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Cloning, expression and characterization of serine protease gene from *Entrococcus hirae*

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ABSTRACT

Enterococcus hirae is a Gram Positive Bacteria. *Enterococcus hirae* has an ability to produce Protease. In this experiment, we attempted for the protease producing gene *in Enterococcus hirae* and transferring the gene to non-Protease producing organism. It means which gene that responsible to produce protease is identified by this experiment. Primer designing tools are used to design the specific primer for the amplification of DNA. The Primers are used for amplification. DNA is isolated from *Enterococcus hirae* using DNA isolation method. The isolated DNA is cross checked by the Agarose Gel Electrophoresis Method. The isolated DNA is further introduced into PCR machine for amplification. The PCR Master Mix and Primers are used for amplification. After the DNA amplification, the cloning vectors are used for Cloning. The Ta plasmid vector (pBZ57RT) are used for DNA cloning, the cloned DNA with the vector is transformed into the Non protease producing organism, such as E.coli and checked for its activity.

KEYWORDS

Protease gene;
Enterococcus hirae;
Casein degradation activity;
DNA isolation;
Amplification;
Agarose gel electrophoresis;
Transformation.

INTRODUCTION

Enterococcus hirae is a Gram Positive Bacteria. *Enterococcus hirae* has an ability to produce Protease. In this experiment, we identified gene expression. It means which gene that responsible to produce protease is identified by this experiment.

Primer designing tools are used to design the specific primer for the amplification of DNA. The Primers are used for amplification. DNA is isolated from *Enterococcus hirae* using DNA isolation method. The isolated DNA is cross checked by the Agarose Gel Electrophoresis Method.

The isolated DNA is further introduced into PCR machine for amplification. The PCR Master Mix and Primers are used for amplification. After the DNA amplification, the cloning vectors are used for Cloning. The TA plasmid vector (pBZ57RT) are used for DNA cloning, the cloned DNA with the vector is transformed into the Non protease producing organism, such as *E.coli*.

Now, the *E.coli* grown with the transformed gene. So the *E.coli* is consist of protease producing gene, it can able to produce protease. By this Process we can identified that the specific gene which is response for

the production of protease[8].

The protease produced from *E.coli* after transformation is always performed by the specific sequence that we transformed into it. Because *E.coli* is non protease producing organism, so the protease produced by the *E.coli* after transformation is provided by gene that isolated from *Enterococcus hirae*[9].

MATERIALS AND METHODS

Skimmed Milk agar plate is made by Dissolving 20 g of dried skimmed milk in 100 cm^3 of distilled water[2]. Sterilize separately. Transfer the milk to the agar aseptically after cooling to 45-50 °C. Dispense aseptically. *Entercoccus hirae* culture (KC 991294.1) was obtained from the Department of Biotechnology, Vel Tech High Tech Dr.Rangarajan Dr.Sakunthala Engineering College, Avadi, Chennai, Tamil nadu 600062, India. The Strain was maintained by Sub-culturing on Agar Medium slants at 24°C for 5 days of Surface cultivation. *Escherichia coli* were employed for sub cloning and plasmid recovery[6].

Isolated CFU's were routinely grown in *Luria-Bertani (LB)* broth medium (composed of (g/l): peptone - 10; yeast extract - 5; NaCl - 5). Media were autoclaved at 120 °C for 20 min. Cultivations were conducted in 25 ml of medium in 250 ml conical flasks maintained at 37°C. Incubation was carried out with agitation at 200 rpm for 24 hr. The cultures were centrifuged and the supernatants were used for isolation of DNA for gene sequencing[5]. ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit was used to sequence the PCR-generated Products. *pBZ57RT* was used as expression vector. Genomic DNA is extracted from *Enterococcus hirae* KC991294.1 using the General Method[1]. Primer is designed and used to clone the genomic sequence.

Agarose gels are easy to cast and is particularly suitable for separating larger DNA of size range most often encountered in laboratories, which accounts for the popularity of its use. Primer3 is a free online tool to design and analyze primers for PCR and real time PCR experiments. Primer3 can also select single primers for sequencing reactions and can design oligonucelotide hybridization probes[7]. TA cloning is a sub cloning technique that avoids the use of restriction enzymes and is easier and quicker than traditional subcloning[12].

Sub culturing and finding the activity of protease

Enterococcus hirae is sub cultured from mass culture using Agar medium. The Subculture is used for further process. The agar medium is pour in the test tube. A loop full of culture is taken and streak in the medium for sub culturing. Identification of protease activity by Protein degradation method[4]. Skimmed milk is used to provide skimmed milk agar plate. Skimmed milk is containing casein; the protease produced from the *Enterococcus hirae* is degrading the casein that present in the Skimmed milk agar plate.

Primer designing

Two types of primers are designed for amplification, they are known as forward primer and reverse primer. The primers are designed by the bioinformatics tools. Normally BLAST, CLUSTAL W and Primer3. The sequence is retrieved from the Genbank (www.ncbi.nlm.nih.gov/ genbank ý) and the FASTA sequence is loaded to get similar sequence from BLAST (www.ncbi.nlm.nih.gov/BLAST). Sequence that are related to the Original Sequence is retrieved[17].

Designing of primer

Primer3 is a free online tool to design and analyze primers for PCR and real time PCR experiments. Primer3 can also select single primers for sequencing reactions and can design oligo nucleotide hybridization probes. The online tool constitutes some important features like primer detection, cloning, sequencing and Primer listing[18].

GC content tabling

The Primer contain G:C content separately for forward and reverse primer.

TABLE 1 : GC content of forward and reverse primer is tabulated

PRIMER	G:C Content
Forward Primer	62.4
Reverse Primer	47.6

The Forward and Reverse Primer is Purchased from Snerrgy Company. The Stock solution is maintained in -20°C and Working solution is maintained in 4°C.

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DNA sequencing

The PCR product is sequenced using ABI PRISM 310 Genetic Analyser. This machine is connected to a System and the result is displayed in the system. The Sequenced product is checked with the original sequence. This sequence 100% matched with the original sequence. The Sequence match are confirmed by checking with MEGA. The Result of PCR Sequenced product is shown below[15].

DNA isolation

DNA is isolated from the Enterococcus hirae by the DNA isolation method. General method is been used and the DNA is extracted.

Running on PCR

The gene coding for the 16S ribosomal RNA from the isolated DNA was amplified across 25 cycles, using Thermal cycle machine.

Transformation

Transform the plasmid into the given E.coli host by transformation technology. Transformation is a technique most widely used gene transfer mechanism. Transformation process is carried out by heat shock method. The plasmid DNA is injected into the *E.coli* (Non Protease producing Organism). The introduction of exogenous DNA into the bacteria is one of the significant experiments in biotechnology. Screening of recombinant and the propagation of the plasmid vector, clones and expression of recombinant proteins. This method involves the transformation of a plasmid vector into a host and study its expression with the production of a blue compound (that act as a indicator) by blue white screening as the name implies. The given host DNA is the ampicillin sensitive containing a gene expression only omega portion of β-Galactosidase gene, that can competent with α peptide of the vector (Plasmid DNA) to produce active functional enzyme.

Treatment of the *E.coli* cells harvested at 0.6 OD with ice cold solution of divalent cations (Cacl2) includes a transition state of competent. The DNA uptake from the extracellular source is enhanced by a sudden heat shock given to a chilled cells.

The expression of genes for the enzyme is induced by IPTG (Iso Propyl β-D Thio Galacto Pyranoside) which induce β-Galactosidase enzyme synthesis. The enzyme then converts the chromogenic substrate in the medium X-Gal (5'Bromo 4 Chloro 3 Indole β D Galacto pyranoside) forming blue colonies. The transformant are selected by plating on the Solid medium with appropriate antibiotic, IPTG, XGal[19].

Screening of isolated transformed colonies

The Blue colonies are collected individually and cultured in the agar plate. Now the agar plate is containing transformed cells. The colonies are cultured in the agar plate and the colonies are identified the activity by casein degradation method.

Finding the activity

The Skimmed milk is added with agar and preparing the skim milk agar plate. The skim milk agar plate containing casein. The transformed colonies is collected in a loop and streak in the casein plate. It incubated for 24-48 hours. The protease produced from the organism degrade the casein in the skim milk agar plate which denotes that the transformed cell containing the ability to produce protease[3]. From this we can identify the expression of gene[16].

RESULT AND DISCUSSION

Sequence retrieved

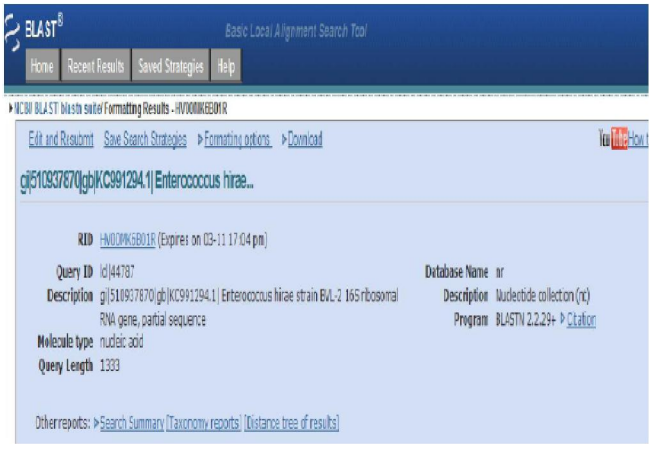

Figure 1 : The sequence is retrieved for the NCBI website[20]

Related sequence

Mega analysis

The analysis is done and conserved sequence are retrieved.

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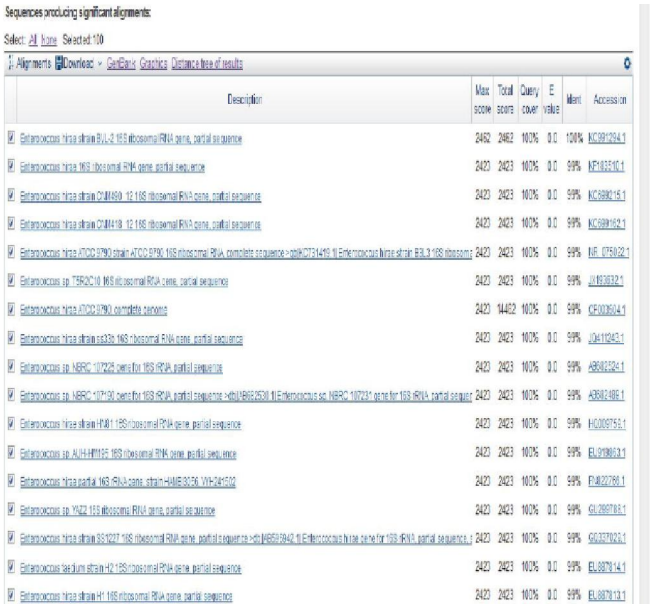

Figure 2 : These are the sequence retrieved from BLAST. 100 sequence that are related to the original sequence is retrieved.

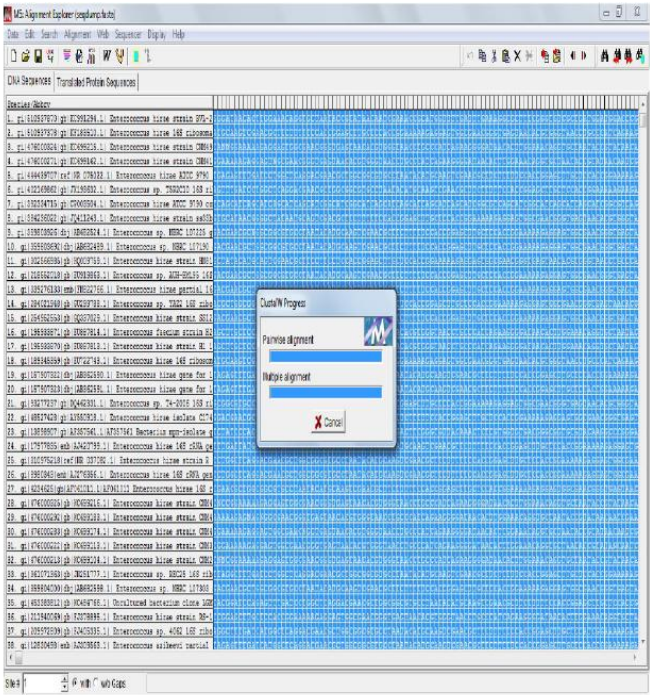

Figure 3 : The sequence are aligned with multiple sequence alignment by CLUSTAL W[21]

Reverse and forward primer

In which the Primer designed are used at specific temperature mentioned. The length of the Primer is mentioned and GC content and Tm is mentioned. (Tm – Melting Temperature.)

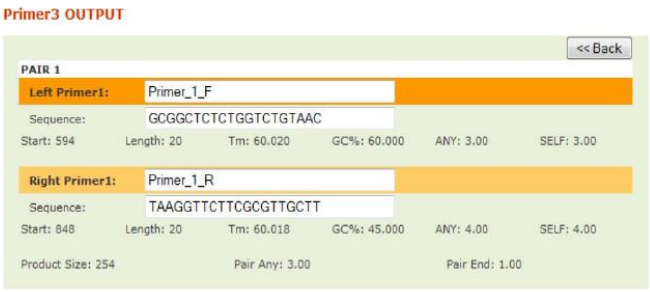

Figure 4 : The primer resulted by this software

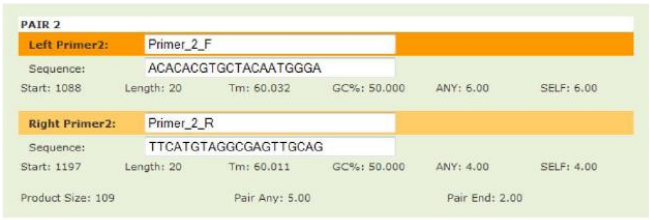

Figure 5 : Other pair of primer

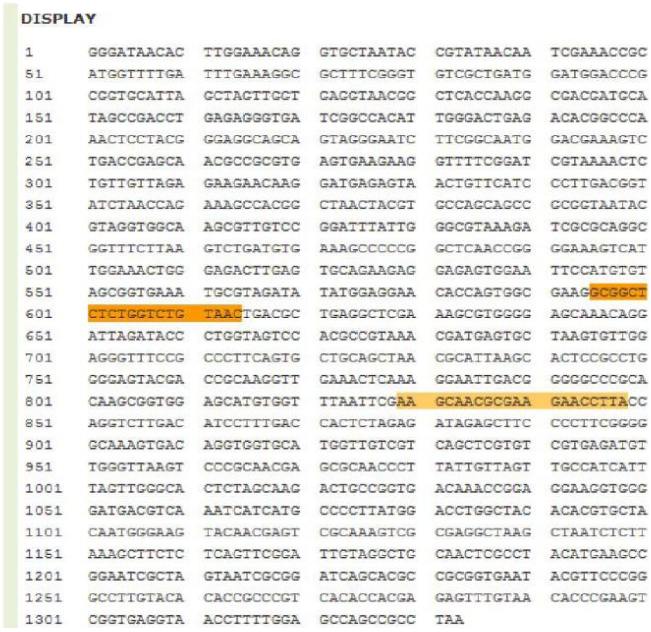

Figure 6 : The main markings denotes the primer and shaded marking denotes the alternate primer

TABLE 2 : The primer contain G:C content separately for forward and reverse primer

PRIMER	G:C Content
Forward Primer	62.4
Reverse Primer	47.6

The overview is

GC content tabling

The Forward and Reverse Primer is Purchased from Snerrgy Company. The Stock solution is maintained in

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   1 gggataacac ttggaaacag gtgctaatac cgtataacaa tcgaaccgc atggttttga
  61 tttgaaaggc gctttcgggt gtcgctgatg gatggacccg cggtgcatta gctagttggt
 121 gaggtaacgg ctcaccaagg cgacgatgca tagccgacct gagagggtga tcggccacat
 181 tgggactgag acacggccca aactcctacg ggaggcagca gtagggaatc ttcggcaatg
 241 gacgaaagtc tgaccgagca acgccgcgtg agtgaagaag gttttcggat cgtaaaactc
 301 tgttgttaga gaagaacaag gatgagagta actgttcatc ccttgacggt atctaaccag
 361 aaagccacgg ctaactacgt gccagcagcc gcggtaatac gtaggtggca agcgttgtcc
 421 ggatttattg ggcgtaaaga tcgcgcaggc ggtttcttaa gtctgatgtg aaagccccgg
 481 gctcaaccgg ggaaagtcat tggaaactgg gagacttgag tgcagaagag gagagtggaa
 541 ttccatgtgt agcggtgaaa tgcgtagata tatggaggaa caccagtggc gaaggcggct
 601 ctctggtctg taactgacgc tgaggctcga aagcgtgggg agcaaacagg attagatacc
 661 ctggtagtcc acgccgtaaa cgatgagtgc taagtgttgg agggtttccg ccttcagtg
 721 ctgcagctaa cgcattaagc actccgcctg gggagtacga ccgcaaggtt gaaactcaaa
 781 ggaattgacg gggcccgca caagcggtgg agcatgtggt ttaattcgaa gcaacgcgaa
 841 gaaccttacc aggtcttgac atccttgac cactctagag atagagcttc cccttcgggg
 901 gcaaagtgac aggtggtgca tggttgtcgt cagctcgtgt cgtgagatgt tgggttaagt
 961 cccgcaacga gcgcaaccct tattgttagt tgccatcatt tagttgggca ctctagcaag
1021 actgccggtg acaaaccgga ggaaggtggg gatgacgtca aatcatcatg ccccttatgg
1081 acctggctac acacgtgcta caatgggaag tacaacgagt cgcaaagtcg cgaggctaag
1141 ctaatctctt aaagcttctc tcagttcgga ttgtaggctg caactcgcct acatgaagcc
1201 ggaatcgcta gtaatcgcgg atcagcacgc cgcggtgaat acgttcccgg gccttgtaca
1261 caccgcccgt cacaccacga gagtttgtaa cacccgaagt cggtgaggta acctttttgga
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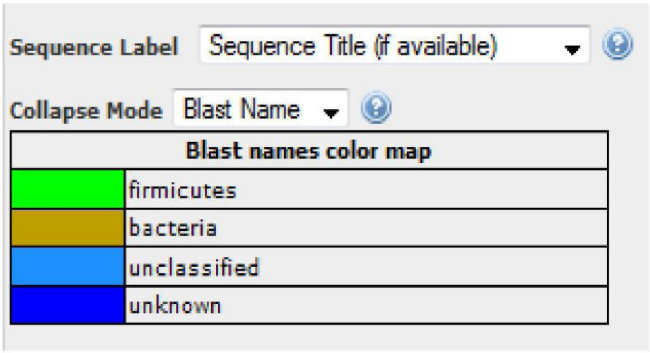
Figure 7 : The result of PCR sequenced product



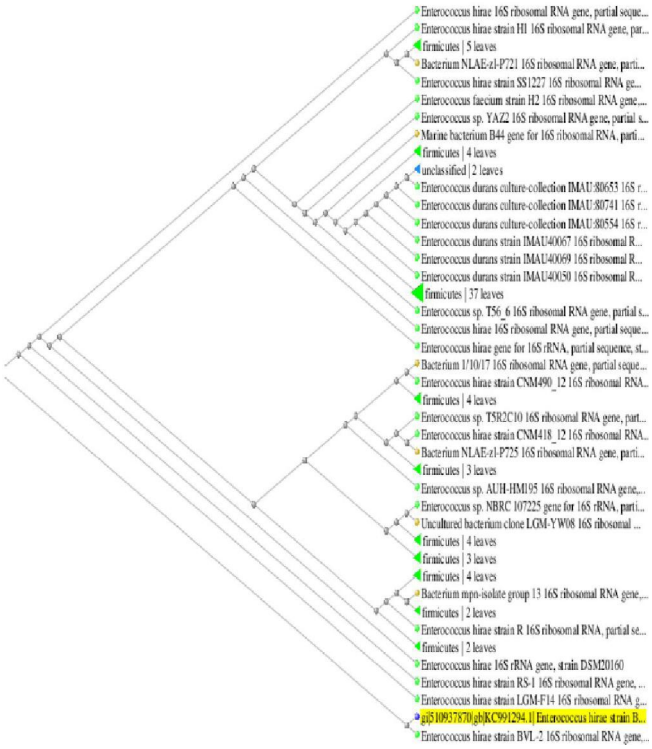


Figure 8 : The highlighted is the species we used and other species are the related sequence that are retrieved through BLAST in NCBI website.

-20°C and Working solution is maintained in 4°C.

PCR product sequenced

Phylogenetic tree analysis

This Phylogenetic tree is Slanted with following denotes following:

Agarose Gel Electrophoresis

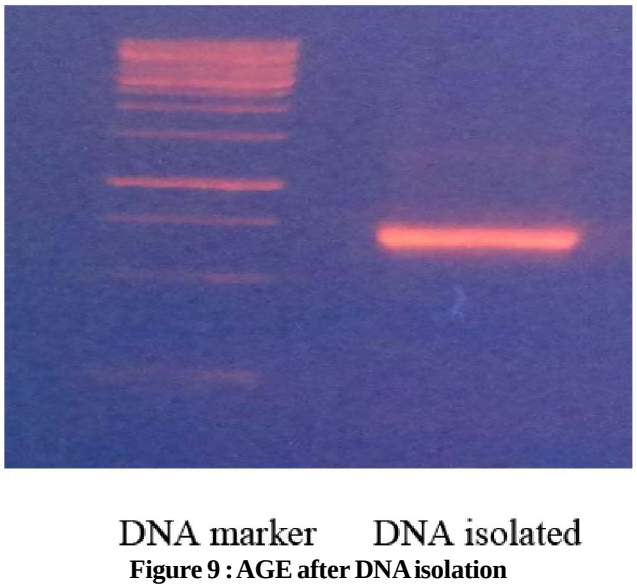


Figure 9 : AGE after DNA isolation

Agarose Gel Electrophoresis

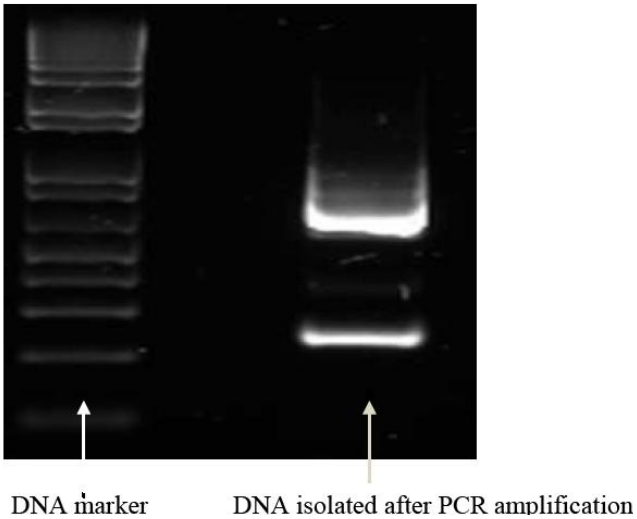


Figure 10 : AGE after PCR process

Screening of transformed colonies

Casein degradation plate

A loop full of transformed culture and normal *E.coli* culture is taken and streak in the casein agar plate and incubated for 24 to 48 hrs. Clear zone of casein degra-

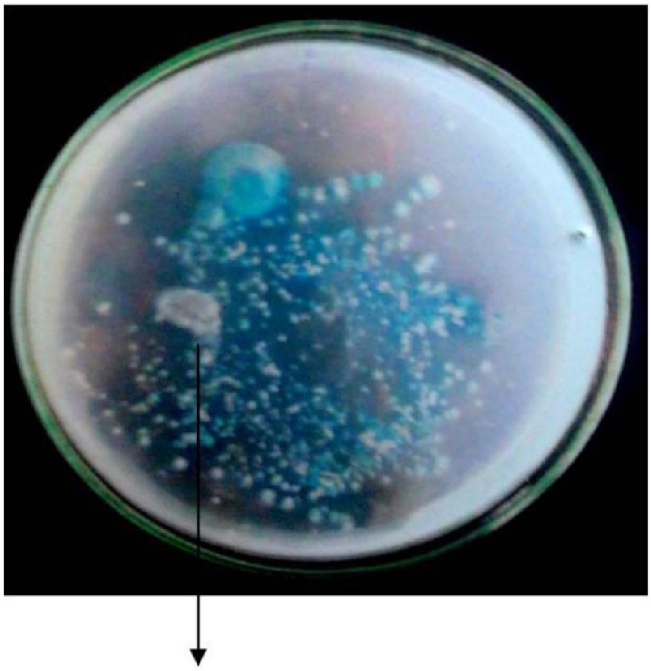


Figure 11 : The white colonies indicates the transformed colonies

Figure 12 : The above plate contain *E.coli* which is not transformed and other strain contain protease gene transformed by the insertion of gene of insert through TA vector. This result showed that *E.coli* that is transformed got the ability to produce protease gene and degraded casein.

dation is identified. The zone is formed by transformed cell in the culture. which produces protease[14], it degrade the casein present in the casein agar plate. This proved the Protease gene being transformed and changed *E.coli* to produce Protease in it.

CONCLUSION

An *Enterococcus hirae* have the ability of producing the protease gene expressing protease enzyme which have many application in different industries. This gene is amplified by PCR. The primer is designed with the Bioinformatics tools. The Sequence is retrieved from genbank with genbank ID KC991294.1 *Enterococcus hirae*. Then this sequence is used for the retrieval of similar sequence from BLAST (NCBI Website). The retrieved sequence is then aligned by multiple Sequence alignment (CLUSTAL W) in MEGA Software. This Software that generated a conserved region. Using that region a primer is designed with Specific parameters by Online Primer designing tool Primer3. This Primer is used for the amplification of the sequence. The amplified sequence is purified and sequenced by ABI PRISM 310 Genetic analyser and then it is checked. The sequenced PCR product gave the same sequenced ensured the PCR product is amplified. Then the DNA isolated and amplified DNA are run in Agarose Gel Electrophoresis and ensured presence of DNA in it.

Then with the help of cloning vector the DNA is inserted by Transformation process. The DNA is taken by *E.coli* by heat shock and cooled. Then the *E.coli* is sub cultured and plated in Casein plate. The Zone formed around the around the organism ensured the Presence of protease in it and ensured transformation is successful and modified *E.coli* to produce protease.

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