UNIVERSITY OF BORÅS

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Chromosomal Integration of *KerA* Gene in *Bacillus megaterium*

For Stable Keratinase Production

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Keywords: *Bacillus lichiniformis*, Chromosomal integration, *Bacillus megaterium*, integration vector, pMUTIN GFP+6192, Keratinase

Abstract

In order to develop a stable strain of *Bacillus megaterium* for Keratinase production, the Keratinase gene (*KerA*) of *Bacillus lichiniformis* ATCC 53757 and *SPlipA* gene from plasmid pHIS1525.SPlipA (*Bacillus megaterium* origin) were PCR amplified and constructed to give a gene cassette called *SPK* . Then the gene cassette *SPK* was cloned into the Integration vector, pMUTIN-GFP+ 6192bps and transformed in *Bacillus megaterium* ATCC 14945. The chromosomal integration was created using homologous single crossing over mechanism. The strong natural promoter from the chromosomal locus of the *SPK* not only produced the increased extracellular enzyme, but also functions as a non inducible promoter which does not require any inducer for the production of the enzyme in the new integrant strain. The integrant strain was subjected to feather degradation test and found that it could totally digest the feather meal in complete seven days, resulting in a rich fermentation broth.

Keywords: *Bacillus lichiniformis*, Chromosomal integration, *Bacillus megaterium*, integration vector, pMUTIN GFP+6192, Keratinase

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List of Abbreviations

Btu…… British thermal unit

COD……..Chemical Oxygen Demand

bp.…….……Base Pairs

Orf…….Open Reading Frame

GFP……...Green Fluorescent Protein

IPTG……..Isopropyl β-D-1 thiogalatopyranoside

rRNA…….Ribosomal Ribose Nucleic Acid

LB……..Lysogeny Broth

BGSC…….Bacillus Genetic Stock Center

TE……...Tris-EDTA

TBE……Tris-Boric-EDTA

EtBr……Ethidium Bromide

PCR……Polymerase Chain Reaction

Tm……..Melting Temperature

GC…….....Guanine Cytosine content

TCA……Trichloroacetic acid

1. Introduction

The increasing world population and the growing world economics has seriously lead to a strong energy crisis, which in turn has created a critical demand for energy recourses because of the constant depletion of the existing fossil fuels. So for the past few decades the world countries have started looking for an alternative renewable resources, which can last forever, such kinds of renewable recourses include sunlight, wind, rain, tides, geothermal heat and biomass[1]. Considering the biomass, chicken feather was taken into the account for biogas production. It has been calculated that an average of about eleven billion pounds of chicken feathers are shredded every year from the poultry industry. Usually chicken feathers are turned into a low value feed stock or dumped in the landfills [2] chicken feathers are made up of keratin, the same tough, tightly wound protein fiber that makes up hair, wool, fingernails and hooves. Based on the tough and hard properties of feathers, it can be directly used for making fibers and composites, which can be further used as a construction material[3]. When the chicken feathers are hydrolyzed, the digested meal can be used as low value feedstock and also it can be used as a promising biomass source for the biogas production[4]. Even though there is lots of hydrolyzing process like thermal and chemical processes available, they cannot be used because of its high economic consideration, environmental situation and lack of recourses for thermal energy. So the enzymatic hydrolysis process was taken into the account, which is considered to be an efficient and successful method to digest the keratin rich feathers, even then the production of pure enzyme and treating with the raw feathers would result in much cost effect, so by treating the raw feathers with keratinase producing bacteria could end up with the better solution. So the naturally keratinase producing bacteria *Bacillus lichiniformis* paved a way for this problem. As *Bacillus lichiniformis* is a wild type strain it is not suitable for the industrial scale pretreatment processes because of its low yield of enzyme production, unstable and improper growth conditions, so to avoid this problem the recombinant DNA technology must be used.

Using the recombinant strain in the pretreatment process for the enzyme production could be reasonably cheap and efficient method. Production of recombinant keratinase by expression plasmids based strains has been largely published and reported [5-10], even though the classical, ‘vector based expression’ can be an efficient enzyme producing system, but it cannot be used in the pretreatment process due to Segregational instability of the vector. To overcome this problem integration of the keratinase coding gene into the main chromosome can be more stable and can lead to high enzyme production system.

Considering the above mentioned problems it was decided to construct chromosomally integrated strain carrying the *KerA* gene and decided to choose the *Bacilluis megaterium* as a host cell because of its industrial application and the known geneticall knowledge. So the ultimate aim of this project is to integrate the keratinase coding *KerA* gene from *Bacillus lichiniformis* into the chromosome of expression host *Bacillus megaterium*. So that it can produce more stable and high enzyme yield, which in turn can be used in the pretreatment process of chicken feathers for biogas production

2. Background

2.1 Energy crisis

The alarming increase in the world population and the rapid economic development of world countries has led to an indomitable energy crisis, in a recent research survey conducted by the U.S Energy ministry, it has estimated that the total world consumption of marketed energy increases by 49 percent from 2007 to 2035 at the rate of 1.4 percent per year; from 495 quadrillion Btu in 2007 to 739 quadrillion Btu in 2035. Fig 1, clearly illustrates the projection of the world marketed energy consumption, 1990-2035[1].

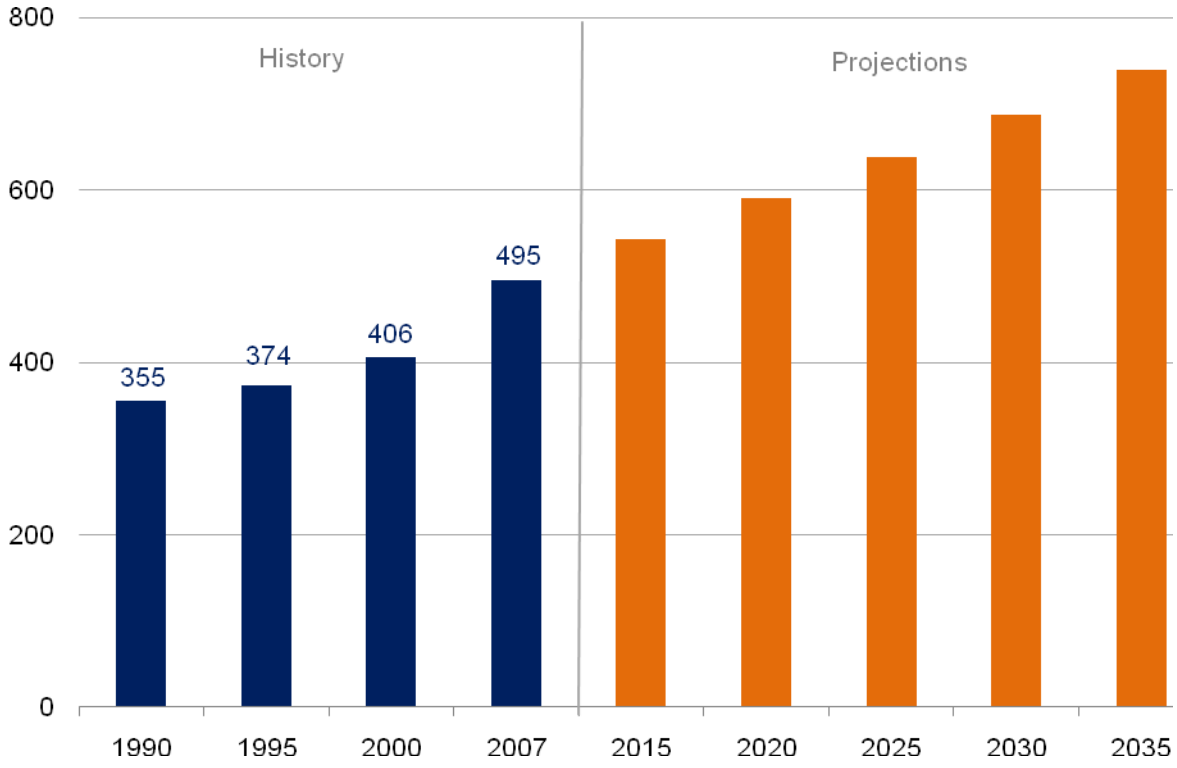


Figure 1 World marketed energy consumption in quadrillion Btu, 1990-2035 [1]

2.2 Renewable Energy

For the past few decades the world is facing a serious energy crisis this is due to the constant depletion of the non renewable energy recourses and so now the world attention has turned towards the renewable energy for the sustainable. Renewable energy can be defined as the kinds of energy that meets the needs of the present without compromising the ability of the future generation to meet their needs, in simple words the 'Energy which strictly follow the

life cycle assessment pathway'. The renewable energy generally comes from the natural recourses such as sunlight, wind, rain, tides, geothermal heat and Biomass. Fig 2, clearly illustrates the U.S Energy consumption by Energy source, 2009[11]; in which the different types of renewable and non-renewable energy sources are clearly differentiated and also the renewable energy part is exaggerated to show the various types of renewable energy consumed.

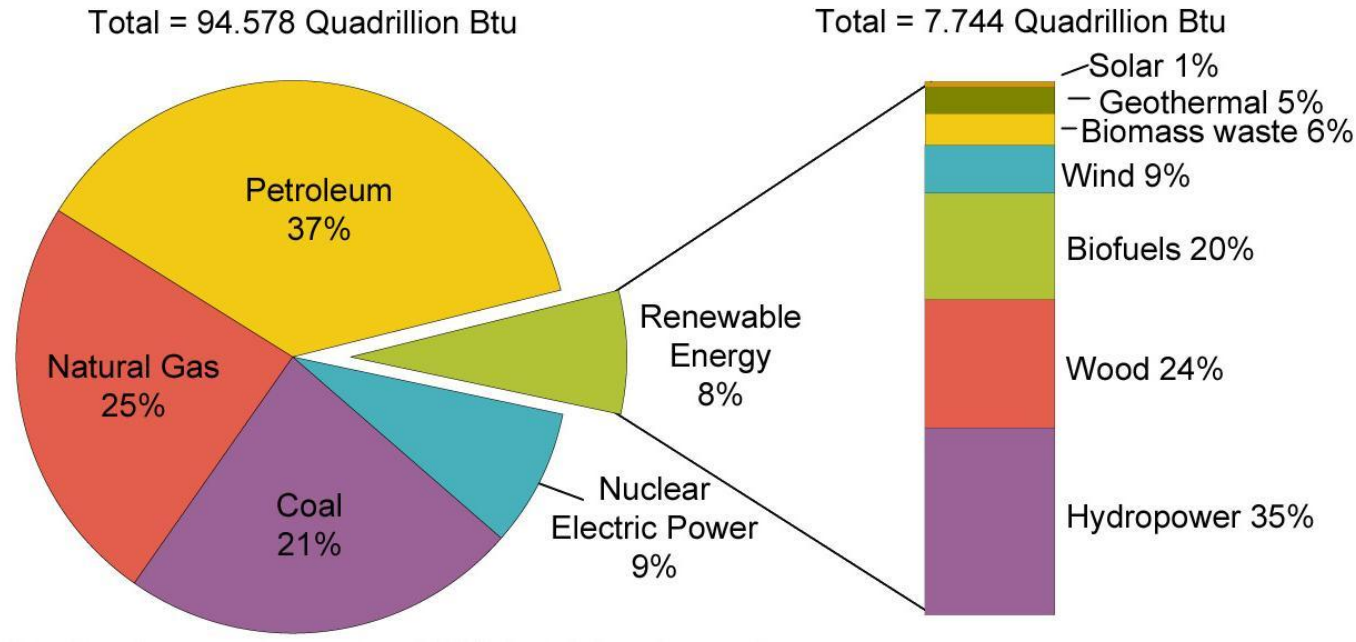


Figure 2 US. Energy consumption by Energy source 2009 [11].

2.3 Biomass

Biomass is one of the most promising renewable recourses for bio-energy harnessing, which in turn can be used for many applications; generally biomass can be a plant, plant derived materials and animal matters, so biomass can exist in a variety of forms like solid stock, herbaceous matter, seeds, algae, bio-waste, crop residues, animal wastes and manure. The bio-energy from the biomass can be obtained in the form of Biogas, Ethanol, Biodiesel, Bio-oil and syngas, through a special pathway which in turn the Bio-energy can be used for heating, power, transportation and Bio-refineries [12]. Fig 3, elaborately illustrates the method of conversion of Biomass into Bio-energy products and its application.

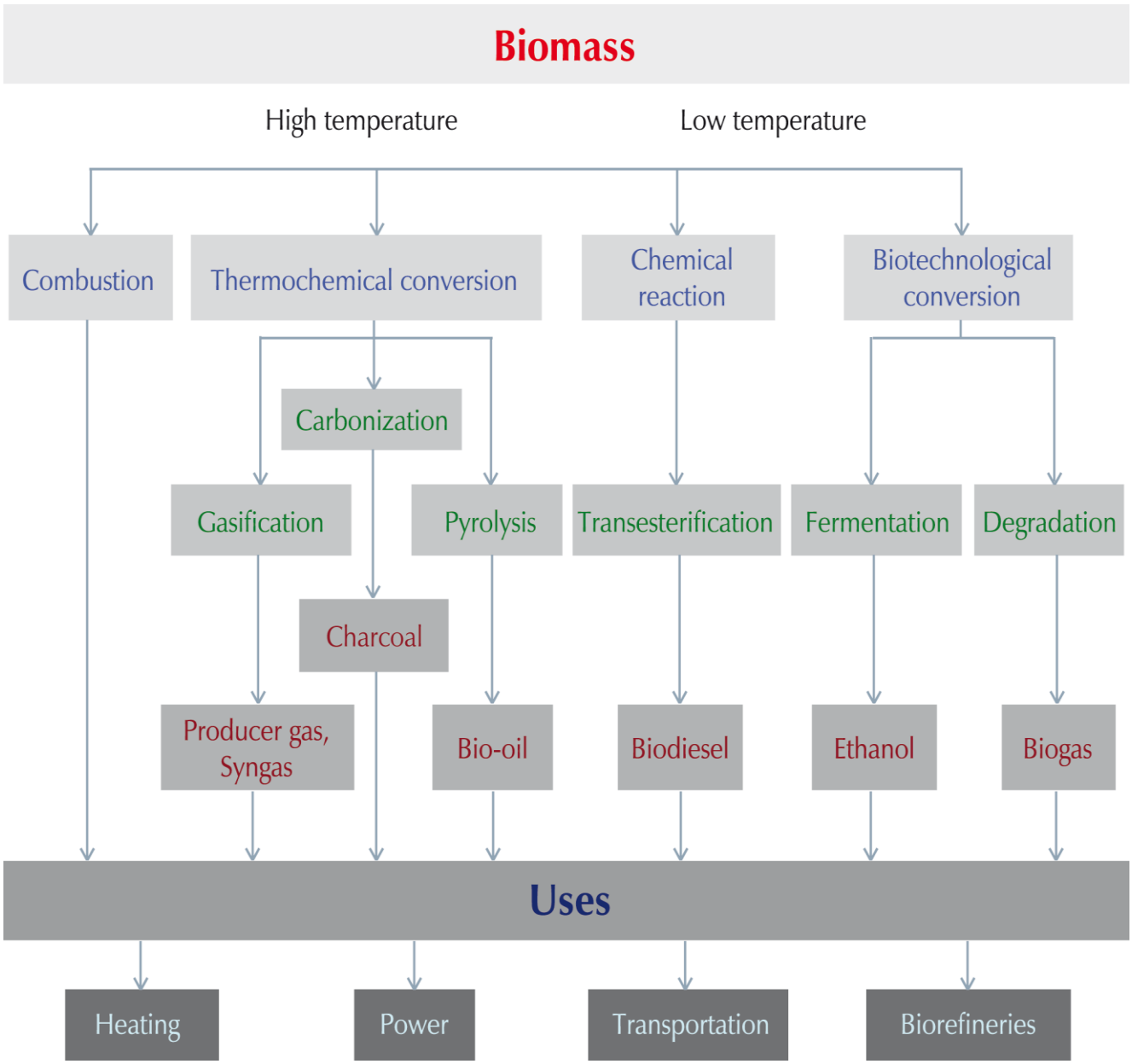


Figure 3 Technologies for converting biomass to energy [12]

2.4 Biogas

Biogas is one of the versatile, sustainable bio-energy obtained through the anaerobic digestion of the biomass. Generally biogas is produced from the sources like plant material, municipal sewage, animal waste and manure. The whole biogas production through anaerobic digestion process takes place in four major steps, which includes Hydrolysis, Acidogenesis, Acetogenesis and Methanogenesis. During the hydrolysis process the insoluble complex

organic compounds such as carbohydrates, proteins and lipids are broken down to simpler organic substances like sugars, amino acids and fatty acids respectively, and then these organic materials passes through the Acidogenesis and Acetogenesis pathway and reaches the Methanogenesis, where the special Methanogenic bacteria converts the intermediate organic matters to complex Biogas. [13] Fig 4, elaborately shows the Anaerobic digestion pathway, such produced biogas usually contains the complex mixture of gases methane, carbon dioxide and other trace gases Table. 1 clearly shows the mixture of gases according to their percentage[14].

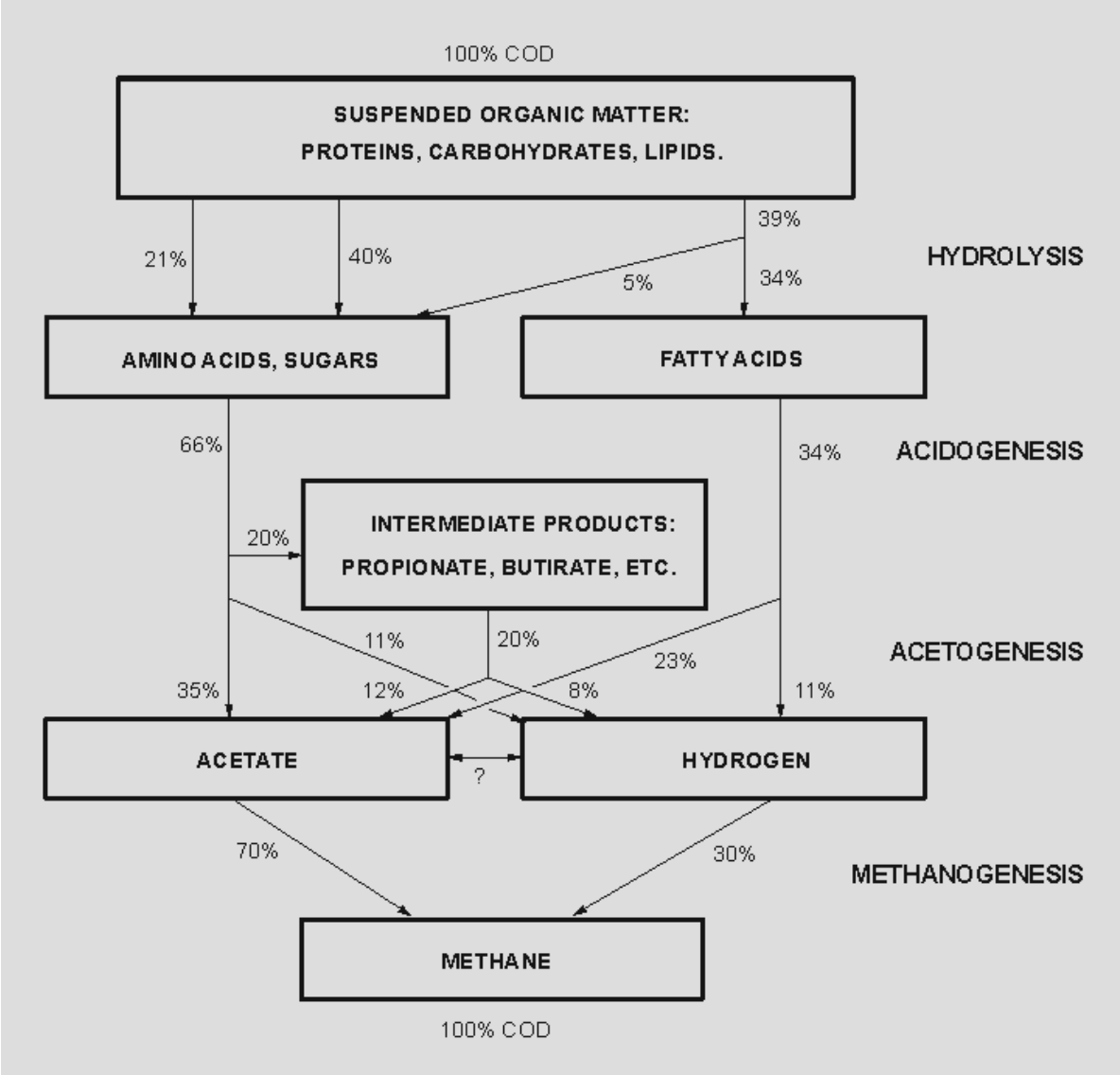


Figure 4 General schema of anaerobic digestion [13]

Table 1 General Composition of biogas [14]

Matter		**Percentage (%)**
Methane	CH_4	50 - 75
Carbon dioxide	CO_2	25 - 45
Water vapor	H_2O	1 - 2
Carbon monoxide	CO	0 - 0,3
Nitrogen	N_2	1 - 5
Hydrogen	H_2	0 - 3
Hydrogen sulfide	H_2S	0,1 - 0,5

2.5 Chicken feathers for biogas production

Annually vast quantities of chicken feathers are shredded from the poultry industry but it is a very inconvenient and troublesome waste product of the poultry farming industry. Usually most of the feathers are ground up and used as filler for the animal however this use has the potential to pass harmful bacteria along with the animal that ingest the feather meal. [15] As the chicken feather is made up of 88% of fibrous protein-keratin, it can be used as a promising biomass source for biogas production when it is hydrolyzed. The special characteristics of chicken feather as a biomass are mainly due to the fibrous protein keratin itself, keratins belong to the family of fibrous proteins, which mainly occur in higher vertebrates like reptiles, birds and mammals, and exercise as a protective in function. The major sources of keratins are hair, skin, feather and nails[16]. Keratins are a group of water insoluble proteins, molecular weight ranges from 49-70 k, which can form a 10-nm tonofilaments in a wide variety of epithelial cells [17]. Keratins are formed through disulphide, hydrogen and other cross linkages. Keratins are structurally classified into two major groups they are, α and β keratins. The chicken feather is a major constituent of β-helix structure mostly twisted and cross linked by disulfide bridges. The amino acid content of keratin is characterized by a high cystine and serine content which is 18% and 15% wt respectively, it lacks from hydroxyproline and hydroxylisine. The chemical activity of the keratin is determined by the

degree of cystine content, the disulphide bonds formed between two molecules makes keratin strong and proteolitic resistance[18] Fig 5, shows the general structure of keratin[19].

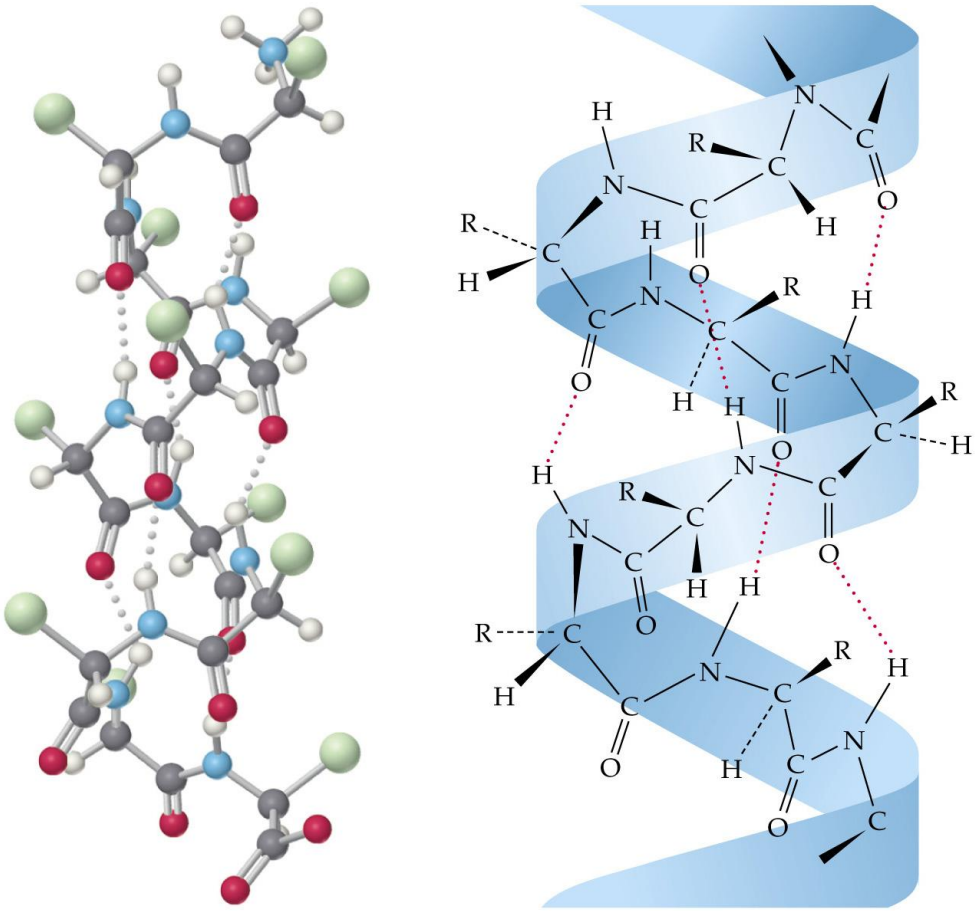


Figure 5 General structure of keratin [19]

2.6 Enzymatic hydrolysis of chicken feathers

Hydrolysis is considered as one of the crucial steps in anaerobic digestion process where the complex polymer substance is broken down to simpler monomers. The major hydrolysis technology includes physical treatment like heat and pressure, chemical treatment like alkali treatment and detergent treatments and biological treatment like enzymatic hydrolysis. Among the all, enzymatic hydrolysis is considered as an important process, because of its high efficiency and economic consideration, the enzymatic hydrolysis of chicken feather is

carried by the enzyme keratinase. Keratinase is a proteolitic enzyme and classified under proteinase, which mainly attacks the disulfide bridges of keratin. The keratinase production by various microorganisms has been already reported. The work includes keratinase production by fungi and bacteria. *Bacillus lichiniformis* PWD-1 was found to contain the *kerA* gene, which has the size of 1441bp in whole length and 1140bp of protein coding region, responsible for the keratinase secretion [20], which is shown in the Fig 6. After the discovery of *kerA* gene, many works based on the keratinase has been carried out using recombinant DNA technology on various microorganisms. The *kerA* gene has been used as a gene source and expressed in different organisms like *Bacillus subtilis*[21] *Pichia pastoris*[8] and *Bacillus megaterium*[7] and found to work efficiently in keratinase production.

```
   1 ctcctgccaa gctgaagcgg tctattcata ctttcgaact gaacattttt ctaaaacagt
  61 tattaataac caaaaaattt taaattggcc ctccaaaaaa ataggcctac catataattc
 121 attttttttc tataataaat taacagaata attggaatag attatattat ccttctattt
 181 aaattattct gaataaagag gaggagagtg agtaatgatg aggaaaaaga gtttttggct
 241 tgggatgctg acggccttca tgctcgtgtt cacgatggca ttcagcgatt ccgcttctgc
 301 tgctcaaccg gcgaaaaatg ttgaaaagga ttatattgtc ggatttaagt caggagtgaa
 361 aaccgcatct gtcaaaaagg acatcatcaa agagagcggc ggaaaagtgg acaagcagtt
 421 tagaatcatc aacgcggcaa aagcgaagct agacaaagaa gcgcttaagg aagtcaaaaa
 481 tgatccggat gtcgcttatg tggaagagga tcatgtggcc catgccttgg cgcaaaccgt
 541 tccttacggc attcctctca ttaaagcgga caaagtgcag gctcaaggct ttaagggagc
 601 gaatgtaaaa gtagccgtcc tggatacagg aatccaagct tctcatccgg acttgaacgt
 661 agtcggcgga gcaagctttg tggctggcga agcttataac accgacggca acggacacgg
 721 cacacatgtt gccggtacag tagctgcgct tgacaataca acgggtgtat taggcgttgc
 781 gccaagcgta tccttgtacg cggttaaagt actgaattca agcggaagcg gatcatacag
 841 cggcattgta agcggaatcg agtgggcgac aacaaacggc atggatgtta tcaatatgag
 901 ccttgggggа gcatcaggct cgacagcgat gaaacaggca gtcgacaatg catatgcaag
 961 aggggttgtc gttgtagctg cagcagggaa cagcggatct tcaggaaaca cgaatacaat
1021 tggctatcct gcgaaatacg attctgtcat cgctgttggt gcggtagact ctaacagcaa
1081 cagagcttca ttttccagtg tgggagcaga gcttgaagtc atggctcctg gcgcaggcgt
1141 atacagcact tacccaacga acacttatgc aacattgaac ggaacgtcaa tggtttctcc
1201 tcatgtagcg ggagcagcag ctttgatctt gtcaaaacat ccgaaccttt cagcttcaca
1261 agtccgcaac cgtctctcca gcacggcgac ttatttggga agctccttct actatgggaa
1321 aggtctgatc aatgtcgaag ctgccgctca ataacatatt ctaacaaata gcatatagaa
1381 aaagctagtg tttttagcac tagctttttc ttcattctga tgaaggttgt ccaatatttt
1441 gaatccgttc catgatc
```

Figure 6 The 1441bp sequence of *KerA* gene. **[20]**

2.7 *Bacillus megaterium* as a host cell

Bacillus megaterium is a gram-positive, rod shaped soil bacteria. Recently it has found a great economic importance because of its commercially important products, such as penicillin amidase and steroid hydalases; it is also the major aerobic producer of vitamin B. The genetic techniques such as transduction, transposition, protoplast fusion and plasmid transformation become developed enough in *Bacillus megaterium*, to apply them to the study of its metabolic and developmental function. *Bacillus megaterium* has proven to be an excellent host for the expression of non-homologous DNA. It can produce high yield and stable protein expression;

it has xylose operon tightly regulated and efficiently inducible by xylose. *Bacillus megaterium* has the advantage, that none of the alkaline protease is present. This fact enables an excellent cloning and expression of foreign proteins production without degradation, suited for industrial large scale protein production [22].

2.8 Segregational instability

The loss of an entire plasmid from its host is termed segregational instability; it has been proposed that only naturally occurring, low-copy-number plasmids are stably maintained by partitioning functions [23]. Even though the host cell is compatible for genetically engineered plasmids or vectors, in most cases the vectors are not stable enough for industrial scale production or pretreatment process. The segregational instability of plasmids can be due to defective partitioning at cell division and also can be due to environmental conditions in which plasmid-bearing cells are grown. Other factors which would be expected to influence the segregational instability of a plasmid include properties of the plasmid itself, in most naturally occurring plasmids have a complex mechanism to control copy number and in the case of constructed low copy number plasmids, it is only the certain sequences, which ensure the efficient partitioning of plasmid DNA molecules at cell division[24]

2.9 Chromosomal integration

The severity of instability problems affecting hybrid plasmids in Bacillus has lead to great deal of interest in developing expression systems, in which the gene of interest is inserted into the chromosome. Several genes which could not be established or stably maintained on conventional plasmid vector have been stabilized by chromosomal integration[23]. One of the easiest ways to achieve chromosomal integration is to use integration vectors. Integration vectors are the plasmids that have a controlled replication along with a selectable marker; if the plasmid is transformed into the respective host, all transformants will have integrated the plasmids into their chromosome without replicating as new plasmid inside the host, and the selectable marker always specifies antibiotic resistance. Usually controlled replication means that the plasmid has replication functions that work only in *E.coli* (carrier cell) but not in the host bacteria[25]. In order to achieve the integration a particular sequence which is homologous (identical) to the chromosomal sequence is cloned into the integration vector. Upon transformation, the cloned homologous sequence from the plasmid will replace the existing identical sequence on the chromosome, as single identical sequence is used, a single crossover will integrate the entire plasmid into the target locus of the chromosome by Campbell-type mechanism [25]. Fig 7 shows the general mechanism of the cloned pMUTIN series of integration vectors, in which the *orfB'* sequence from the vector integrates in to the homologous locus of the chromosome by replacing the *orfB* and also shows that the whole vector is getting integrated into the chromosome.

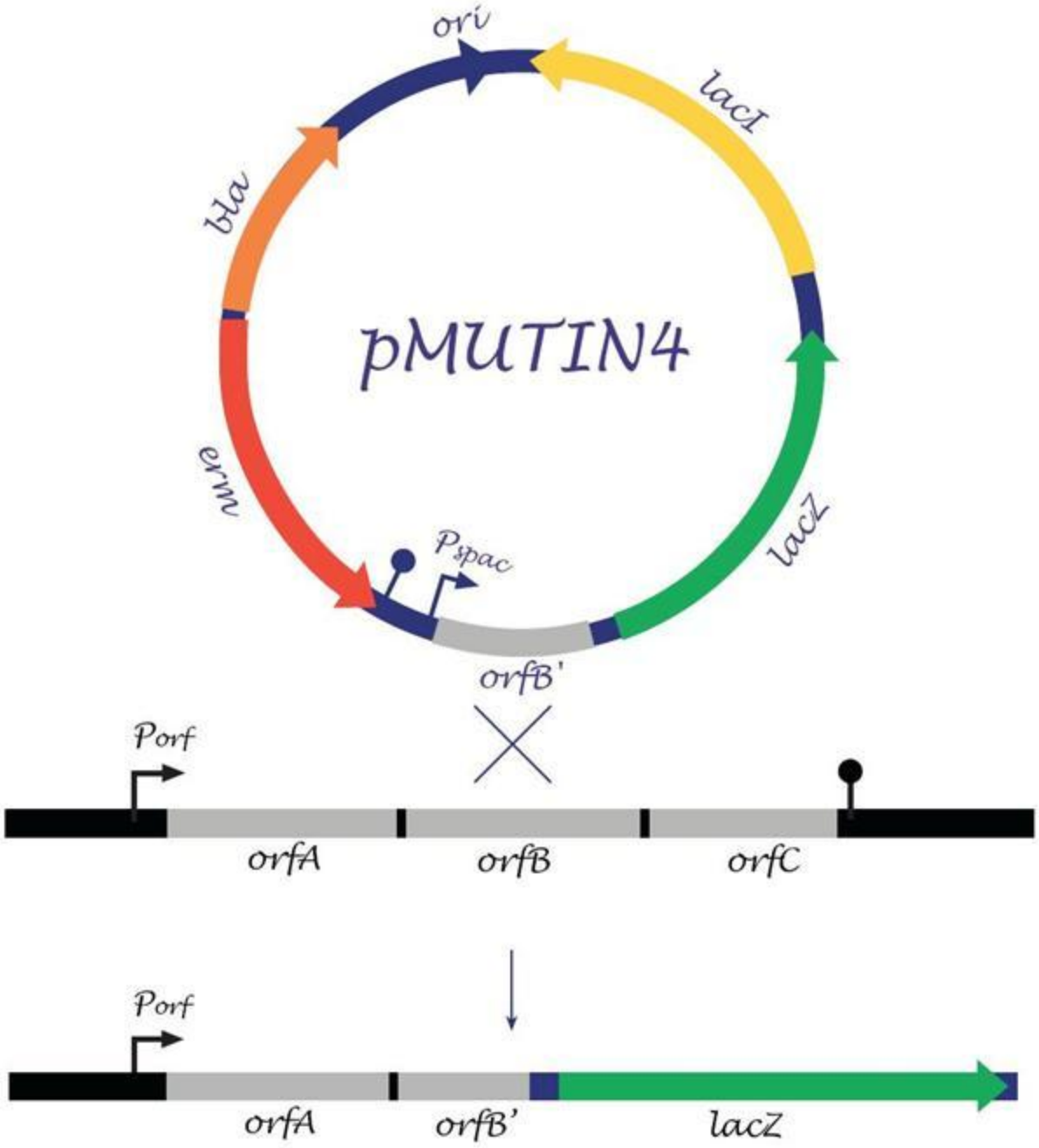


Figure 7 The general mechanism of the pMUTIN series of integration vectors. [25]

2.10 SPlipA gene

SPlipA Gene is basically a signal peptide **A** gene, belongs to the family of secreational peptides originally from *Bacillus megaterium*. It functions as signal peptide to communicate with the transmembrane protein of the cell wall to produce any fusion proteins secreted out of the cell as an extracellular protein. In this project *SPlipA* gene is employed with dual functions, as it is an origin of Bacillus megaterium it is used as a homologous gene for integration, when coupled with the *KerA* gene, it produces fusion protein secreted outside as an extracellular Keratinase [22].

2.11 Integration vector, pMUTIN GFP+6192 bp

The integration vector used in this experiment is pMUTIN GFP+6192 bp, which was purchased from the Bacillus Genetic Stock Center, transformed in *E.coli* ECE149 (carrier bacteria). pMUTIN GFP+6192 bps are actually an integration vector exclusively constructed for *Bacillus subtilis* and other gram–positive host. The salient features of the vector are as follows, it consists of a large multiple cloning site with various different restriction sites, it has GFP+ sequence, which encodes for green fluorescent protein, it consist of Pspac hybrid promoter, inducible by IPTG, it has lac l-encodes lac repressor, with modified ribosome binding site for Gram-positive expression, it has erm- encodes rRNA adenine N-f methyltransferase, selectable in gram- positive bacteria only(erythromycin 0.3µg/ml), it also consist of bla-encodes β-lactamase, selectable in E.coli only(ampicillin 100µg/ml), ori-colE1 origin of replication in *E.coli* only, trp AT T_1 T_1 T_0 transcription terminators. As it consist of *E.coli* origin only, it cannot replicate in any gram positive bacteria, but the vector can integrate very well and selected using erythromycin resistance marker(2). One of the most important characteristic features of the pMUTIN GFP+ vector is that it can integrate into the chromosome up to 50 times, so the vector has a ability to make up, maximum of 50 copies of itself, but the copy number is also depended on the host and its physiology. Fig 8, shows the pMUTIN GFP+6192 bps vector map and its features [26].

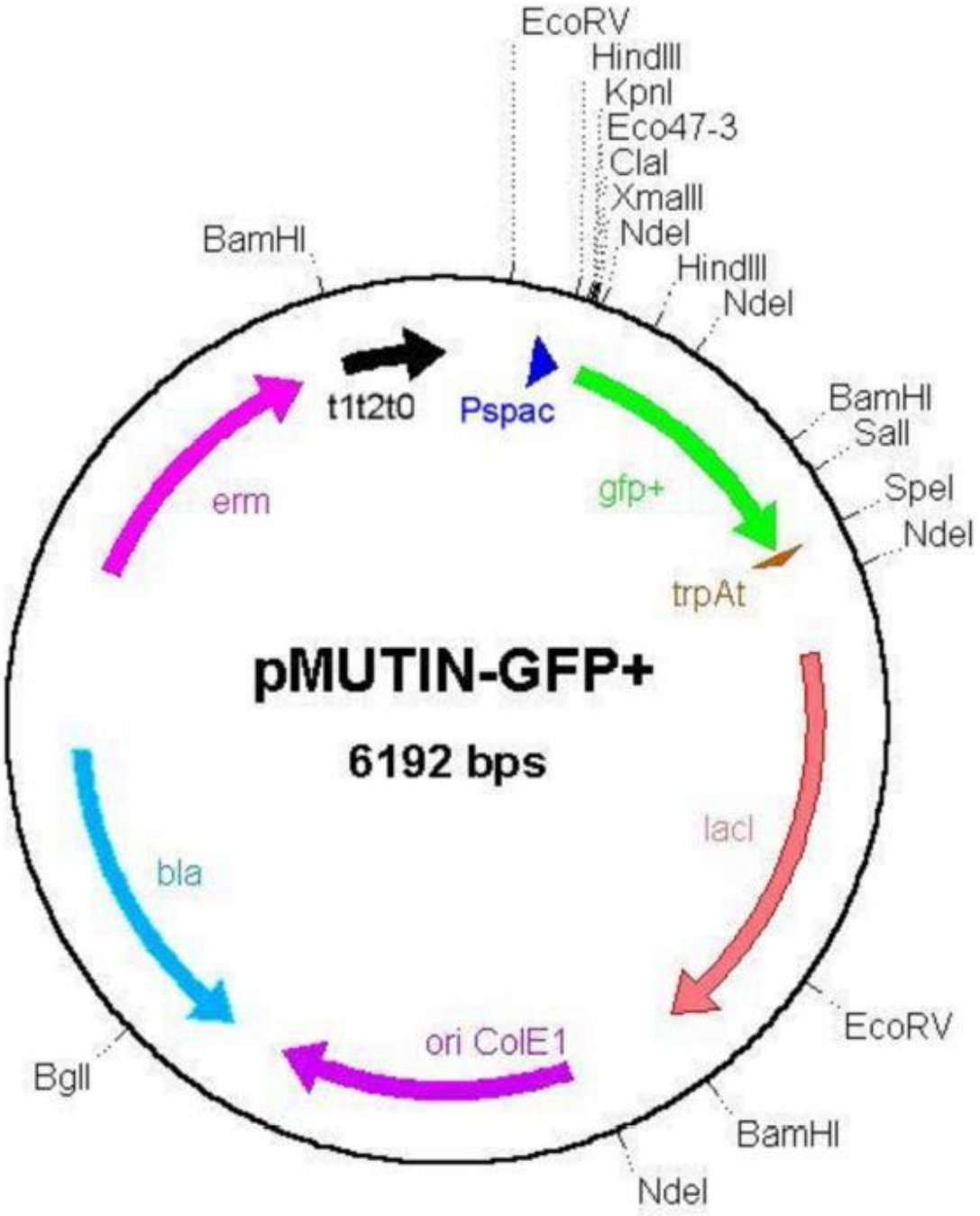


Figure 8 The pMUTIN GFP+6192 bps vector map and its features. [26]

3. Materials and Method

3.1 Bacterial strains and growth condition

Bacillus megaterium ATCC 14945 was used as the wild type host strain in this study, *Bacillus lichiniformis* ATCC 53757 and *E.coli* ECE 149 BGSC were the *KerA* gene and pMUTIN GFP+6192 integration vector donor strains respectively. Most strains were grown overnight, unless otherwise noted, at 37ºC. *E.coli* ECE149 is the only strain of the three that grows with the antibiotic containing media (ampicillin 100µg/ml). Lysogeny broth (LB), a nutritionally rich medium, and the most common media used for maintaining and cultivating strains of bacterium was prepared according to the recipe for LB formulated by Giuseppe, Bertani[27], recipe as follows.

10g Bacto-tryptone
5g yeast extract
10g NaCl
pH 8 by NaOH

The above materials were resolved to, makeup a 1 liter media and sterilized in the autoclave at 121ºC, prior to sterilization the pH of the media were adjusted to 8 by sodium hydroxide. After an overnight incubation, the cell cultures were analyzed under the microscope to ensure the morphological structure of the organisms. Bacillus lichiniformis and Bacillus megaterium were analyzed under the fluorescent microscope (ZEISS, Axiostar, 956830 plus). Viewed through, A-plan 100X, applying phase contrast ph3, with oil immersion. *E.coli* ECE149 was viewed through A-plan 100X, ph3-oil immersion with fluorescent filter, ranging excitation wavelength of 450-490 nanometers and emission wavelength of 500-550 nanometers.

3.2 DNA preparation

3.2.1 Total DNA preparation from *Bacillus lichiniformis*

Isolation of the total DNA from the *Bacillus lichiniformis* overnight culture was followed by the modified protocol of Sambrook[28]

1. 10 ml overnight culture of *Bacillus lichiniformis* cell culture was taken and centrifuged at 4000 rpm for 5 minutes
2. Then the pellet was collected and resuspended in 3 ml of lysis buffer

   Lysis buffer recipe

   10 mM NaCl - pH 8.0
   50 mM Tris-HCl - pH 8.0
   10 mM $MgCl_2$
   20 mM EDTA - pH 8.0

3. Then 0.5 ml of 10mg/ml Lysozyme was added to the lysis buffer and incubated at 37ºC for 60 minutes in water bath (JULABO, U3)
4. To the mixture, a 2µl RNase (500 µg/ml) was added and incubated again for 15 minutes.
5. To the mixture of the solution, 18µl of 10 mg/ml of proteinase K and 180 µl of 10% SDS solution was added and incubated at 37ºC for 60 minutes
6. Then the solution mixture was transferred to new phase lock gel and 400 µl of TE-saturated phenol: chloroform: isoamylalchol reagent (24: 24: 1) was added and vortexed.
7. Then the mixture was spun at 6000 rpm and the upper aqueous layer was transferred to a new microfuge tube.
8. To the solution, 2 volume of 100% ethanol was added, centrifuged at 10,000 rpm to collect the pellet of DNA.
9. In order to wash the DNA, the pellet was again washed with 70% ethanol this step was repeated twice to achieve a pure DNA.
10. The DNA pellet was finally vacuum dried and resuspended in 50 µl of TE buffer (10: 0.1).

3.2.2 Mini-preparation of pMUTIN GFP+ from *E.coli* ECE149

In order to isolate the integration vector pMUTIN GFP+ from *E.coli* ECE149, the Gen Elute™- Plasmid Miniprep kit –PLN10 was used[29], the kit includes

Resuspension solution
RNase A solution
Lysis solution
Neutralization solution
Column preparation solution
Elution solution
Gen elute miniprep binding columns
2ml collection tubes

Initially, the *E.coli* ECE149 was grown in the LB medium for overnight, with the ampicilin to the final concentration of 100µg/ml.

1. Initially 2ml of overnight culture of ECE149 was centrifuged at 10,000 rpm and completely resuspended in 200µl of Resuspension solution.
2. To the resuspended cell, 200µl of lysis solution was added and mixed well by inverting it by 6-8 times.
3. After lysis, the cell debris was precipitated by adding 350µl of neutralization solution and mixed by inverting it for 4-6 times, and then the solution was centrifuged at 10,000 rpm to precipitate the pellet.
4. Simultaneously the Gen Elute Miniprep Binding column was washed with 500µl of column preparation solution and centrifuged at 10,000 rpm for 30 seconds, and then the flow-through liquid was discarded.
5. After binding, the column was washed with 500µl of optional wash solution by centrifuging it at 10,000 rpm for 30 seconds and flow-through was discarded.

6. Then 750µl of wash solution was added to the column and centrifuged at 10,000 rpm for 30 seconds and the flow-through was discarded.
7. After washing, 100µl of elution solution was added to the column and eluted by centrifuging it at 10,000 rpm for 1 minute. Finally the eluted solution containing DNA was stored at -20°C.

3.2.3 Chromosomal DNA preparation of integrant *Bacillus megaterium*

To analyze the new integrant of *Bacillus megaterium*, it was cultured overnight in the antibiotic containing LB medium (erythromycin 0.3 µg/ml). Then again 3ml of fresh LB medium was inoculated from the overnight culture and allowed to grow up to OD 600 of 1.0-1.5

1. The culture was centrifuged at 7000 rpm, in two microfuge tubes and then 250 µl of lysis buffer was added to each tube and vortexed.

   Lysis buffer recipe

   0.05 M EDTA
   0.1 M Nacl, pH- 7.5

2. The cell suspension was transferred to a single tube and 60µl of 10mg/ml Lysozyme made in fresh lysis buffer was added and incubated at 37ºC for 30 minutes.
3. Then 30 µl of 15% sarkosyl detergent was added and placed on the ice for 5 minutes
4. To the solution mixture, 500 µl of Tris buffered phenol was added and vortexed.
5. Then the suspension was centrifuged at 14,000 rpm for 5 minutes and upper aqueous layer was transferred to a new tube.
6. To the aqueous layer 500µl of chloroform: isoamylalchol (24: 1) reagent was added, vortexed and centrifuged again to collect the upper aqueous layer.
7. In order to harvest the DNA, 200µl of 3M sodium acetate and 500µl of 100% ethanol was added to the aqueous layer the solution was mixed well by inverting the tube.
8. Then the suspension was centrifuged for 3 minutes at 14,000 rpm and the supernatant was poured off and the pellet was washed with 500µl of 70% ethanol, this step was repeated twice to get pure DNA and the pellet was dried for 5 minutes at room temperature.
9. Then DNA pellet was resuspended in 50µl of TE (10:0.1) and stored at -20ºC.

3.3 PCR primers for Gene isolation and Cassette Construction

A – SPlipA -ClaI-(F): 5' GCCCATCGATGTTCACTTAAATCAAAGGGG 3’

B – SPlipA-MluI-(R): 5' CGACGCGTGAGCTCCGGAGATCTTCAAT 3’

C – KerA - MluI-(F): 5’CGACGCGTATGATGAGGAAAAAGAGTTTTTGG 3’

D – KerA - SpeI-(R): 5’CTAGACTAGTTGAGCGGCAGCTTCGAC 3’

The sequences of the primers used were selected from the sequenced Bacillus lichiniformis KerA operon and pHIS1525.SPlipA, The homology of the SPlipA gene was sequenced using the EMBL-EBI's FASTA- nucleotide similarity search tool[30], and found to be SPlipA gene origin of Bacillus megaterium. After confirmation, the SPlipA gene was isolated from the pure Bacillus megaterium-expression vector pHIS1525.SplipA, which was already available in lab. OligoperfectTM - design software (Invitrogen) was used to deduce Tm, GC, primer-dimer, hairpins and palindromes of potential primers, there were several important considerations in primer designs[31]. First, primers A and B would define the isolation of *SPlipA* gene from pHIS vector, primer C and D would define the isolation of *KerA* gene from *Bacillus lichiniformis* and primers A and D would represent the *SPK* gene cassette construction. Primers A and D were designed with *Cla*I and *Spe*I restriction sites respectively on the 5' prime end of the primers, where as primers B and C were designed with *Mlu*I restriction site. To avoid any deletion of primer near the restriction site during PCR and restriction digestion, a few nucleotide bases were added next to 5' prime end of the primers, which were highlighted in green, while the restriction sites were underlined. In fact the primers A and D were again used for analyzing the integrant *SPK*.

3.4 PCR Amplification

Dream Taq polymerase (Fermentas), 1µl was used in PCR reactions, 50 µl reactions were set up using 10X Dream Taq buffer containing Mg^{2+} 5 µl of 2.5mM final concentration[32]. 5 µl of dNTPs were added to a final concentration of 0.25mM each, while 3 µl of appropriate primers were added to a final concentration of 0.5 µM each, and 4 µl of template DNA to the reaction. The cycling conditions as follows: Initial denaturation 95ºC, 2 min; denaturation 95ºC, 30 sec; annealing 57ºC , 30 sec; extension 72ºC, 40 sec, 2 min and 2 min for *SPlipA gene*, *KerA* gene and *SPK* gene cassette respectively; then final extension 72ºC, 10 min, with 30 cycles. The genes *SPlipA* and *KerA* were amplified in separate tubes. After restriction and ligation, the ligated *SPlipA* and *KerA* genes were used as a template to create the *SPK* gene cassette which was further subjected to downstream processing.

3.5 Agarose Gel electrophoresis

DNA agarose gel electrophoresis was carried out using scientific DNA plus electrophoresis system. The gel was prepared according to the following recipe, 1% agarose was resolved in 100ml of TBE buffer and then heated until the agarose dissolved completely, then 10 µl of 10mg/EtBr (Ethidium bromide) was added to gel. Later on the gel was casted in the casting tank with the wells comb, when the gel was ready; the wells were loaded with 1Kb ladder (invitrogen) and the PCR products. To run the setup, 90 voltage power supplies was applied to the gel of 2 hours, and then the gel was viewed under the UV trans-illuminator(Ultra-Violet Products Pvt Ltd, Cambridge UK).

3.6 Gene Purification from the Gel

After gel electrophoresis, the PCR products were purified from the Gel using TAKARA's EASY TRAP Ver-2 Kit[33]. The kit includes

Glass powder - 1000µl

NaI solution - 100ml
Concentrated buffer - 25 ml

In order to recover the PCR products from the gel, the following protocol was followed as directed by the kit provider.

1. Gel slices' three time weight of the NaI solution was added to the piece of the gel in the microfuge tube.
2. Then the gel was incubated at 55ºC to melt the gel
3. To 1 µg of DNA, 5 µl of glass powder was added to the solution to absorb the DNA.
4. After a vigorous vortex, the tube was left at the room temperature for 5 minutes, and it was centrifuged at 3000 rpm to precipitate the glass powder.
5. To the precipitated glass powder, the concentrated buffer from the kit was added and suspended properly by pipetting up and down; this step was repeated twice to get a perfect wash.
6. Then the suspension was centrifuged at 3000 rpm and the washing buffer was poured off.
7. Then the glass powder was completely suspended in the sterilized water and incubated at 55ºC to get the DNA separated out from the glass powder.
8. After incubation the suspension was centrifuged at 3000 rpm, and the DNA was collected in the supernatant.

3.7 Restriction digestion and Inactivation

As restriction digestion is one of the tedious and crucial steps in the molecular cloning, it needs more important consideration to note, so the software NEB Cutter V2.0[34] from New England BioLabs was used to determine the appropriate restriction enzyme. After confirming with NEB Cutter tool, that the selected enzymes do not cut through the gene, it was then included in the PCR primers. The restriction enzymes used in these experiments are *Cla*I, *Mlu*I and *Spe*I Fast enzymer® (Fermentas). All the enzymes were used as dictated by the service provider (Fermentas)[32]

3.7.1 Restriction using *Mlu*I

In order to cleave the *Mlu*I restriction site on the *SPlipA* and *KerA* gene, it was subjected to restriction digestion on separate tubes. The reaction was prepared in microfuge tube to the final volume of 30µl, by the following order as listed below.

10X buffer - 3 µl
DNA (*SPlipA* and *KerA*) - 10µl
Enzyme (*Mlu*I) - 2µl
Water - 15µl

After restriction digestion, the reaction was inactivated by phenol – chloroform method and then both the genes were ligated together and used for the *SPK* gene cassette construction.

3.7.2 Double digestion

In order to make an easy cloning, double digestion restriction reactions were followed with *SPK* gene cassette and the pMUTIN vector. Double digestion is nothing but the restriction digestion reaction, using two different compatible restriction enzymes in a same reaction tube. To confirm the reliability of the double digestion method the double digestion reaction between *Cla*I and *Spe*I were checked with double digestion data sheet provided by the Fermentas and the following recipe was followed[32].

Double digestion of SPK

SPK gene cassette - 20µl
Enzyme –I (*Cla*I) - 2µl
Enzyme - II (*Spe*I) - 2µl
Buffer - 6µl

The reaction mixture was incubated at 37ºC for 15 min.

Double digestion of vector

pMUTIN vector DNA - 20µl
Enzyme –I (*Cla*I) - 2µl
Enzyme - II (*Spe*I) - 2µl
Buffer - 6µl
The reaction was incubated at 37ºC for 30 minutes

3.7.3 Inactivation.

As the restriction enzymes used were not heat sensitive, it was unable to follow the heat treatment inactivation method, so the phenol-chloroform method DNA extraction was introduced between the restriction digestion and ligation reactions[32]. The *KerA*, *SPlipA* and pMUTIN vector were subjected to inactivation protocol, prior to ligation. The protocol as follows

1. To the digested mixture, double the volume of phenol-chloroform-isoamylalchol reagent was added and vortexed.
2. Then the solution was centrifuged at 10,000 rpm and the upper aqueous phase was transferred to new microfuge tube.
3. To the aqueous phase, double the volume of 100% isopropanol was added to precipitate the DNA, then it was centrifuged at 10,000 rpm and DNA pellet was collected.
4. To the DNA pellet 70% ethanol was added and washed, this step was repeated twice and the DNA was air dried at room temperature.

3.8 Ligation and Cassette Construction

Ligation is one of the crucial steps in molecular cloning, which actually clone different segments of DNA together, in this experiment the T4 DNA Ligase from Fermentas was used as a ligation enzyme. After the inactivation of *Mlu*I restricted *SPlipA* and *KerA* they were further subjected to ligating it together. For this ligation, the reaction was setup to 30µl and incubated at 22ºC for 30 min, as per the following recipe

Ligation of SPlipA with KerA

10X ligation buffer	- 3µl
T4 DNA Ligase	- 1µl
DNA (SPlipA and KerA)	- 8+8µl
Water	- 10µl

3.8.1 SPK Cassette Construction

One of the most important experiment conducted on this project was the construction of *SPK* gene cassette, As two different genes were employed in cloning of the vector, it was unable to restrict and ligate the genes easily, because continuous process of restriction, inactivation and ligation of the two different genes would totally dilute the cloning process, so to avoid the dilution of genes, a single cassette was constructed. By using a cassette, the probability of the cloning in to the vector was increased. The *SPK* gene cassette was constructed by the following protocol.

1. The ligated *SPlipA* and *KerA* gene was subjected to phenol- chloroform inactivation to inactivate the T4 DNA Ligase.
2. After inactivation, the genes were used as a template DNA for the primers A and D and amplified with PCR
3. The amplified *SPK* cassette was then subjected to double digestion with *Cla*I and *Spe*I to cleave the 5' and 3' end of the cassette.
4. After the double digestion the cleaved *SPK* cassette was loaded on the agarose gel electrophoresis. this method was introduced to isolate the cassette from the cleaved 5' and 3' pieces
5. Then the *SPK* cassette was purified from the gel using TAKARA EASY TRAP V2.0 kit and kept ready for cloning.

3.8.2 Cloning of *SPK* with pMUTIN

As the *SPK* cassette was gel purified, it was free from the cleaved phospo 5' and 3' ends, which would totally affect the cloning process by ligating back to the cleaved ends, the cloning process was carried by ligating the cleaved *SPK* cassette with the restricted pMUTIN vector as per the following recipe.

Vector DNA – pMUTIN	- 15µl
Insert DNA – SPK	- 15µl
T4 DNA Ligase	- 3µl
Buffer	- 7µl

3.9 Transformation

Transformation of wild type host *Bacillus megaterium* ATCC 14945 into a integrant strain was performed using protoplast transformation method as described by Mc Cool and Cannon[35]. In this protoplast transformation method a special medium called RHAF (Royal Hellenic Air Force) was used. Prior to protoplast transformation the overnight culture of *Bacillus megaterium* was prepared in the LB medium.

Table 2 Composition of RHAF medium [35]

Chemicals	**Concentration(g/l)**
NH_4Cl	1.0
Tris base	2.0
KCl	0.035
NaCl	0.058
Na_2SO_4.10 H2O	0.30
KH_2PO4	0.14
$MgCl_2$. 5H2O	4.26
Yeast extract	5
Tryptone	5
Sucrose	68.46
Glucose	200

1. Then 20 ml of fresh LB was inoculated with 50µl of overnight culture and incubated at 37ºC AT 150 rpm.
2. After reaching the OD of 0.40 at 600 nm the culture was centrifuged at 4000 rpm and resuspended in 2 ml of RHAF.
3. The cell suspension was again centrifuged and resuspended in 2 ml of RHAF media containing 600µg/ml Lysozyme and incubated at 37ºC for 15 minutes to make protoplast cells.
4. Then the protoplast cells were harvested by centrifugation at 4000 rpm for 5 minutes and the supernatant was poured off, the protoplast cells were slowly washed with 2 ml of RHAF media.
5. After washing, 200µl of the protoplast suspension was transferred to new tube and 10 µl of the cloned integration vector DNA was added to the solution.

6. Then 200µl of 35% polyethylene glycol (MW=8000) was added and gently mixed and incubated at 37ºC for 3 minutes.
7. Then again the solution was diluted with 3ml of RHAF media and centrifuged at 4000 rpm for 5 minutes.
8. After centrifugation, the pellet was resuspended in 1ml of RHAF and incubated at 37ºC for 1 hour and 30 minutes.

Then 50µl of the suspension was taken and spreaded on the RHAF 1.2% agar plates and incubated at 37ºC for overnight.

3.10 Selective plating method

To select the transformed integrants a special selective plating method was followed, which included the milk agar plates containing antibiotic (erythromycin 0.3µg/ml) this method was followed, as the new integrants can lyse the milk casein and produce clear zones around the colonies so that they can be easily selected, the selection procedure as follows.

1. Initially 1.5% agar was prepared separately in 80ml of LB media and the pH was adjusted to 8 and sterilized
2. Then 20 ml of water containing 2g of skimmed milk powder was sterilized separately
3. Then the solutions were mixed together when they reached 60ºC, and made to final volume of 100ml
4. Finally, erythromycin of final volume, 0.3µg/ml was added at hand bearable temperature and poured on the petridishes and allowed to cool.
5. Prior to plating , the colonies from the RHAF plates were recovered by washing the colonies with 5 ml of LB media
6. Then the recovered colonies were spreaded on the selective milk agar plate and incubated at 37ºC for overnight.
7. Then the colonies that produced the clearing zones were recovered by freeze media and stored under -80ºC for further analysis.

3.11 Verification by PCR amplification

To verify the integrant of the pMUTIN vector, the strain was subjected to the PCR amplification; the chromosomal DNA from the new integrant was isolated and subjected to PCR amplification of the three genes *SPlipA*, *KerA* and *SPK*. The principle behind the method is, if genes would have integrated, then the PCR would amplify them. Considering this the primers AB, CD and AD was used in *SPlipA*, *KerA* and *SPK* respectively.

3.12 SDS-PAGE

SDS-PAGE was performed to detect the protein produced by the integrant strain; it was performed according to Lammli[36]. The recipe of the gel making as follows

Table 3 SDS-PAGE, separating gel recipe [36]

10 ml separating gel (15%)	**volume (ml)**
40% Acrylamide /bis	3.75
1.5 M Tris-Hcl (Ph-8.0)	2.5
10% SDS solution	0.1
Super Q	3.65
10% APS	0.05
TEMED	0.005

Table 4 SDS-PAGE, concentration gel recipe**.** [36]

10ml concentrating gel (4%)	**volume(ml)**
40% Acrylamide/bis	1
0.5M Tris-Hcl (pH-6.8)	2.5
10% SDS solution	0.1
10% APS	0.05
Super Q	6.4
TEMED	0.01

Prior to SDS-PAGE the protein samples were prepared according to following protocol

1. The overnight culture of the recombinant *Bacillus megaterium* and *Bacillus lichiniformis* was centrifuged at maximum rpm and the supernatant was transferred to a new microfuge tube.
2. Then the protein containing samples were subjected to acetone precipitation in the ratio of 200µl of protein and 800µl of acetone.
3. Then the precipitated sample was washed and mixed with Bromophenol blue and boiled to get attached to the protein.
4. After polymerization of the gel , the samples were loaded on the gel along with the protein molecular weight ladder (BioRad) ranging 10-250KDa
5. Then the gel was allowed to run for 1hour 30 minutes.

6. Then the gel was stained with Coomasie brilliant blue in 40% methanol, 10% acetic and 50% water, for 1 hour.
7. After staining, the gel was subjected to de-staining to visualize the protein bands by washing with de-staining solution, which was nothing but a staining solution except the Coomasie brilliant blue.

3.13 Zymogram staining

Zymography is an electrophoretic techniques used to detect the enzyme activity, it is nothing but an SDS-PAGE system that includes a substrate usually gelatin, so that it can copolymerize with polyacrylamide gel. The principle behind the method is that, a protease could lyse the gelatin present in the Acrylamide gel and can make clear band on the respective location, if only the enzyme is active. This experiment was conducted, to show that the enzyme produced is a protease; a modified protocol of SDS-PAGE was followed for Zymogram [37].

Gelatin solution was prepared by adding 2.65mg/ml of gelatin to the water.

Table 5 Zymogram, gelatin gel recipe [37]

10% Acrylamide gelatin gel	**volume(ml)**
Gelatin solution	8.3
Acrylamide/bis 30%	7
Glycerol 50%	0.105
TEMED	0.010
APS 10%	0.100
1.5M Tris (pH- 8.8)	5.25

Table 6 Zymogram, concentrating gel recipe [37]

10ml concentrating gel (4%)	**volume(ml)**
40% Acrylamide/bis	1
0.5M Tris-Hcl (pH-6.8)	2.5
10% SDS solution	0.1
10% APS	0.05
Super Q	6.4

2X SDS sample buffer

0.5 M Tris-Hcl – 2.5ml
Glycerol - 2ml
10% (w/v) SDS - 4ml
0.1% (w/v) Bromophenol blue - 0.5ml

The volume was made up to 10 ml with sterilized dd water

Washing solution:

25% Triton X-100 in
50mM Tris pH- 7.4
5mM $Cacl_2$
1µM $Zncl_2$

Incubating solution:

50mM Tris pH- 7.4
5mM $Cacl_2$
1µM $Zncl_2$

Staining solution

Coomasie G250 in
Ethanol 30%
Acetic acid 10%
Water 60%

De-staining solution

Ethanol 30%
Acetic acid 10%
Water 60%

1. After the gel got polymerized, the protein samples from *Bacillus megaterium* wild type, *Bacillus megaterium* recombinant and *Bacillus lichiniformis* were prepared with the sample buffer and loaded in the gel
2. Then the gel was allowed to run for 1 hour 30 minutes, after electrophoresis the gel was carefully removed from the frame and washed with washing solution for 1 hour in the rotating shaker.
3. After washing, it was briefly rinsed with dd water and incubated overnight at 37ºC in the incubating solution
4. Then the gel was stained with staining solution for 30 minutes and then de-stained with de-staining solution for 2 minutes
5. Then the reaction was stopped with 2% acetic acid.

3.14 Protease and keratinase assay

The assay was conducted in order to find the protease and the keratinase activity of the enzyme, initially the samples were collected from the feather meal broth for every sixth and twelfth hour and stored at -20°C.

The proteolitic enzyme activity was determined by method of Secades and Guijarro[6], employing azocasein (Sigma Aldrich Chimie) as substrate. Proteolytic assay was carried out briefly by proper dilution of enzymes to 480µl of azocasein (1% w/v) in Tris buffer (50mM, pH 7.5). The mixture then was incubated at 37ºC for half an hour. The reaction was essentially terminated with 600µl of 10% (w/v) trichloroacetic acid. Then the mixture was put on ice for half an hour and followed by centrifugation at 1500g at 4ºC for 10 minutes. 800 µl from the supernatant was neutralized by 200µl of 1.8 N NaoH, and the finally the absorbance (A_{420}) was assayed using a spectrophotometer (UV-160A). One unit of Keratinase activity was defined as the amount of enzyme required to yield an increase in absorbance (A_{420}) of 0.01 in 30 minutes at 37ºC.

Simultaneously the Keratinase assay was also conducted along with protease assay in order to find the Keratinase activity of the integrant strain. Keratin azure (Sigma-Aldrich, USA) was used as the substrate. It was first frozen at -20ºC and then ground into a fine powder. The 5 mg keratin azure powder was suspended in 1ml 50mmol/L Tris–Hcl buffer (pH-8.0). The reaction mixture contained 1 ml keratin azure suspension and 1 ml appropriately diluted enzyme. The reactions were carried out at 50ºC in a water bath with constant agitation of 200 rpm for 30 min. after incubation, the reactions were stopped by adding 2 ml 0.4 mol/L trichloroacetic acid (TCA) and followed by centrifuging at 10000 rpm for 20 min to remove the substrate. The supernatant was spectrophotometrically measured for release of the azo dye at 595 nm. The 1 ml keratin azure suspension in the same buffer (like that of the sample) was agitated for 30 min at 50ºC , then was added 2 ml 0.4 mol/L TCA and 1 ml enzyme solution as a control. One unit (U) Keratinase activity was defined as the amount of enzyme causing 0.01 absorbance increase between the sample and control at 595 nm under the conditions given[38].

3.15 Feather degradation test

Phosphate buffer

K_2HPO_4 - 0.3 g/L
KH_2PO_4 - 0.4 g/L
Nacl - 0.5 g/L
$Mgcl_2$ - 0.05 g/L

Chicken feather degradation by the integrant strain was carried out in the Erlenmeyer flask in phosphate buffer [39]. Initially chicken feathers were chopped, washed and air dried, then 2g of chicken feathers were added to the 100ml of sterilized phosphate buffer solution. The antibiotic, erythromycin was also added to a final concentration of 0.3µg/ml and finally the solution was inoculated with integrant Bacillus megaterium by the inoculation ratio of (10:90)

then the culture were incubated at 37°C with continuous shaking at 140 rpm, then everyday samples were collected from the feather broth for enzymatic assays.

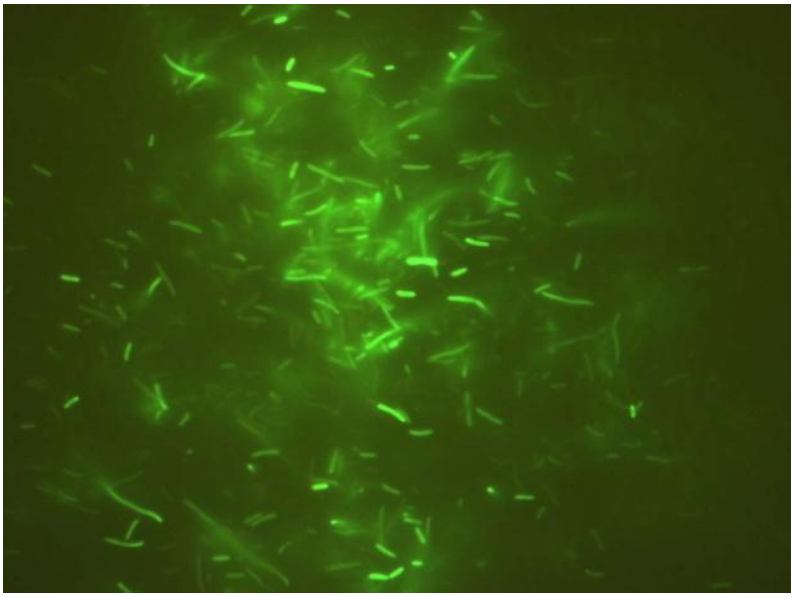
3.16 Microscopical analysis of feather degradation

To study the degradation of the chicken feathers by the integrant strain, the microscopical analysis of the degraded chicken feather was conducted. Two microscopical slides were prepared from the undegraded chicken feather and the degraded chicken feather, which was collected from the degraded chicken feather meal after seven days from the date of inoculation. Then both the samples were analyzed under the microscope (ZEISS, Axiostar, 956830 plus). Viewed through, Achroplan 40X, applying phase contrast ph2 and analyzed using software Axiovision 3.1 through Axiocamera (MRc. ZEISS).

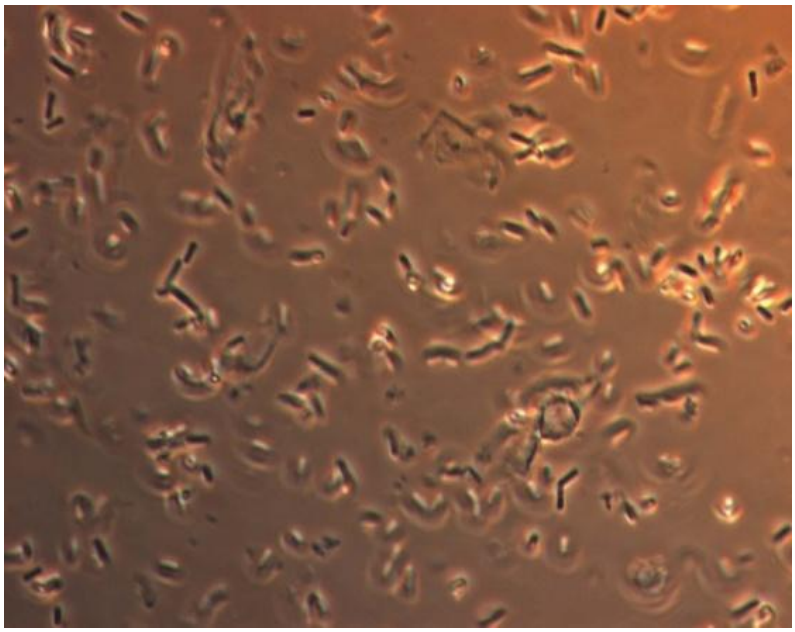
4. Results and Discussion

4.1.1 Morphology of the bacterial culture

All the cell cultures used in this study can be seen in Fig 9. They are all rod shaped bacteria. Fig 9A shows the microscopic view of *E.coli* ECE 149 BGSC containing pMUTIN GFP in fluorescent green color; Fig 9B shows the microscopic view of *Bacillus lichiniformis* and Fig 9C shows the microscopic view of *Bacillus megaterium* wild type strain.

(A) 

(B)



(C)

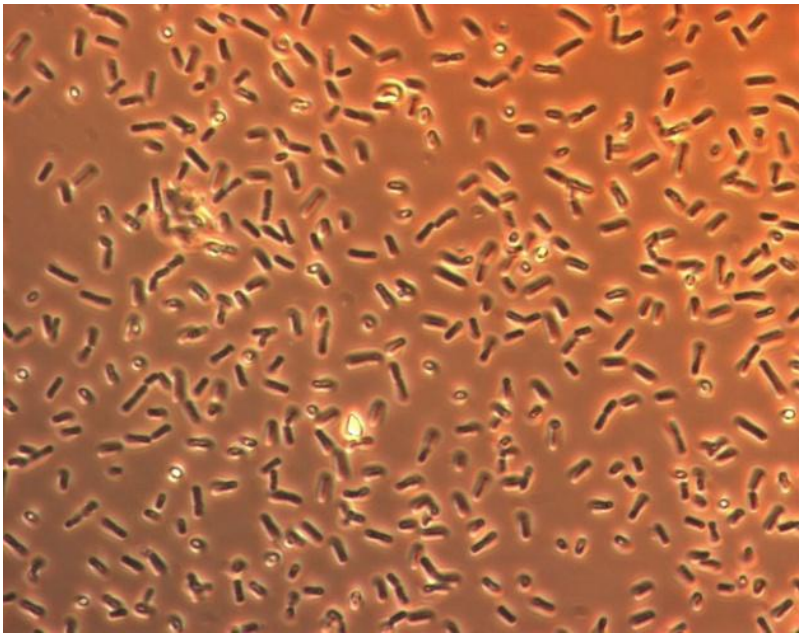


Figure 9 Morphology of the bacterial culture

4.1.2 Isolation and construction of genes and gene cassette

To create the *SPK* cassette, the *KerA* gene from *Bacillus lichiniformis* of length 1.14 kbp and *SPlipA* gene from pHIS1525 of length 135 bp were separately PCR amplified and isolated using the primes AB and CD respectively. Then the *SPK* gene cassette of length 1275 bp was constructed with PCR by using the primers A and D and subsequently cloned into the integration vector pMUTIN-GFP+. Fig 10 shows the 1 kb ladder, pMUTIN, *KerA* 1140 bp, *SPK*-1275bp, *SPlipA*-135 bp and 100 bp ladder in the order from left to right.

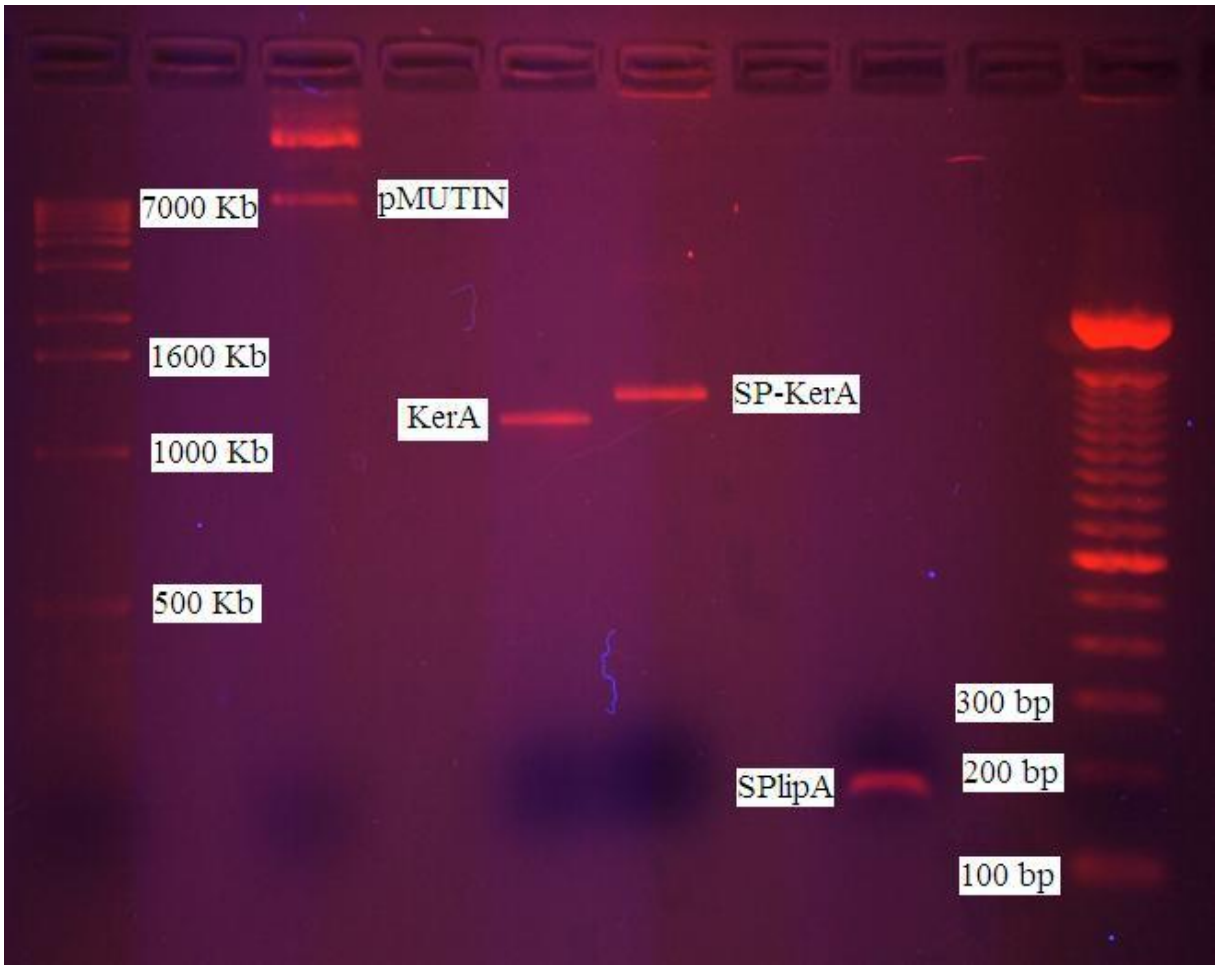


Figure 10 Isolation and construction of gene and gene cassette

The construction of the *SPK* gene cassette was considered as one of the major problem faced during this project, because it involves the series of restriction digestion and ligation processes and PCR amplification. To this addition, the restriction enzymes used were not heat sensitive and they could not be heat inactivated, so all the enzymes were inactivated by the phenol: chloroform method, which was considered as a tedious technique, as it completely diluted the nucleic acid mixture.

4.1.3 Cloning and integration of the SPK into the pMUTIN-GFP+

The *SPK* cassette was restricted and digested to cleave the *Cla*I and *Spe*I restriction ends then subsequently cloned into the pMUTIN-GFP+ integration vector on the corresponding *Cla*I and *Spe*I sites. The gene cassette *SPK* was ligated to pMUTIN by removing out the GFP function. It can be seen in the Fig 11, that the *SPlipA* gene and *KerA* gene are shown in green and violet colors respectively, between the *Cla*I and *Spe*I restriction sites.

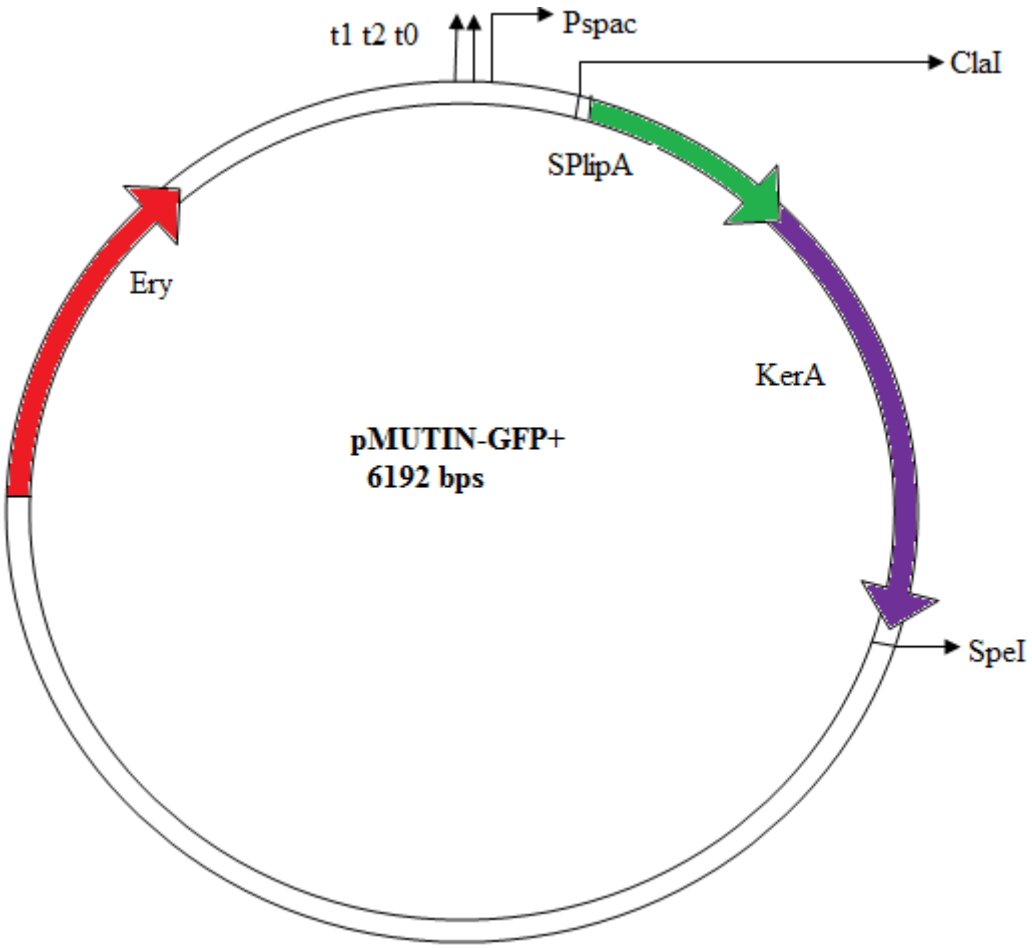


Figure 11 shows the map of cloned integration vector pMUTIN SPK

The Homologous single cross over strategy is elaborated in the Fig 12; and it shows the possible mechanism of integration, in which the homologous *SPlipA* gene along with the *KerA* and the whole vector is getting integrated into the corresponding chromosomal locus.

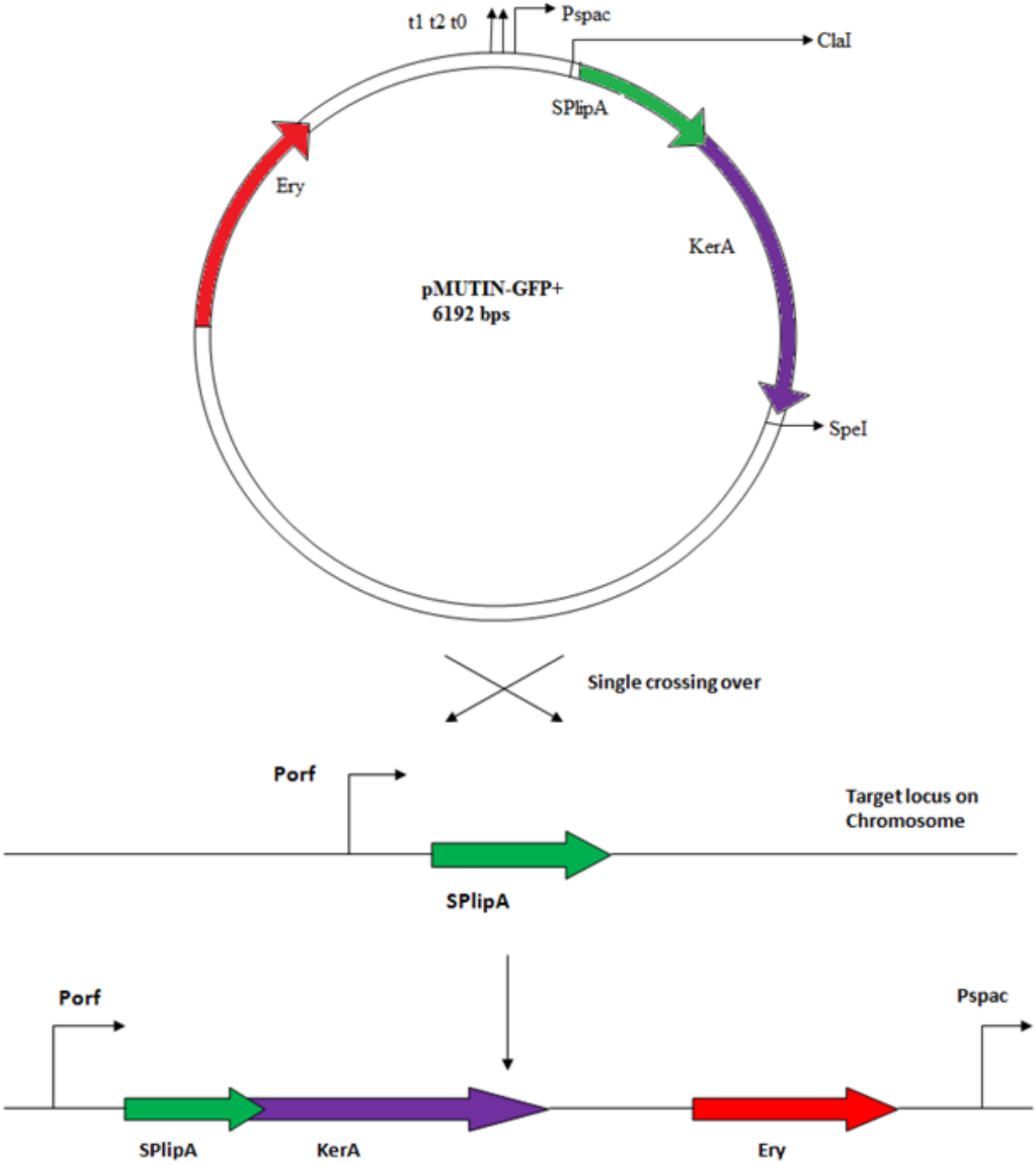


Figure 12 Homologous single crossing over strategy

In order to favor an easy transformation, protoplast formation and transformation method was carried out. As the integration involves the homologous crossing over methodology, this completely decreased the transformation frequency. This was a problem, when the transformation aliquot was spread over 20 plates, but where the transformed colonies were found only on one plate.

4.1.4 Expression of the Integrant Strain

To check the expression of keratinase in the developed integrant strain, bacteria carrying the *SPK* were selected on the milk agar plates containing erythromycin. The integrant strains clearly displayed the clearing zones around colonies, which were due to the lysing of the milk casein by the protease secreted from the integrant strain which can be seen in Fig 13.

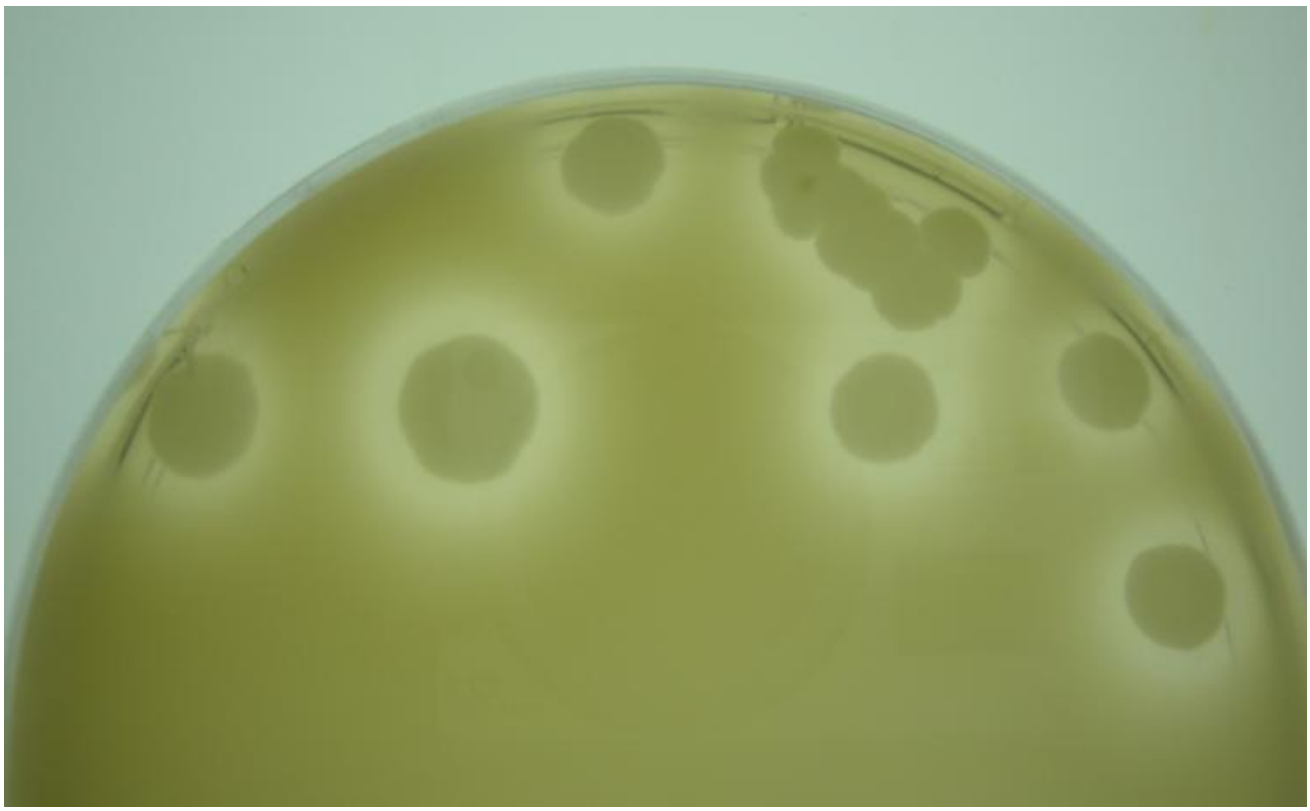


Figure 13 Expression of the integrant strain, indicated by clear zone around the colonies.

4.1.5 Gene verification

To check the chromosomal integration of the vector, the recombinant strain was tested for integrants by PCR. The chromosomal DNA was isolated and amplified by PCR using the primers C and D, which was then resolved in the 1% agarose gel. Fig 14 shows the 1.14 kb length of *KerA* gene from the chromosomal DNA in lane 3 and 5 compared with the DNA ladder in lane 1.

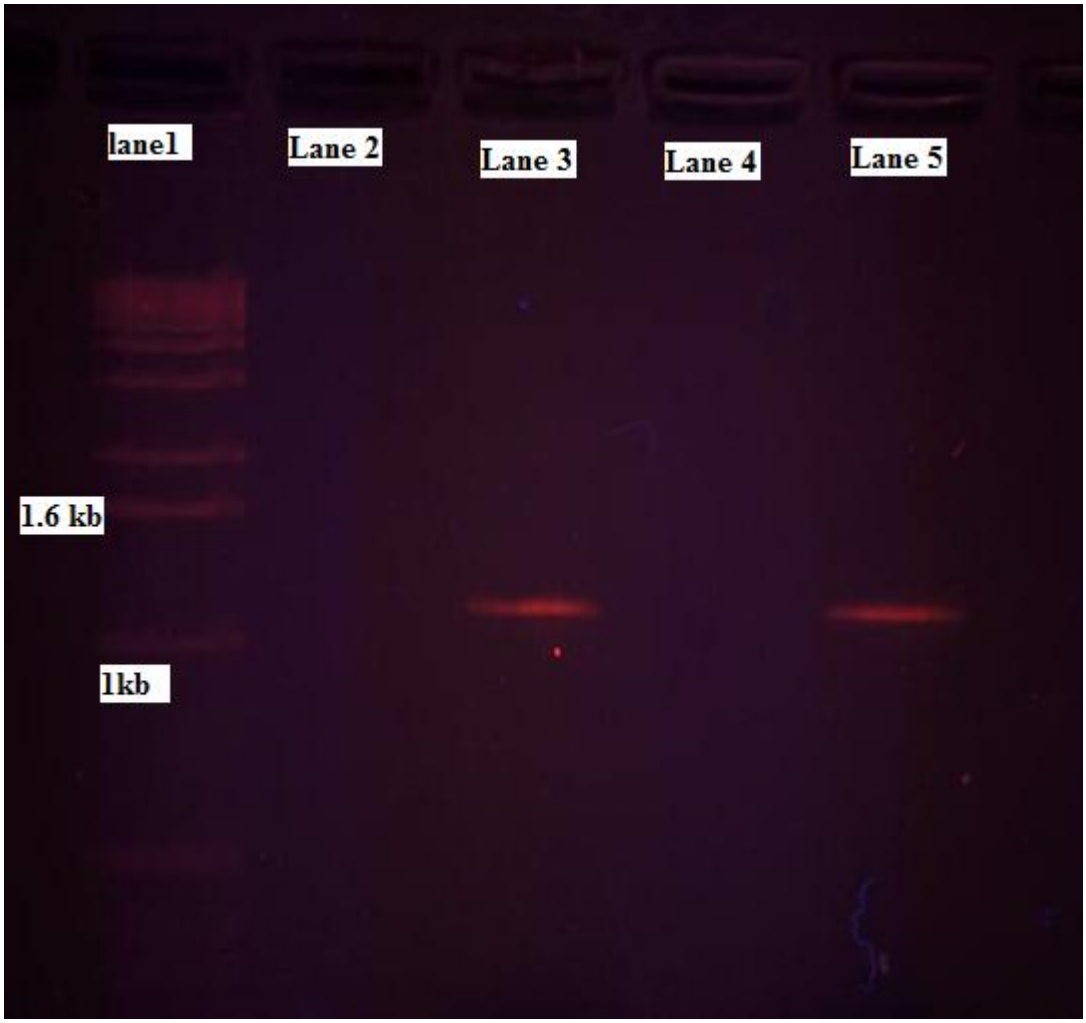


Figure 14 Gene verification by PCR

4.1.6 SDS-PAGE

The SDS-PAGE separates the protein molecules based on their molecular weight, so the secretion and the molecular mass of the enzyme were confirmed with SDS-PAGE. Fig 15 shows protein bands of molecule weight ladder, *Bacillus lichiniformis* keratinase (B.L.W) and *Bacillus megaterium* integrant keratinase (B.M.R) in the order from left to right. It is clearly seen that the keratinase enzyme band in the B.M.R approximately coincides and corresponds to mass of of keratinase produced by the *Bacillus lichiniformis* PWD-1 at 33kDa.

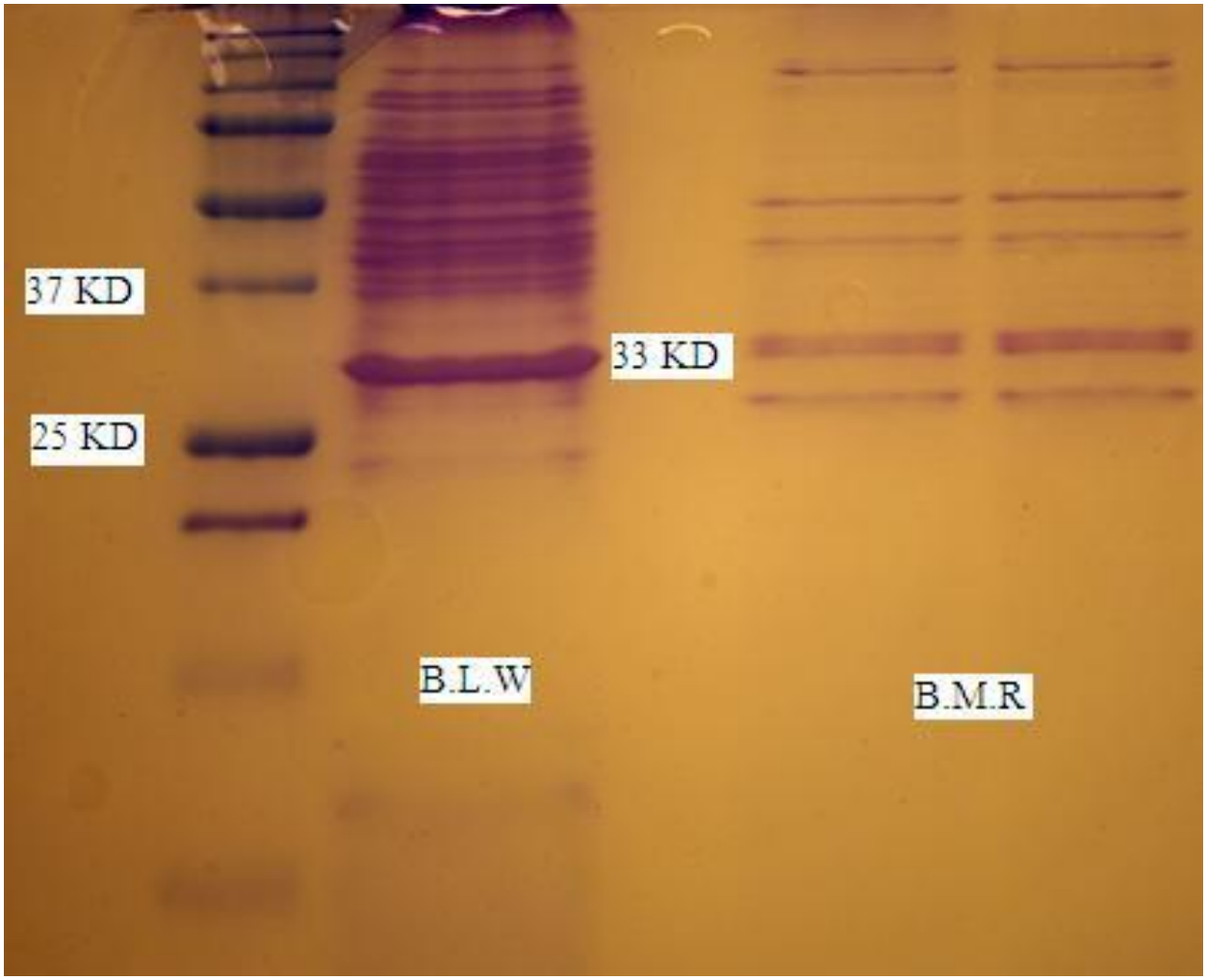


Figure 15 SDS-PAGE: showing the keratniase sample of B.L.W and B.M.R

4.1.7 Zymography

The enzyme activity of the keratinase was analyzed with the Zymogram staining. The zymography of the integrant was conducted on the gelatin based separation gel, which was used to identify the activity of the enzyme. Fig 16 shows the bands of, protein molecular weight ladder, *Bacillus megaterium* wild type keratinase (B.M.W), *Bacillus lichiniformis* wild type keratinase (B.L.W) and *Bacillus megaterium* integrant keratinase (B.M.R), in the order from left to right.

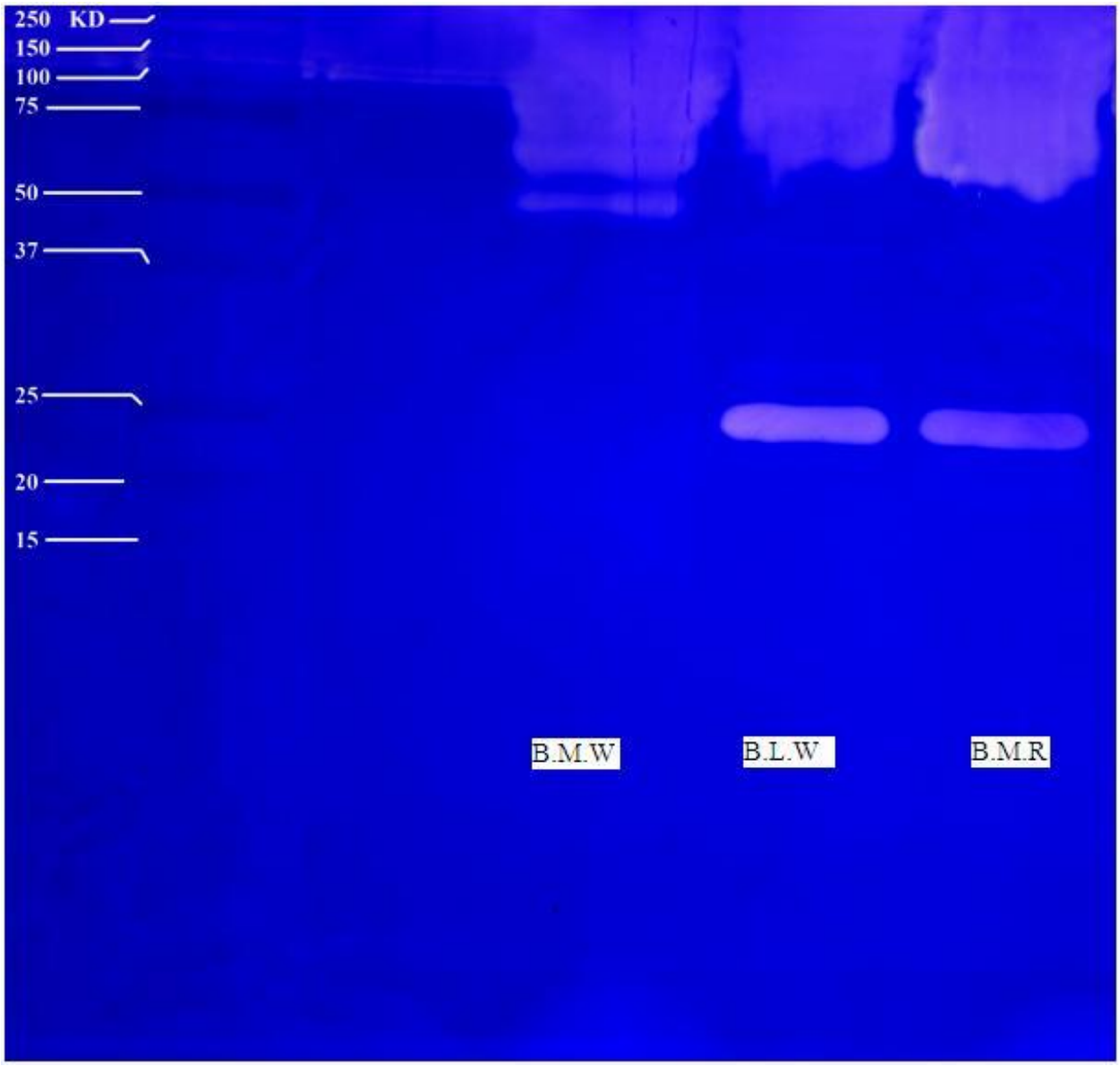


Figure 16 Gelatin Gel Zymography

The zymography result is a good evidence to show the protease activity of the enzyme, as the B.L.W keratinase and B.M.R keratinase has created the clearing zone on its corresponding location; it is confirmed that the enzyme produced by the B.M.R is protease active, because clear bands occurs due to, lysing of the gelatin in the gel by protease enzymes only. As the *Bacillus megaterium* integrant can make a clear band, while the *Bacillus megaterium* wild-type cannot; this proves that the secreted enzyme is protease active.

4.1.8 Enzymatic assay

The enzymatic assay was conducted in order to find the protease and keratinase activity of the enzyme, so the sample from every sixth hour was collected and assayed. The protease and the keratinase activity units, which were calculated from the spectrometric absorbance values, were listed in the Table 7, and its corresponding graph was plotted against the time and activity unit which is shown in the Fig 17.

Table 7 The protease and the keratinase activity units against time

Time in Hours	**Protease Activity Unit**	**Keratinase Activity Unit**
0	0	0
6	17.9	2
12	18.1	12.3
24	8.1	9.5
30	9.4	9.3
36	7.6	9.3
48	5.4	3
54	8.3	4.5

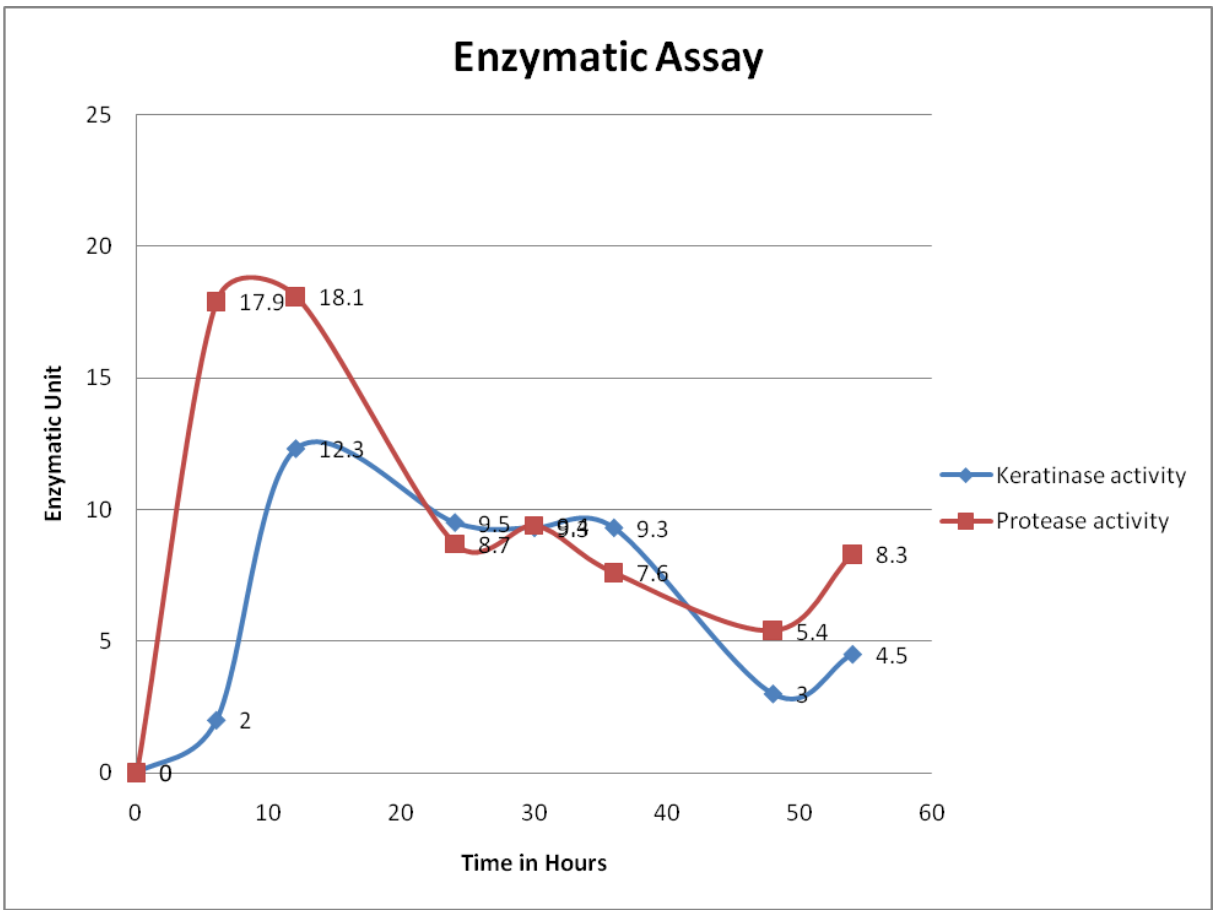


Figure 17 Enzymatic assays proteolytic and Keratinolytic activity against time

As it can be seen in Fig 17, it was found that the strain has the ability of both the proteolytic and keratinolytic activity, in fact at the 12th hour, both shows their highest enzyme activity of 18.1 Unit and 12.3 Unit respectively and then both declines at the 30th hour. In the enzyme activity it was also found that the protease activity is higher than keratinase, this can be due to the wild type strain, which naturally contains some proteases secreted and they may cleave the newly secreted keratinase, which results in lower keratinase activity.

4.1.9 Feather degradation test

Feather degradation test was conducted to check the activity of the enzyme on feathers. Figure 18 shows that the raw feathers could be digested in 3 days and the complete digestion was achieved in 7 days leading to a yellowish peptide rich broth.

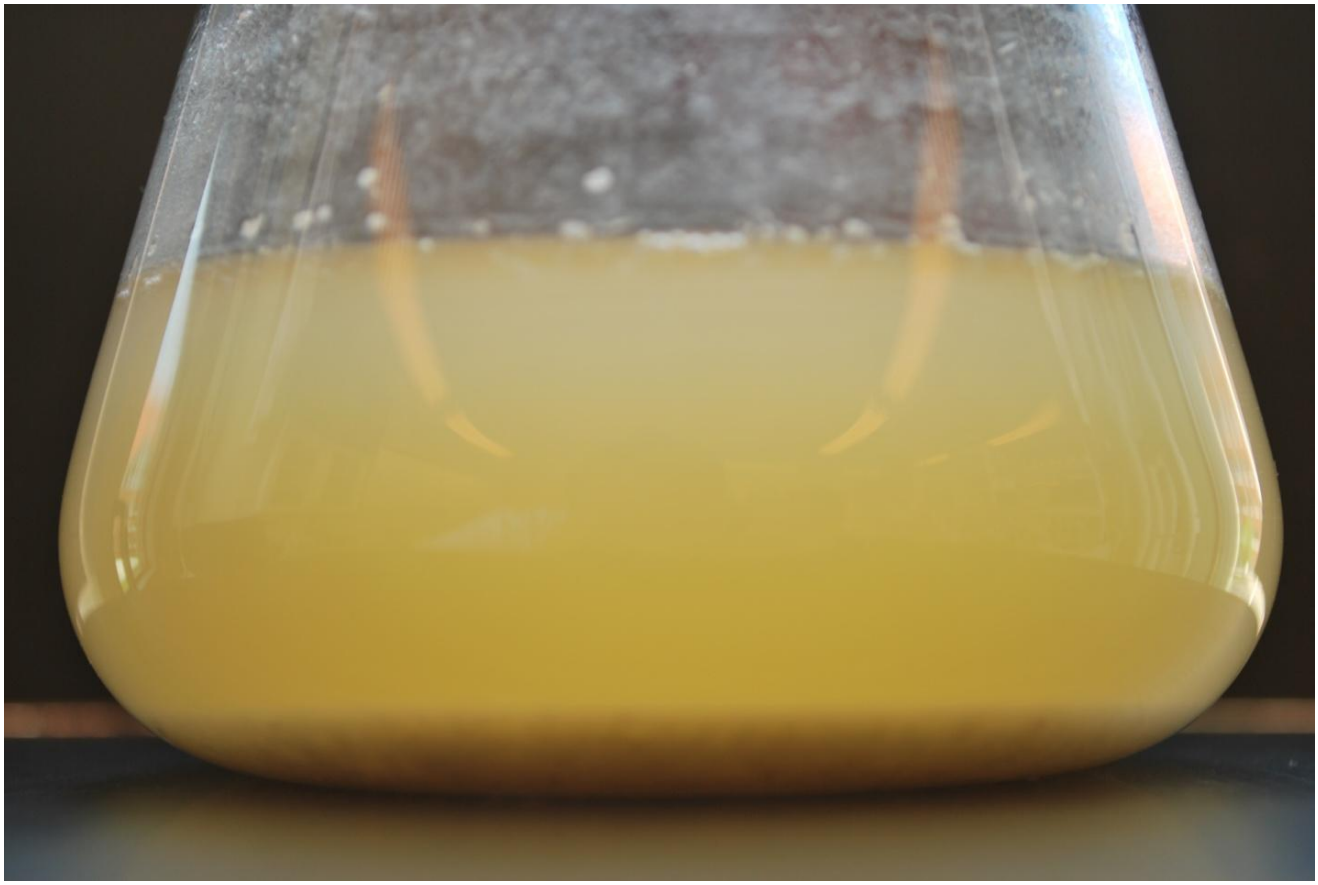
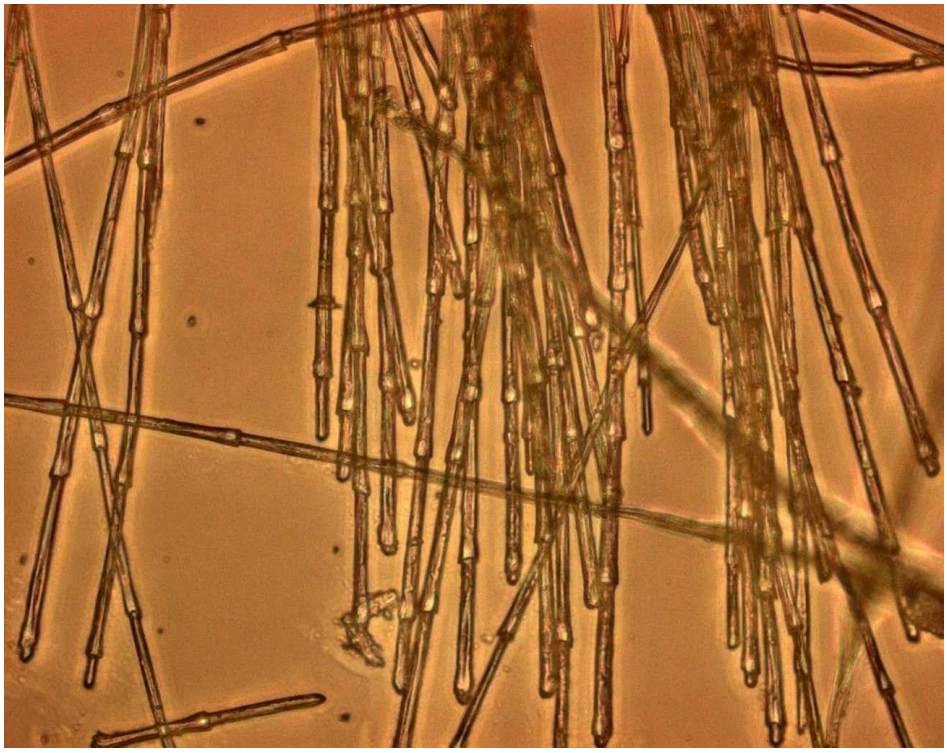


Figure 18 Feather degradation test showing the peptide rich broth of digested feather meal.

4.1.10 Microscopical analysis of feather degradation

Microscopical analysis was conducted to analyze the feather degradation Figure 19 clearly shows the picture of non-degraded feathers (A) and degraded feathers (B). The non-degraded feathers can be seen as a cluster of long, cylindrical, slender and several segments with jointed units, while the degraded feathers are seen as segregated pieces of broken segments from the jointed units, and also the broken segments are seen as spongy appearance instead of perfect cylindrical shape.

(A)



(B)

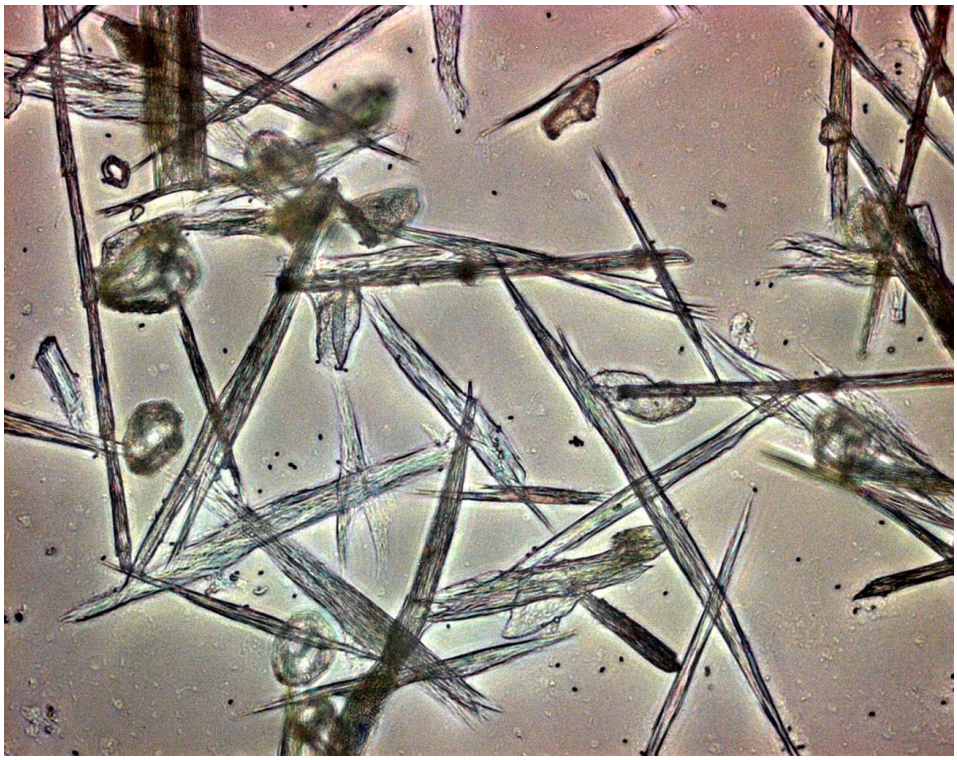


Figure 19 Microscopical analysis of feather degradation. Figure (A) shows perfect structure of non-degraded feathers while figure (B) shows microscopical view of degraded feathers.

5. Conclusions

In summary the method employed in this study created a successful integrant strain of *Bacillus megaterium* carrying the *KerA* gene inducible under natural promoter, which could produce an extracellular Keratinase on the chicken feathers and digest them completely in 7 days. As the *SPK* gene operates from the natural promoter of the *SPlipA* locus in the chromosome. In this way it does not need any external inducer to start the secretion of the enzyme, instead it is secreted out of the cell and constantly when keratin is present in the environment, which is considered as an advantage of this strain.

As it was already mentioned, that pMUTIN-GFP+ has the ability to integrate and make several copy of itself, a preliminary gene amplification test was conducted by the elevation of antibiotic; and found that the integrant strain from 1st generation could grow even in the presence of 100x antibiotic concentration. From the literatures it was found that the chromosomal integration has already been experimented on various bacterial strains and the integration was also achieved. Some of the major problems faced by the researchers were the determination of copy number of integrated gene, expression of the integrated target gene and the enzyme yield corresponding to copy number. A research group under the guidance of Jason C.H Shih, who worked on chromosomal integration of *KerA* gene into the *Bacillus lichiniformis*, has found that their copy number of the *KerA* gene was between 3-5 copies [40], astonishingly they also found that increased copy number (>5) has considerably reduced the processing and secretion of the extracellular keratinase in their strain *Bacillus lichiniformis* DB104.

So considering the results from the primary gene amplification test, a similar but elaborate gene amplification through elevation of antibiotic experiment could next be used in the future works. Further studies will be, to find the copy number of the integrated gene inside the chromosome, expression level of the integrated gene and also to find the optimum growth condition of the integrant strain; which could lead to a creation of an advantageous expression host good for pretreatment of poultry feathers for biogas production.

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