequence identities with our protein sequence were 98.54%, 97.81% and 98.18% respectively. Models simulating 4gi3.1.A, 1yu6.1.A and 1c3l.1.A were named as Model 01, Model 02 and Model 03 respectively. QMEAN scores estimated for Model 01, 02 and 03 were 0.60, 0.11 and -0.08 and were predicted with none, 1× Ca^{2+} and 2× Ca^{2+} ligand binding sites respectively. Among these three models, Model 03 was considered to be the correct one because it was observed that the $CaCl_2$ has a positive influence over the keratinase activity of MZK05. Addition of 0.5 mM $CaCl_2$ caused an increase of 20% in enzyme activity of the MZK05 wild strain (Nizam *et al.*, 2006). Again, there are reports that Ca^{2+} plays an important role in stabilizing several members of the subtilisin protein family including subtilisin Carlsberg, subtilisin BPN or Proteinase K through two different Ca^{2+}-binding sites in these proteins (Pantoliano *et al.*,1988). Thus, based on the ligand binding sites, Model 03 best fits the criteria.

In comparison to this, the absence of disulfide bonds in the enzyme was established in the present study which would facilitate easy and increased contact with the substrate resulting improved enzyme activity. The results were in conformance with the idea when the enzyme activity in shake flask and the specific activity were compared to the reports (Liu *et al.*, 2014). A 46% higher yield in keratinase activity (196 U/ml) was obtained in shake flask in this study than the reported activity (134 U/ml) by recombinant *Escherichia coli* BL21.

On the other hand, they reported highest ever keratinase activity from recombinant *B. subtilis* (3,010 U/mL) and a moderate activity from *P. pastoris* (1,050 U/mL), when cultured in a 3.0 L bioreactor. Based on the specific enzyme activity, these results were compared with our study as it was done in shake flask. The reported specific activities of purified kerE (*E. coli* BL21), kerB (*B. subtilis*), and kerP (*P. pastoris*) were almost similar i.e. 1,206.8, 1,286.7, and 1,245.6 U/mg respectively, an indication of efficiency of the enzyme, whereas it was 1647 U/mg in our study which is 24% higher than their average activity. Actually, enzyme activity does not necessarily represent the efficiency of an enzyme unless the specific enzyme activity is determined.

From this point of view, expression of a highly efficient keratinase enzyme was successful in this study which can be scaled up into a bioreactor for industrial level production. Regarding the protein expression, presence of six potential N-Glycosylation sites in the mature protein sequence also suggests expression at higher level in the eukaryotic expression systems such as yeast and also enhanced expression by engineering additional glycosylation sites near the N-termini of the target protein.

Cloning, expression and purification of keratinase in pET-30a(+) system

Bacillus licheniformis MZK05 keratinase gene was successfully cloned into pCR2.1 TA cloning vector. The TA Cloning vector (pCR2.1) provides a quick, one-step cloning strategy for the direct insertion of a PCR product into the vector. TA vector was employed for cloning PCR products amplified using Taq DNA polymerase which adds adenine (A) to the 3′ end of its PCR products. Subcloning of *ker*A gene into pET-30a(+) expression vector was successful.

The pET-30a(+) vector was chosen as the expression vector because of its tight and inducible high expression capacity, purification of the His-tag protein by affinity chromatography in mild condition that minimizes the effects on the antigenicity and functional activity of the protein. The expression is under the control of *taq* promoter which is chemically inducible with lactose and also engineered with an internal *lac*I^q gene, product of which acts as a repressor protein. It binds to the operator region of *tac* promoter that prevents expression until induction by lactose. Thus a tight control over expression of insert is maintained.

A good number of transformants were found for the ligation mixture (vector: insert-1:3) and 10 clones were selected randomly to check for insert by PCR. PCR positive clones were then used for the propagation and extraction of pET-30a(+)-kerA.

The transformants of *E. coli* BL21 was prepared transforming the pET-30a(+)-kerA vector and was induced with lactose in LB-ampicillin broth. Expression of a 40 kDa His-tag kerA protein took place when induced with lactose at a concentration of 2 mM and maximum expression level was attained at 3 h. A variety of commercially important enzymes, such as cyclodextrin glycosyltransferase (Lee and Tao, 1994) and isoamylase (Lin and Chu, 1997) have been expressed in recombinant *E. coli.* The expression of those genes is initiated by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium and the recombinant proteins were often deposited within the host cell in the form of insoluble aggregates. The use of lactose as the inducer can result in the expression of biologically active protein (Hoffman *et al.*, 1995). Although lactose is readily available in large quantities as a by-product of the dairy industry, only a limited number of studies have examined the induction expression of foreign genes by lactose (Donovan *et al.*, 1996). Due to the cost of IPTG compared to lactose, the use of the latter may offer an inexpensive alternative for the induction of heterologous gene expression from the lac promoter.

In the present study, purification of KerA protein was performed by affinity chromatography by Ni^{2+}-NTA agarose. A widely employed method utilizes immobilized metal-affinity chromatography (IMAC) to purify recombinant proteins containing a short affinity tag consisting of polyhistidine residues. IMAC is based on the interactions between a transition metal ion (Co^{2+}, Ni^{2+}, $Cu^{2+}$and Zn^{2+}) immobilized on a matrix and specific amino acid side chains. Immobilized metal affinity chromatography is a versatile method that can be utilized to rapidly purify polyhistidine affinity-tagged proteins, resulting in 100-fold enrichments in a single purification step. Affinity-tagged protein purities can be achieved at up to 95% purity by IMAC in high yield (Hochuli *et al.*, 1988). Purification using polyhistidine tags has been carried out successfully using a number of expression systems, including *Escherichia coli,* (Van *et al.*, 1992) *Saccharomyces cerevisiae,* mammalian cells and baculovirus-infected insect cells.

After immobilized metal affinity chromatography purification, 39 kDa KerA purified protein was found but some low molecular weight protein also observed. A 10-30 kDa centricon tube was used for purification. This centricon remove low molecular weight protein and only 39 kDa purified KerA protein was found. The concentration of purified protein was found to be 190 mg/L which is an appreciable yield. The keratinase activity of the purified keratinase was found to be 358 U/mL. A 4.5 fold increase in keratinase activity have been obtained by heterologous expression of keratinase than the wild-type organism.

Characterization of the recombinant keratinases

Most enzymes are proteins that accelerate the chemical reactions by decreasing the activation energy. But activity of the enzyme can be affected by the several environmental factors including temperatures and pH (Eed, 2012). Effect of temperature and pH on keratinase activity has been included in the present study. *Bacillus licheniformis,* showed to be more adapted to keratinase production using keratin as substrate since maximum kerotinolytic activity of the isolate was observed during early growth and the strain displayed a higher total activity during incubation.

The enzyme activity was studied for over a broad range of temperature (30-80°C). The optimum temperature of keratinase from *Bacillus licheniformis* 60°C was slightly higher than that of other *Bacillus* kerotinolytic protease (50-55°C) (Lin *et al.*, 1992; Cheng *et al*.,1995; Balaji *et al*., 2008). The high thermo stability allows performance of the industrial process at high temperature and the risk of microbial contamination. Keratinolytic bacteria often exhibit optimal growth and activity at higher temperature (Lin *et al.*, 1992). Some mesophilic bacteria exhibit the optimal enzymes production and activity from 20-30°. Williams and Shih, 1989 also reported that maximum growth of *B. licheniformis* PWD-1 was observed at 50°C. In the present work, both recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA had optimum temperature at 40^{o}C. After 40^{o}C, it became to decrease. The stability of temperature was (20-40)^{o}C. The strain *Bacillus licheniformis* grew well and completely degraded poultry feather in the nutrient medium and achieved in 40°C (Vigneshwaran *et al*., 2010). Laxman *et al*., 2004 observed that the enzyme showed broad temperature specificity with a maximum activity at 60°C.

The relative activity of enzyme was found to be higher in the temperature range of 50–70°C than at the normal assay temperature (40°C), while the enzyme retained nearly 50% of its activity at 80°C.

Second most important factor that can affect the enzyme activity is pH and each enzyme function best at certain pH value. Different organisms show maximum enzyme production at different pH levels. For example, *Bacillus cereus, Bacillus subtilis* and *Bacillus pumilus* produce maximum enzyme at pH levels of 7.0, 5.9 or 5.6 respectively. In present study, both recombinant pGEX-6P-2-kerA and pET-30a(+)-kerA showed the highest activity at pH 8 and the stability was (6-8). After pH 8, it began to decrease. Khardenavis *et al*., 2009 showed very little activity at pH 4.0–6.0 after which an increase in activity was observed up to pH 10.0. Hossain *et al.,* 2007 and Najafa *et al.,* 2006 reported that the bacterium could grow over a wide pH range (6–12) while keratinase production is limited to pH 7-10 with maximum production at pH 8. The active range of pH 5.0 and 7.0 whereas the optimum pH for the keratinase was found to be at pH 7.0, the enzyme was stable at the range of 6-8 (Cheng *et al.*, 1995).

A metal ion probably act as salt or ion bridge to maintain the structure conformation of the enzyme of the enzyme or to stabilize the binding of the substrate and enzyme complex. Therefore, this enzyme could be applied to digest keratin substrate under various metal ion surroundings (Vigneshwaran *et al.,* 2010). During comparison of the effect of metal ions among keratinolytic activities of recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA, it was noticed that in presence of $Ca^{2+,}$ Mn^{2+} and Mg^{2+}, the keratinolytic activity was increased. On the other hand, Zn^{2+} and Co^{2+} showed less inhibitory effect on recombinants. Earlier, $Ca^{2+,}$ Mg^{2+} and Mn^{2+} increased the activity of keratinase produced by BlM9 in the same way by Mamun *et al*., (2015). Shimongaki *et al*., (1991) found stimulatory effect on Mn^{2+} serine alkaline protease. Tsujibo *et al*., (1990) and Aretz *et al.*, (1989) obtained best results with Ca^{2+} with an increase in activity around two-fold.

Results of sensitivity of purified recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA to various inhibitors showed that strong inhibition 62% and 73% respectively was achieved by pre-incubation with PMSF indicating that the enzyme belongs to serine group proteases. However, EDTA and DDT were only slightly inhibited the keratinase with 26% and 45% on pET-30a(+)-kerA, respectively, whereas 19% and 33% on pGEX-6P-2-kerA, respectively.

Whole genome sequencing of *Bacillus licheniformis* MZK05

Whole genome sequencing of *Bacillus licheniformis* MZK05 strain was performed using Illumina MiSeq technique. From NCBI genome data blast, the annotated whole genome sequence had 99.99% similarities with existing whole genome of *Bacillus licheniformis* (Accession no CP21507.1, CP000002). The genome of *Bacillus licheniformis* MZK05 is 4,145,737 bp long. There is high and low GC region present with a GC content of 43.05%. The number of tRNA genes and rRNA operons are 85 and 6, respectively. The position of *ker*A gene in whole genome sequencing of *Bacillus licheniformis* MZK05 is (1211369-1212344) bp. The number of known protein coding gene is 2796 and hypothetical gene is 1282. Annotations by RAST revealed a total of 419 subsystems with 48% subsystem coverage. The most abundant of the subsystems are related to amino acids and derivatives (n=423), followed by carbohydrates (n=517), cofactors, vitamins, prosthetic groups, pigments (n=219), protein metabolism (n=233), RNA metabolism (n=161), fatty acids, lipids and isoprenoids (n=116).

Optimization of fermentation conditions for enhanced production of keratinase by recombinants of *Bacillus licheniformis* MZK05

Temperature is one of the important parameters that have a significant impact on the growth of microbes and needs to be controlled at optimum one. Cultivation temperature affects protein synthesis by influencing rate of biochemical reactions within the cell and consequently inducing or repressing enzyme production (Bakermans and Nelson, 2004). However, studies by Frankena *et al.*, (1986) showed that a link existed between enzyme synthesis and energy metabolism in Bacilli, which was controlled by temperature and oxygen uptake. To determine the effect of temperature on keratinase production by recombinant pGEX-6P-2-kerA or pET-30a(+)-kerA, the fermentation experiments were carried out in LB medium at various temperatures (30^{o}, 35^{o}, 40^{o} and 45^{o} at 150 rpm for 24 hrs. The optimum temperature was revealed on 37^{o}C and a positive relationship between the increase in incubation temperature and enzyme production up to 37^{o}C was found. However, about 75% of the enzyme production was obtained at 40^{o}C but decreased rapidly at its beyond, these results were in harmony with the findings of Asokan and Jayanthi, (2010) and Mabrouk *et al.*, (1999), Rahman *et al.*, (2005) also observed optimum temperature for enzyme production in *Pseudomonas aeruginosa* strain

K at 37^{o}C and by increasing to higher temperature upto 40^{o}C and 45^{o}C used more than 24.0% and 53.0% loss of enzyme activity.

The metabolic activities of the microorganisms are very sensitive for pH variation (Sher *et al*., 2012) and optimum pH has important role in enzyme yield (Ali *et al.*, 1998). Effect of pH on the enzyme production by recombinant pGEX-6P-2-kerA and pET-30 a(+)-kerA was carried out in 250 ml of Erlenmeyer flasks containing 100 ml of LB medium with various initial pH ranging from 6.5 to 9.0 with an interval of 0.5 adjusted by using 0.1 N NaOH and 0.1 N HCl. The optimum pH for production of keratinase by recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA were pH 7.5 with highest enzyme activity 122 U/ml and 215 U/ml at 37^{o}C. The decrease in enzyme production was observed clearly by decrease and increase in pH beyond the optimum level. Similar result was also observed by Seifzadeh *et al*., 2008. The optimal pH for other *Bacillus* bacteria reported in literature are pH 7.0 for *Bacillus* spp. K2 and *B. licheniformis* ATCCC 21415 (Mabrouk *et al*., 1999), pH 9-9.5 for *Bacillus* spp. P-2 (Kaur *et al.,* 2001) and *Bacillus subtilis* strain AKRS3 (Ravishankae *et al.,* 2012). The important characteristics of most alkaliphilic microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production (Aunstrup, 1980). *Bacillus* spp. JB99 (Johnvesly and Naik, 2001), *Bacillus* spp. SSR-1 and *Bacillus brevis* MTCCC B0016 (Banerjee *et al.*, 1999) were reported to have 10.0-10.5.

There is a complex relationship between the growth of microbes and enzyme productivity (Lipson, 2015). Thus present study also described how the enzyme production by *Bacillus licheniformis* MZK05 was affected by its growth over 10 hours of time period in both shake flask and lab scale bioreactor. The result showed that, there was an increase in enzyme production with increase in microbial growth and as the bacterium entered into the stationary and decline phase of its growth cycle, still there was the continual increase in enzyme production until the end of the incubation period in both shake flask and bioreactor studies. The recombinants pGEX-6P-2-kerA and pET-30a(+)-kerA showed highest keratinase production at 5 h which were 240 U/ml and 310 U/ml in bioreactor.

Oxygen diffusion in shake flask was superficial while in bioreactor the employed mechanical system permits a higher retention time of the gas in contact to the liquid phase.

Bioreactor study also revealed the typical relationship between the bacterial growth and change in dO_2 concentration. Soon after the fermentation began, as the keratinase was produced with maximum growth rate, the concentration of the dO_2 dropped drastically until the cell entered into the stationary period, after which, there was an increase in dO_2 concentration as when cell entered into the decline phase oxygen requirement become less. This indicate a good correlation with growth, the enzyme synthesis and dO_2 utilization.

keratinase productivity was compared between shake flask and bioreactor under the similar conditions in terms of media composition, inoculum volume and physico-chemical parameters. Keratinase productivity were increased for pGEX-6P-2-kerA was 1.5 and pET-30a(+)-kerA was 1.9 fold respectively as compared to shake flask.

Use of purified keratinase for some technical applications

Biodegradation of feather keratin by microorganisms producing keratinases represents an alternative method to improve the nutritional value of feather waste and to prevent environmental contamination. There is growing interest in microbial proteases of commercial importance in many areas as environmental sciences, biomedicine and biotechnology. The use of microorganism to produce natural products and processes that benefits and improve our socio-economic lifestyles had been a part of our human history (Jayalakshmi *et al*., 2012). Since volatile and rising cost factors such as energy, raw materials and market competitiveness have a significant impact on the economic efficiency of biotechnological bulk productions for applications, industrial processes need to be steadily improved and optimized. Most of the hard-to-degrade keratin containing substrates like feathers or animal hairs is either discarded or incinerated, resulting in energy consumption and environmental pollution. Thus, an innovative eco-friendly method to deal with these wastes is urgently needed. Microbial degradation of keratin by keratinases has gained biotechnological interest in applications and represents a great opportunity to manage waste materials with no adverse impacts. This article deals with the importance of keratinase and its ongoing applications that can be further used in future to sustain environment. To demonstrate this approach, keratinase enzyme has been developed as an alternative platform for the use of some technical applications by *Bacillus licheniformis* MZK05.

Hair is composed mainly of keratin protein (90%) and a small amount of lipid (1–9%). The diameter of hair fibers varies between 40 and 150 μm and its major structure consists of a cuticle, cortex and medulla (Kajiura *et al*., 2006). Most hair fiber mass is in the cortex which is responsible for the great tensile strength of hair fiber. Protein hydrolysates, in particular those with low molecular weight distribution have been known to protect hair against chemical and environmental damage. Keratinase is a very good substitute for chemicals on hair treatment. Keratinase use in different type of hair treatment and hair rebonding. For hair rebonding test, hair was washed with a mild shampoo. A flat iron was used to straighten hair. Purified keratinase enzyme with moisturizer (1:5) was applied on bunch of curly hair and another bunch of curly hair was treated with only rebonding cream and the result indicated that purified keratinase applied hair showed better comparing to rebonding cream used hair. The main function of keratinase enzyme is to break down the di-sulfide bond of hair, particularly of curled hairs become straight and soft.

Feathers are largely produced as a waste by-product at poultry plants (Williams *et al*., 1991). They are insoluble structural proteins cross-linked by disulfide, hydrogen and hydrophobic bonds but could represent a rich protein resource because they contain over 90% (w/w) keratins. Keratins cannot be degraded by the usual proteolytic enzymes such as pepsin, trypsin and papain. Nevertheless, feathers do not accumulate in nature because keratins could be degraded by keratinases (EC 3.4.21/24/99.11) produced by some microorganisms (Onifade *et al*., 1998). For feather hydrolysis test, whole feather was immersed into 5 ml of purified keratinase enzyme of pGEX-6P-2-kerA and pET-30a(+)-kerA and incubated in shaking incubator for 24 to 48 hrs at 37° C. It was tested for 24 and 48 hrs that how much it was hydrolysed. After 48 hrs, pGEX-6P-2-KerA and pET-30a(+)-kerA both enzymes were completely hydrolysed comparing to wild MZK05. No hydrolysis occurred in control and partial hydrolysis observed in wild MZK05. Compared to pGEX-6P-2-kerA and pET-30a(+)-kerA, pET-30a(+)-kerA which was better hydrolysed compared to pGEX-6P-2-kerA.

Conventional dehairing of animal skins/hides in leather manufacturing, involves the generation of hydrogen sulfide with highly alkaline effluent due to the use of toxic chemicals like lime and sodium sulfide, can cause a serious health hazard to tannery workers and contributes to aquatic pollution.

Thus the present work was performed to find out the possible eco-friendly dehairing process of animal skins using keratinase preparation, obtained from the recombinant of *Bacillus licheniformis* MZK05. Investigation into the dehairing of skins and hides upon treatment with the enzymes were carried to measure the hair removal potential of the enzymes and as well as to determine visually the degree of its smoothness. The result exhibited that maximums area yield was found rapidly when skins were treated by conventional lime-sulfide method. However this treatment might have denatured and coagulated the skin proteins at high pH and subsequently the skin become wrinkled and lost its smoothness. In dehairing test, conventional dehairing process using CaO and Na_2S needed 26 hrs for complete dehairing of the goat skin. Both enzyme assisted and enzyme mediated also needed 26 hrs, but in comparison of enzyme assisted and enzyme mediated method, 95% hair removed in enzyme mediated method and 85% enzyme removed in enzyme assisted. In case of enzyme assisted method i.e. treatment with 5% Cao for 6 hrs followed by enzymatic action produced area yield comparable to the conventional method.

This may be possible because of soaking the goat skin in 5% Cao solution, during which collagen fibers swelled osmotically by taking up water from the lime solution contributed by removing the electrical charge from the basic groups in collagen and changing the dimentions of its structure and this decreased the cohesive forces between the fibers by breaking H-bonds, causing the fiber structure to become looser (Herfeld and Schubert 1969, Menderes *et al*., 2000) and opens up the collagen fiber bundles (Alexander *et al.*, 1986). Thus the keratinase enzymes can enter more easily into the skin and open the fibrous structure by degrading the inter febrile substances (Sivasubranani *et al*., 2008). This is followed by loosing of hair with an attack by enzymes on the outmost sheath and subsequent swelling and breakdown of the inner root sheath (Alexander, 1988).

8.2 CONCLUSION

The aim of this research was to clone the gene *ker*A encoding keratinase from the most potential strain of *B. licheniformis* in two plasmid systems, pGEX-6P-2 and pET-30a(+) and expression in heterologous system in *E. coli* BL21. This will follow the investigation into purification of the enzymes, their characterization and some technical applications. A comprehensive study was, therefore, carried out and the following outcomes were obtained which will definitely facilitate the application of keratinae enzyme in Bangladesh.

- *Ker*A gene was successfully amplified from potential strain of *B. licheniformis* by Polymerase Chain Reaction (PCR).

- *Ker*A gene encoding keratinase in *Bacillus licheniformis* MZK05 was cloned into plasmid vectors, pGEX-6P-2 and pET-30a(+) and expressed in heterologous system (*E. coli* BL21).

- In pGEX-6P-2 system, a 58 kDa GST-KerA fusion protein was expressed, subsequently cleaved by PreScission protease to produce the full length 39 kDa protein. On other hand, A 40 kDa His-KerA protein was purified by IMAC process using Ni^{2+}-NTA resin in pET-30a(+) system.

- The concentration of purified protein was found to be 119 mg/L which is an appreciable yield. The keratinase activity of the purified keratinase was found to be 312 U/ml (pGEX-6P-2-kerA) and 358 U/ml (pET-30a(+)-kerA) respectively. A 4 fold (pGEX-6P-2-kerA) and 4.5 fold (pET-30a(+)-kerA) increase in keratinase activity have been obtained by heterologous expression of keratinase than the wild-type organism.

- Sequencing and prediction of 3D model of the expressed protein were performed. *ker*A gene of *B. licheniformis* MZK-05 showed 100% identity with thermostable keratinase of *Bacillus licheniformis* DS23, *Ker*A of *Bacillus licheniformis* OWU 1411T and 98.4% identity with *ke*rA of *B. licheniformis* PWD-1

- Purified keratinase was characterized for various parameter such as temperature, pH, metal ions and nitrogen sources.

- The whole genome sequencing of *Bacillus licheniformis* MZK05 was performed. The genome of strain *Bacillus licheniformis* MZK05 is 4,145,737 bp long with a GC content of 43.05%, containing 85 tRNA genes and 6 rRNA operons.

- The bioreactor produced elevated productivity for pGEX-6P-2-kerA (48000 U/L/h) and pET-30a(+)-kerA (62000 U/L/h) when compared to the shake flask cultures. The result showed that the productivity of pGEX-6P-2-kerA and pET-30a(+)-kerA in bioreactor were 1.5 and 1.9 times higher as compared to the shake flask.

- Successfully used for technical applications like dehairing, rebonding and feather degradation.

Therefore, the cloning of *kerA* gene from *B. licheniformis* MZK05 into pGEX-6p-2 and pET-30a(+) vectors, its expression in *Escherichia coli* BL21 host and purification, characterization and its applications were performed which will be the basis for industrial production of keratinase in Bangladesh.

CHAPTER 9

REFERENCES

9.0 REFERENCES

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PUBLICATION AND CONFERENCE PRESENTATIONS

Publication:

Nahar M, Shishir MA, Waliullah S, Haque S, Ilias M, Karim MM, Khan SN and Hoq MM. 2016. Cloning, expression and structure simulation of keratinase from *Bacillus licheniformis* strain MZK05. Malaysian Journal of Microbiology. Vol. 12(2) **p.** 182-190.

Conference presentation:

1. **Mukitu Nahar**, Shakila Nargis Khan, Muhammad Manjurul Karim, Md. Mozammel Hoq. **High level expression of recombinant keratinase from *Bacillus licheniformis.*** Presented at the "The 13th Asian Congress on Biotechnology,'' July 23-27, 2017, Khon kaen, Thailand.

2. **Mukitu Nahar**, Shakila Nargis Khan, Muhammad Manjurul Karim, Md. Mozammel Hoq. **Gene cloning and expression of a recombinant protein, keratinase from *Bacillus licheniformis* for industrial applications.** Presented at the 30th Annual Conference, Bangladesh Society of Microbiology, April 29, 2017, Dhaka University, Dhaka.

3. **Mukitu Nahar,** Shakila Nargis Khan, Muhammad Manjurul Karim, Md. Mozammel Hoq. **Gene cloning and expression of a recombinant protein, keratinase from *Bacillus licheniformis* for industrial applications.** Presented at the 1st International GCSTMR Congress, Nawab Ali Chowdhury Senate Bhaban, February 4-5, 2017, Dhaka, Bangladesh.

4. **Mukitu Nahar,**Md. Asaduzzaman shishir, Muhammad Manjurul Karim, Shakila Nargis Khan, Md. Mozammel Hoq. **Cloning, expression and structure simulation of keratinase from *Bacillus licheniformis* MZK05.** Presented at the "The12th Asian Congress on Biotechnology,'' November 15-19, 2015.

5. **Mukitu Nahar**, Md. Asaduzzaman shishir, Muhammad Manjurul Karim, Shakila Nargis Khan, Md. Mozammel Hoq. **Cloning, expression and structure simulation of keratinase from *Bacillus licheniformis* MZK05.** Presented at the 2nd GNOBB Conference, "International Conference of Biotechnology in Health and Agriculture (ICBHA)", January 9-10, 2015, Nawab Ali Chowdhury Senate Bhaban, Dhaka, Bangladesh.

APPENDICES

APPENDIX – A

CHEMICALS AND REAGENTS

All chemicals were of analytical grade and were purchased from a variety of suppliers.

Name of chemicals/reagents/substrates	**Source**
Acrylamide	Carl Roth, Germany
Agar	Sigma, USA
Agarose	Promega, USA; Carl Roth, Germany
Aluminium sulfate	Merck, India
Ammonium persulphate	Wako, USA
Ammonium sulfate	Merck, India
Azo-casein	Sigma, USA
BCIP/NBT solution	Thermo Fisher Scientific, USA
Bis-acrylamide	Carl Roth, Germany
Boric acid	Merck, India
Bromophenol Blue	Wako, USA
Bovine serum albumin (BSA)	Sigma, USA
Calcium chloride ($CaCl_2$)	Sigma, USA
Coomassie Brilliant Blue G250	Thermo Scientific, USA
Di potassium hydrogen phosphate	Merck, Germany
Di sodium hydrogen phosphate	Merck, Germany
Dithiothreitol (DTT)	American Bio analytical, USA
EDTA	BDH, England
Ethanol (EtOH)	Merck, Germany
Ethidium bromide (EtBr)	Sigma, USA
Ferric ammonium citrate	Sigma, USA
Glacial acetic acid	Merck, Germany
Glucose	Sigma, USA
Glycerol	Sigma, USA
Glycine	Wako, USA
Goat Anti-Mouse IgG+HgM	Thermo Fisher Scientific, USA
Hydrochloric acid (HCl)	Merck, Germany
Immersion oil	Merck, Germany
IPTG	Sigma, USA
Imidazole	Thermo Fisher Scientific, USA
Potassium chloride	Sigma, USA
Potassium dihydrogen orthophosphate	Merck, Germany
Lactose	Sigma, USA
Lysozyme	Wako, USA
Methanol	Sigma, USA
Magnesium chloride	Sigma, USA
Magnesium sulfate	Sigma-Aldrich
Manganese chloride	Merck, Germany
Sodium chloride	Sigma, USA

Sodium sulfide	Sigma-Aldrich
Peptone	Oxoid, England
Phosphoric acid	Merck, Germany
Phenol red	Sigma, USA
Potassium hydrogen phosphate	Sigma, USA
Protein marker	Precision plus protein standards (All blue), Bio-Rad, USA; Pre-stained protein Marker, NEB, England
Sodium acetate	Merck, Germany
Sodium dodecyl sulphate (SDS)	Wako, USA
Sodium hydroxide (NaOH)	Sigma, USA
Tri-chloro acetic acid	Sigma, USA
Tris base	Sigma, USA
Triton X-100	Dae-Jung chemicals
Tryptone	BD, USA
Tryptose	BD, USA
Tween-80	Merck
Zinc chloride	Sigma-Aldrich
6x-His Tag Antibody	Thermo Fisher Scientific, USA

APPENDIX – B

MEDIA COMPOSITION

LB broth and agar

Ingredients	**Amount (g/L)**
NaCl	10.0
Tryptone	10.0
Yeast extract	5.0
pH	7.0
Agar (for LB agar)	15
Distilled water	Up to 1000 ml

Directions: Ingredients are dissolved in distilled water by stirring with gentle heating. Medium is sterilized by autoclaving at 121°C for 15 min.

Nutrient Agar

Ingredients	**Amount (g/L)**
Peptone	5.0
NaCl	5.0
Beef extract	3.0
Agar	15.0
pH	7.0
Distilled water	Up to 1000 ml

Directions: Ingredients are dissolved in distilled water by stirring with gentle heating. Medium is sterilized by autoclaving at 121°C for 15 min.

APPENDIX - C

BUFFERS AND SOLUTIONS

Agarose Gel loading dye (6×, 200ml)

Bromophenol Blue (0.5 g), Xylene Cyanol (0.5 g) and Glycerol (30 ml) were mixed well and the volume was adjusted to 200 ml with deionized water.

Ampicillin stock solution (10mg/ml)

100 mg in 10 ml dH_2O, filter sterilized and stored at 4°C in 1 ml aliquots. 100 µl from the stock was used for each plate.

Azo-casein solution (1.0%)

Azo-casein solution was prepared by dissolving 1.0 g of azo-casein in 100 ml 0.05 M Tris-HCL buffer (pH: 8.5). The solution was preserved at 4^oC.

Bradford Reagent

100 mg of Coomassie blue G250 in 50 mL of 95% ethanol and the solution was then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Whatman No. 1 filter paper and stored in an amber bottle at RT. The reagent was also filtered before use as the dye might precipitate from the solution.

EDTA (0.5 M)

186.1 g of Na_2EDTA. 2H2O (disodium ethylene diamine tetra-acetic acid) and 20g of NaOH pellets were added to 800 mL distilled water and dissolved by stirring on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at RT.

Elution buffer

Ingredients were mixed in a way so that the final concentration becomes 20 mM Tris-HCl (7.7)/KOH 7.4, 100 mM KCl, 250 mM Imidazol.

Ethidium bromide solution (staining solution)

Thirty (30) µl of Ethidium bromide (EtBr) was dissolved in 150 ml 1×TBE buffer and kept in the dark place.

Lysis buffer

Ingredients were mixed in a way so that the final concentration becomes 20 mM Tris-HCl (7.7)/ KOH 8.0, 100 mM KCl, 20 mM Imidazol.

1× Laemmli buffer

Ingredients were mixed in a way so that the final concentration becomes 50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 100 mM DTT and 0.1% bromophenol blue.

NaOH (10 M)

40 g of NaOH pellet was dissolved in distilled water to final volume of 100 mL. The solution was stored in an airtight bottle at RT.

Normal saline

Normal saline was prepared by dissolving 0.85g NaCl in 100ml of distilled water and sterilized by autoclaving.

Phosphate buffer

Na_2HPO_4 was dissolved in distilled water to make a 0.05 M solution and the pH was adjusted to appropriate value with 0.05 M Na_2HPO_4.

PreScission cleavage buffer

PreScission cleavage buffer was prepared by adding 50mM Tris-HCl (pH 7.0), 150mM NaCl, 1mM EDTA and 1mM dithiothreitol.

PreScission protease mixture

40 µL (80 units) of PreScission Protease was added into 960 µL of PreScission Cleavage Buffer.

SDS (10%)

10g of SDS (Sodium dodecyl sulfate) was added to 80 mL of distilled water and dissolved by stirring on a magnetic stirrer slowly to avoid foaming. The final volume was adjusted to 100 mL with distilled water and stored at RT.

Tris-HCl (1.0 M)

121.1 g tris-base was dissolved in 800 mL of distilled water. The pH was adjusted to the desired value by adding concentrated HCl and the final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature (RT).

TBE buffer (10×)

108.8 g of Tris base and 55 g boric acid were mixed and dissolved in 800 ml of deionized water and 40 ml of 0.5 M EDTA (pH 8.0) was added into the solution. The volume was adjusted to 1000 ml with deionized water.

TE buffer (pH 8.0)

10 mM tris-Cl (pH 8.0), 1 mM EDTA was prepared by diluting concentrated stocks of 1M tris-Cl (pH 8.0) and 0.5 M EDTA. The buffer was stored at 4^{o}C.

Reagents for SDS-PAGE

1. 30% acrylamide-bisacrylamide solution:

Acrylamide	29.0 g
Bisacrylamide	1.0 g
Distilled water	100 ml

2. 10% ammonium persulphate (APS)

APS	1.0 g
Distilled water	10 ml

Stored at 4^{o}C

3. 0.1% BPB (Bromophenol blue solution) or tracking dye

Bromophenol blue	0.1 g
Distilled water	100 ml

4. Staining solution

Coomassie brilliant blue G-250	0.20 g
Phosphoric acid (85%)	20 ml
Aluminium sulfate	50 g
Absolute Ethanol	100 ml

Deionized water was added to adjust the volume up to 1000 ml.

5. Sample loading buffer

0.5 M tris-Cl (Upper gel buffer)	10 ml
10% SDS	10 ml
1.0 M DTT	5 ml
Glycerol	10 ml
Distilled water	14 ml

6. Electrophoresis buffer (pH 8.3)

Tris-base	3.0 g
Glycine	14.4 g
10% SDS	10 ml
Distilled water	1000 ml

7. Upper gel buffer (pH 6.8)

Tris-base	18.17 g
SDS	0.4 g
pH adjusted to 8.8 by adding HCl	
Distilled water	Up to 100 ml

8. Lower gel buffer (pH 8.8)

Tris-base	18.17 g
SDS	0.4 g
pH adjusted to 8.8 by adding HCl	
Distilled water	Up to 100 ml

Reagents for Western Blot

1. Transfer Buffer (10X)

Glycine	144 g
Tris-base	30.2 g
Methanol	200 ml
Distilled water	Up to 900 ml

2. Blocking solution

Skim milk	2 g
PBS	100 ml

3. Washing solution

Tween-20	100 µl
PBS	100 ml

Wash buffer

Ingredients were mixed in a way so that the final concentration becomes 20 mM Tris-HCl (7.7)/KOH 7.4, 100 mM KCl, 20 mM Imidazol.

APPENDIX – D

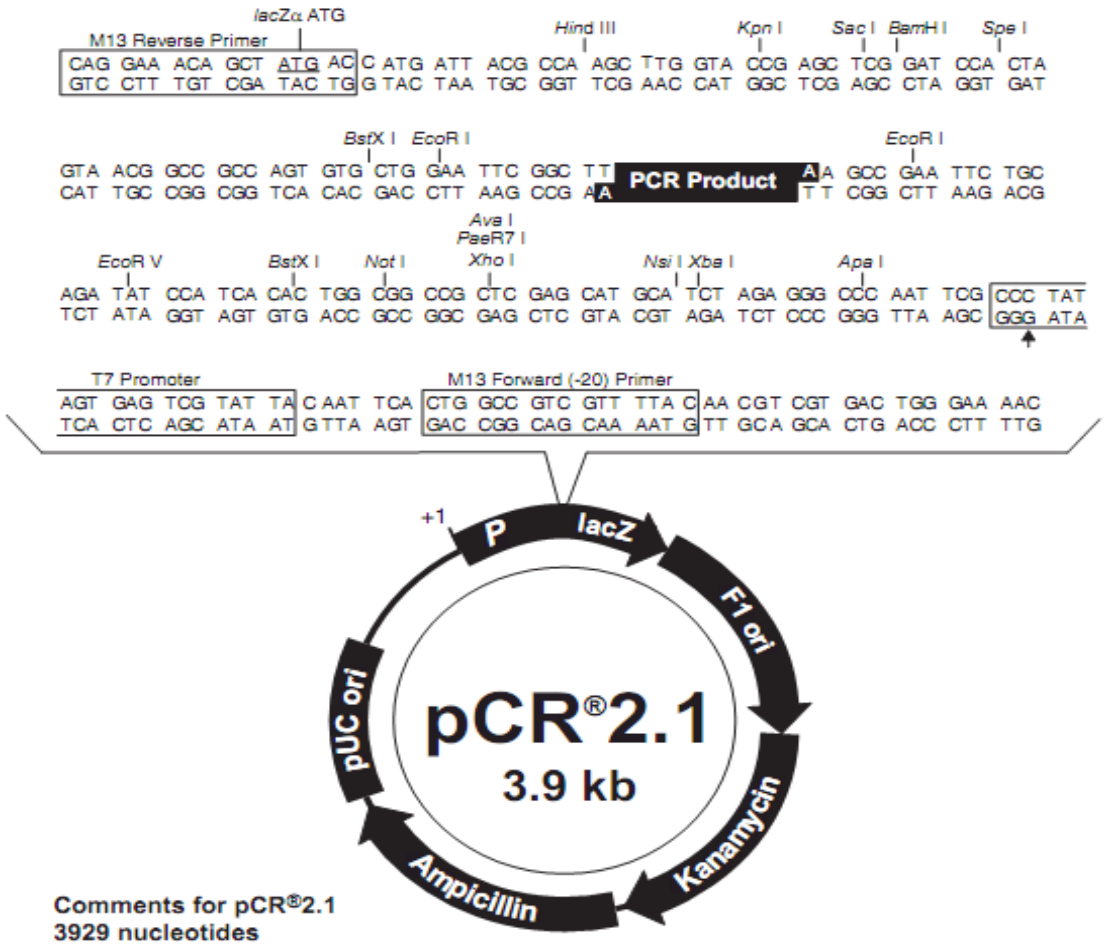
LIST OF EQUIPMENTS

Equipment	**Model/ Company**
Autoclave	Hirayama model HL-42, AE, Japan
Biosafety cabinet	ESCO Class II BSC, USA
Centrifugation	Biofuge Primo (Heraeus) and Hittch Mikro-Rapid
Electronic balance	SHIMADZU, ELB200
Electrophoretic chamber	BIORAD, USA
Filters	0.45μm pore size; Millipore corp, Whatman filter paper
Glassware sterilizer	Redline, Blinder, Germany
Incubator	Redline, Blinder, Germany
Magnetic stirrer	CIMAREC, Barnsted Thermolyne
Micropipettes	Eppendorf research and Nichiryo
Nano drop spectrophotometer	Nanodrop 2000, Thermoscientific
Orbital shaker	Excella E25 Incubator Shaker series, New Burnswick Scientific :
pH meter	INOLAB WTW series, pH 720
Power supply	BIORAD, USA
Refrigerator (4^oC)	Royal Frestech
Spectrophotometer	Genesys 5, Thermospectronic
Thermal cycler	MJ, Bio-Rad
Thermo stated shaking water bath	Water Bath 1083, GFL
Vortex mixture	VM-2000, DIGISYSTEM

APPENDIX – E

GENOMIC MAP OF VECTORS

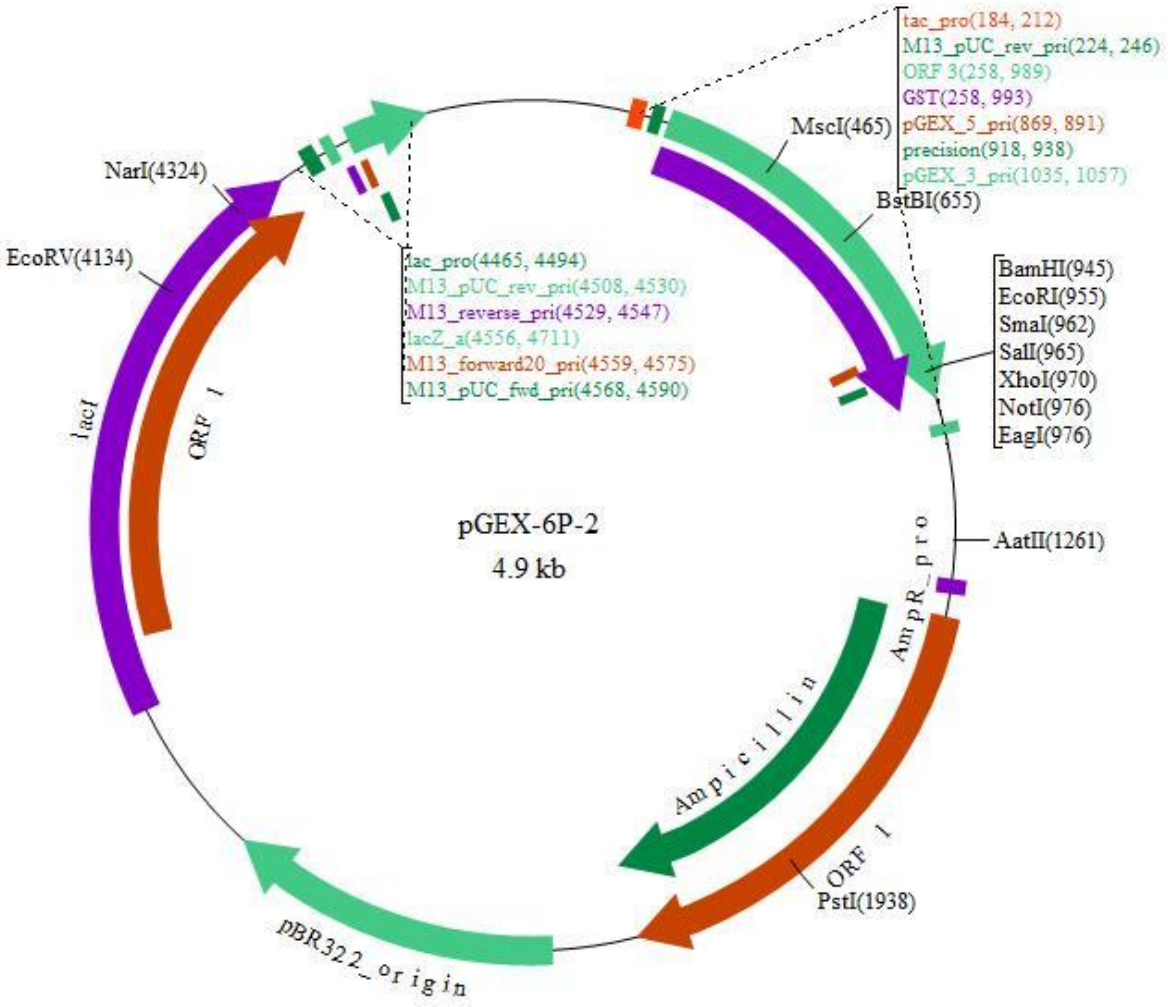
A) Map of linearized pCR2.1 vector:



Comments for pCR®2.1
3929 nucleotides

*Lac*Zα gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

B) Map of pGEX-6p-2 vector:



C) Map of pET-30 a(+) vector: