Cloning and Expression of genes belonging to GH family of enzymes in *Bacillus subtilis* and *Aspergillus oryzae*

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Submitted By

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CANDIDATE'S DECLARATION

I, ARUSHE TICKOO, Roll No. 2K16/IBT/03 of M. Tech. (Industrial Biotechnology), hereby declare that the project Dissertation title "Cloning and Expression of genes belonging to GH family of enzymes in *Bacillus subtilis* and *Aspergillus oryzae*" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

Place, Delhi ARUSHE TICKOO

Date:

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CERTIFICATE

I hereby certify that the Project Dissertation titled "Cloning and Expression of genes belonging to GH family of enzymes in *Bacillus subtilis* and *Aspergillus oryzae*'' by ARUSHE TICKOO, Roll No. 2K16/IBT/03, Department of Biotechnology, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is a record of the project work carried out by the student under my supervision at novozymes. To the best of my knowledge this work has not been submitted or full for any Degree or Diploma to this University or elsewhere.

Place, Delhi
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ABSTRACT

GH enzymes are one of the most important and oldest industrial enzymes. These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, pharmaceutical, and detergent, textile and paper industries. GH enzymes are used in detergents to facilitate the removal of starch containing stains. This experimental study aimed at generating recombinant *Bacillus subtilis* variants expressing significant amount of amylase enzyme. Site directed mutagenesis and site saturation libraries were used to impart mutations in various backbones (wild type strains). This method consists of C-fragment generation and giga PCR (mega primer based PCR). Mutated amylase genes were cloned in *Bacillus subtilis* and *E.coli* and transformed colonies were selected based on the presence of zone of clearance. The amylase gene of these clones was amplified by colony PCR and sent for DNA sequencing. The sequence confirmed variants were then fermented in different media for optimization. Expression study of GH enzymes was done using Sodium Dodecyl PolyAcrylamide Gel Electrophoresis (SDS PAGE). Change in the level of expression in different amylase backbones with respect to change in temperature and media volume was also studied. We have used a method for the fast and efficient cloning of GH enzyme genes from various sources by combining the ability of *Bacillus subtilis* to clone amplify and express the respective genes and of *Aspergillus oryzae* to efficiently express the heterologous genes. Recombinant protein production was screened in *Aspergillus oryzae*. Based on the characterization of the enzymes by expression analysis, purification and assay studies, large scale production may be undertaken.

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ABBREVIATIONS

°C	Degree Celsius
PCR	Polymerase Chain Reaction
bp	Base Pairs
DNA	Deoxyribonucleic Acid
dNTP's	Deoxynucleotide Triphosphate
Kb	Kilo Base Pairs
M	Molar
Mg	Miligrams
mM	Millimolar
RPM	Revolutions Per Minute
µL	Microlitre
µg	Microgram
SDS	Sodium Dodecyl Sulphate
PAGE	Polyacrylamide Gel Electrophoresis
kDa	Kilodalton
GOI	Gene Of Interest
RT	Room Temperature
CFU	Colony Forming Units
FP	Forward Primer
RP	Reverse Primer
CAM	Chloramphenicol
KAN	Kanamycin
CC	Competent Cells
PP	Polypropylene
O D	Optical Density
PDA	Potato dextrose Agar
LB	Luria broth

CHAPTER 1: INTRODUCTION

The existence of enzymes has been known for well over a century. Compounds are the bio-impetuse assuming a vital part in all phases of assimilation and biochemical responses. Certain enzymes (catalytic compounds) are of unique intrigue and are used as natural stimuli in various procedures on a modern scale. Microbial enzymes are known to be predominant proteins acquired from various microorganisms, especially for applications in productions on industrial scales. (Gupta R *etal.,* 2002).

Enzymes are protein fragments that are essential to maintain life structure. GH enzymes are the class of enzymes that are responsible for the break-down of complex carbohydrates molecules. There are distinctive sources from which GH enzymes can be harvested. Plants, animals and microbes are the major producer of the enzymes including amylases (Aiyer 2004). These compounds represent around 30% of the world's catalyst production and have an extraordinary importance with broad biotechnological applications in bread making, nourishment, and textile & paper enterprises. (Gupta R *etal.,* 2003). Today, amylases are accessible financially in the huge number and they have totally superseded chemical hydrolysis of starch and reduced the conception of synthetic mixtures utilized as a part of sugar hydrolysis. Microorganisms create various types of modern proteins (enzymes) due to their biochemical, ecological and hereditary diversity. *Bacillus subtilis* is a standout amongst the most generally utilized microscopic organisms for the making of particular catalytic compounds. (Anwar A *etal.,* 2000). The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics.

Amylases are enzymes that catalyzes the hydrolysis of starch to smaller oligosaccharides by acting on α-1,4 glycosidic bonds. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. It is among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 20-35% of the world enzyme market. Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries.

In this study we have used a method for the fast and efficient cloning of GH enzyme genes from various sources by combining the ability of *Bacillus subtilis* to clone amplify and express the respective genes and of *Aspergillus oryzae* to efficiently express the heterologous genes. Recombinant protein production was screened in *Aspergillus oryzae*. Based on the characterization of the enzymes by expression analysis, purification and assay studies, large scale production may be undertaken.

The *Bacillus subtilis* genome vector is a novel cloning system for large DNA fragments in which the entire 4.2 MB genome of *B. subtilis* functions as a vector. It has several actractive properties like large cloning capacity of upto 3 MB stable propogation of cloned DNA and various modifications stratigies using homologous recombination. The transformation was carried out in *Aspergillus oryzae* as well due the low background expression that allows easy filteration and specific band of protein while *bacillus* has large amount of proteins that gets resolved in the SDS-PAGE

AIM AND OBJECTIVES

1.1 Aim

- To enhance the properties of amylase for industrial applications

1.2 Objective

- To generate DNA variants by engineering mutations through PCR based Site Directed Mutagenesis, Site-saturation libraries and Splice overlap extension to the gene of interest.
- To transform DNA variants into *B. subtilis* and *Aspergillus oryzae* expression host.
- To ferment hits obtained from the supernatant assay

CHAPTER 2: REVIEW OF LITERATURE

2.1 Enzymes

The existence of enzymes has been known for well over a century. In the 1850's, Louis Pasteur concluded that fermentation of sugar into alcohol by yeast is catalyzed by "ferments." He postulated that these ferments were inseparable from the structure of living yeast cells; this view, called vitalism, prevailed for decades. Then in 1897 Eduard Buchner discovered that yeast extracts could ferment sugar to alcohol, proving that fermentation was promoted by molecules that continued to function when removed from cells. Kuhne called these molecules enzymes. All enzymes are proteins. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or separated into its subunits, the catalytic activity is typically lost. If an enzyme is broken down into its amino acids, its catalytic activity is always destroyed. Thus the primary, secondary, tertiary, and quaternary structures of protein enzymes are essential to their catalytic activity. (Binod *etal.,* 2013.)

The enzyme can be classified in to six main classes **Table 1:** Classification of enzymes (Trevor Palmer, 1991)

No.	Class	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Transfer of functional groups to water (Hydrolysis reaction)
4	Lyases	Addition and formation of double bonds by removal of groups
5	Isomerases	Generation of isomeric forms due to transfer of groups within molecules
6	Ligases	Formation of CO-C, CO-S, CO-O, and CO-N bonds by condensation reactions

2.2 Glycoside hydrolases

Glycoside hydrolases also known as glycosidases or carbohydrases comprise a widespread group of enzymes catalyzing glycolytic cleavage of O-glycosidic bond. They are attributed to catabolic enzymes of carbohydrate metabolism; Glycoside hydrolase genes are found in almost all living organisms the only exception being some Archaeans and some unicellular parasitic eukaryotes Some viruses also encode glycosidases. (Coutinho *etal*., 1999) Evolutionarily closely related glycosidases often differ in substrate specificity while enzymes with very different enzyme activities as well as proteins devoid of enzyme activity are found among glycosidase homologs Small changes in the primary structure of glycoside hydrolases are able to change their substrate specificity (Ohmiya,K *etal*., 1999)

Figure 1: Working mechanism of GH enzymes

Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market. Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. However, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medical and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries. (Binod *etal.,* 2013) Amylases can be obtained or purified from different sources like animals, plants and microorganisms. Microbial sources are generally preferred for the mass production of amylase because of reduction in time, labor and cost. The levels of microbial enzyme to be incorporated can be enhanced by well-established hereditary systems, continuous culture selection or improvement of development conditions for the compound of interest.

Detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to

remove tough stains and making the detergent environmentally safe. Amylase is used in the formulation of enzymatic detergent. It is used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custard, chocolate, etc. to dextrins and other smaller oligosaccharides .The enzymes are mainly added to the detergents to improve its cleaning ability. Amylases with desirable properties, such as high thermostability, low pH stability, raw starch digestibility, and the utilization of a high concentration of starch, can be very useful in industrial applications; especially in detergents. When used in detergents, amylases remove starch-based food stains. (Linares-Pastén *etal.,* 2014)

The chief amylase generating bacteria are the species of *Bacillus, Halomonas, Arthrobacter,* and *Serratia*. Amid of all the bacterial sources, most commonly used are *Bacillus subtilis*, *B. stearothermophilus, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus acidocaldarius, Bifidobacterium bifidum*, and *Bacillus macerans* play an important role in production of amylase. Several species of *Bacillus* are industrially employed to produce thermostable amylase. *Bacillus subtilis* is a gram positive, catalase positive, rod shaped bacterium. It is heavily flagellated, which gives it the ability to move quickly in liquids. It is amenable to genetic manipulations, can produce large quantities of desired product, is not pathogenic or toxic to humans and, very significantly, can secrete proteins into the medium.

2.3 Protein Engineering

Catalysts found in nature have been misused in industry basically because of their characteristic catalytic properties. They catalyze complex reactions under mild and natural conditions. It is often difficult to achieve the desired industrial goal using the native form of the enzyme. Recent advancements in protein engineering have initiated the development of commercially available enzymes into better industrial catalysts. Protein engineering aims at modifying the arrangement of a protein structure thus altering its structure which leads to a new construct of enzymes with upgraded practical properties like stability, specific activity, and inhibition by reaction products, and selectivity towards non-characteristic substrates. (Warren etal., 1997).

2.3.1 Strategies involved in Protein Engineering

Site Directed Mutagenesis

Site-directed mutagenesis is an in vitro method for creating a specific mutation in a known DNA sequence. It is also called site-specific mutagenesis or oligonucleotide-directed mutagenesis and is used for investigating the structure and biological activity of DNA, RNA, and protein molecules, and for protein engineering. Presently, several PCR-based mutagenesis approaches are available for site directed mutagenesis but among them Megaprimer method and the overlap extension method has been used widely. (Meijuan Xua *etal.,* 2013)

Megaprimer Method/ Giga Primer Method

In this strategy PCR amplification is done in two steps using two flanking primers with one core mutagenic primer (internal). During the first round of PCR, flanking primers combine with the internal mutagenic primer (having preferred base substitutions) to generate a megaprimer. This megaprimer is then purified and used along with the other flanking primer in the second round of PCR that generate a complete DNA sequence with the anticipated mutation.

Overlap extension PCR

This method involves a pair of overlapping mutagenic primers and two flanking primers. Two different combinations of one flanking primer and one mutagenic primer are used to generate two DNA fragments having overlapping ends by two separate PCR amplifications. A subsequent "fusion" reaction combines these fragments in which the overlapping ends anneal to each other, allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. (Zheng, L *etal.,* 2004) The resulting fusion product is amplified further by PCR. In certain cases, however, the annealing of overlapping ends often fails to occur, which could be due to the complementary re-association of double-stranded DNA in the fusion reaction and the adoption of secondary structure in single-stranded DNA produced in the subsequent PCR. The megaprimer method is simpler and quicker than overlap extension as there is a reduction in complexity by 30%. The overlap extension strategy is particularly useful when

1. Small insertions are required. This can be achieved by flanking overlap in the primer pair.
2. Deletions are required. This can be achieved by generation two fragments that overlap with each other excluding the region of insert for deletion.
3. Multiple mutations can be combined if they are at the N/C terminal and in the middle. (Hunt *etal.,* 1989)

Site Saturation Mutagenesis

Site-saturation mutagenesis (SSM) is a similar method with an additional dimension of complexity, in which a single amino acid can be substituted to any of the other 19 possible substituents. As a result, the mutagenesis product is a collection of clones, each having a different codon in the targeted position, so it is called ‘‘saturated’’. This is an advantage over SDM since all possible substitutions can be obtained with similar effort, allowing a more comprehensive analysis of the function of the original amino acid in 19 the targeted position. The design of a SSM experiment varies substantially according to the application. (Siloto *etal.,* 2012)

2.4 *Bacillus subtilis* as a cloning host

Bacillus subtilis is a Gram-positive, sporulating, aerobic bacterium phylogenetically very distant from *E. coli*. *Bacillus subtilis* cloning system parallel to that developed for *E. coli* permits studies of the interaction of the same genes with two, possibly very different, cellular environments, which would offer greater insight into the diverse processes that convert genetic information to the corresponding phenotype. The development of the *Bacillus subtilis* cloning system has been mainly hindered by the absence of suitable vector replicons. Several plasmids can be introduced and maintained in the highly transformable *Bacillus subtilis* strains however, they lacked genetic markers for selection and DNA cloning. More recently, plasmids originally isolated from Staphylococcus aureus that code for resistance to tetracycline or chloramphenicol have been transfected into *Bacillus subtilis.* (Ehrlich, 1977)

Bacillus subtilis are secretory bacteria where the protein is excreted into the broth easing the process of downstream purification. Due to ease of handling, induction of competent state, understanding of genome they make ideal hosts for protein expression. Bacillus subtilis was

the first organism to have a secretome constructed. DNA is a hydrophilic molecule and cannot pass through the cell membrane of the bacteria and to make them competent, bacterial cells are suspended in a hypotonic solution of Calcium chloride. Exogenous DNA binds to the cell membrane; on uptake, it is fragmented and the DNA integrates to the host by homologous recombination. On recombination, the genotype of the host is altered leading to genetic diversity. (Guglielmetti *etal.,* 2007)

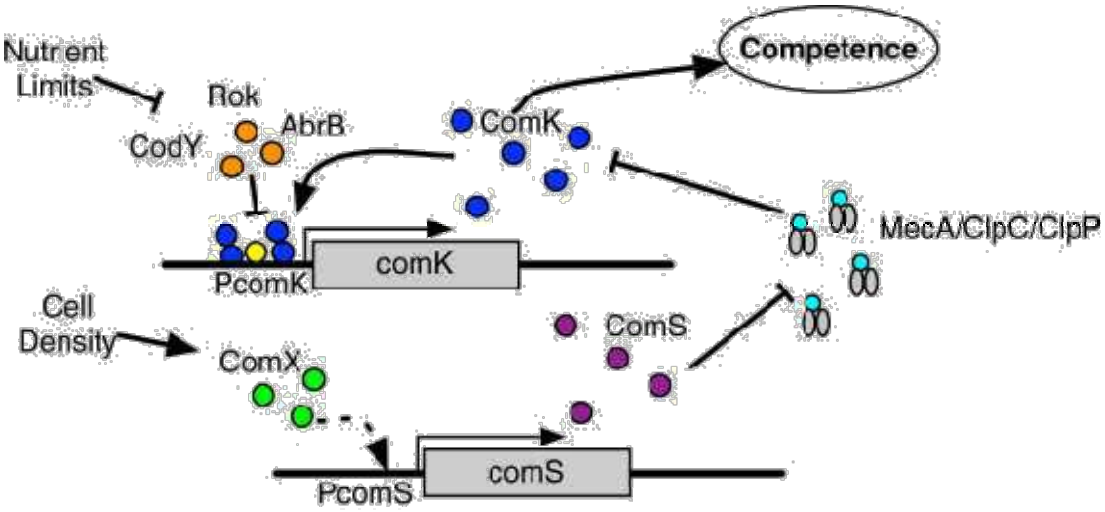


Figure 2: Transformation into competent Bacillus subtilis

E.coli is an efficient cloning host, but not always an ideal expression host. There are several limitations to using E.coli as an expression host for the genes. These include- a deficiency in post-translational modifications such as glycosylation, protein expressed in large amounts often precipitates into insoluble aggregates called inclusion bodies, from which it can only be recovered in an active form by solubilization in denaturing agents followed by careful renaturation, release of endotoxins, which must be removed from the final product.

Choosing a cloning vector:

Vectors must be relatively small molecules for convenience of manipulation. They must be capable of prolific replication in a living cell, thereby enabling the amplification of the inserted donor fragment. Another important requirement is that there be convenient restriction sites that can be used for insertion of the DNA to be cloned. Unique sites are most useful because then the insert can be targeted to one site in the vector. (Avakov *etal.,* 1990).

There are numerous cloning vectors in current use, and the choice between them often depends on the size of the DNA segment that needs to be cloned.

2.5 *Aspergillus oryzae* as an expression host:

Aspergillus is the genus of moulds that reproduce only by asexual means. *Aspergillus oryzae* is a member of the Deuteromycetes fungi, which is a group with no known sexual state. The morphology of the conidiophore, the structure that bears asexual spores, is the most important taxonomic character used in *Aspergillus* taxonomy. *Aspergillus oryzae* is an industrially important fungus used for the production of industrial enzymes and fermented foods such as sake and soy sauce. Fungi combine the advantages of a microbial system such as a simple fermentability with the capability of secreting proteins that are modified according to a general eukaryotic scheme- an advantage of which is the well-developed post-translational modification systems. *Aspergillus oryzae* is an attractive organism because of its high secretion capacity and is frequently used as a model organism.

In recent years, recombinant DNA technology has been used to enhance industrial enzyme production by *Aspergillus oryzae*. Production strains of this organism have been generated by means of classical mutagenesis and protoplast fusion methods. DNA-mediated transformation of *A. oryzae* is achieved by complementation of auxotrophic mutants as well as through the use of dominant selectable markers conferring resistance to Phleomycin or Acetamide. In general, transformation is the introduction into cells of exogenously added DNA and the subsequent inheritance and expression of that DNA. The most important advances in the molecular characterization and controlled modification of *Aspergillus* genes have relied on the use of shuttle vectors which can be used to transform both *Aspergillus oryzae* and *E. coli.* (Minetoki, *etal.,* 1996)

Christensen et al. have applied their findings on the transformation of *A. oryzae* to the development of an expression system for homologous and heterologous gene sequences in this organism. The genome of *A. oryzae*-specific is enriched for genes involved in metabolism, particularly those for the synthesis of secondary metabolites. Specific expansion of genes for secretory hydrolytic enzymes, amino acid metabolism and amino acid/sugar uptake transporters supports the idea that *Aspergillus oryzae* is an ideal microorganism for fermentation. Fungal cell wall acts as a major barrier in the transformation process, thus the

protoplast needs to be prepared for successful transformation into *Aspergillus oryzae*. (Tada *etal.*, 1991) This is done enzymatically. All protoplast preparations have to be protected by the presence of an osmotic stabilizer in the suspending medium.

CHAPTER 3: MATERIALS AND METHOD

Steps in Molecular Cloning

Gene cloning is a method for making many identical copies of a gene by inserting the gene into a living host cell. Under appropriate conditions, the host cell will replicate the "foreign" DNA along with its own DNA whenever it divides. With each cell division, the number of copies of the "foreign" gene doubles. This technique permits us to produce large quantities of a single gene so that they we can study the gene in detail.

In standard molecular cloning experiments, the cloning of any DNA fragment essentially involves seven steps:

(1) Choice of host organism and cloning vector

(2) Preparation of vector DNA

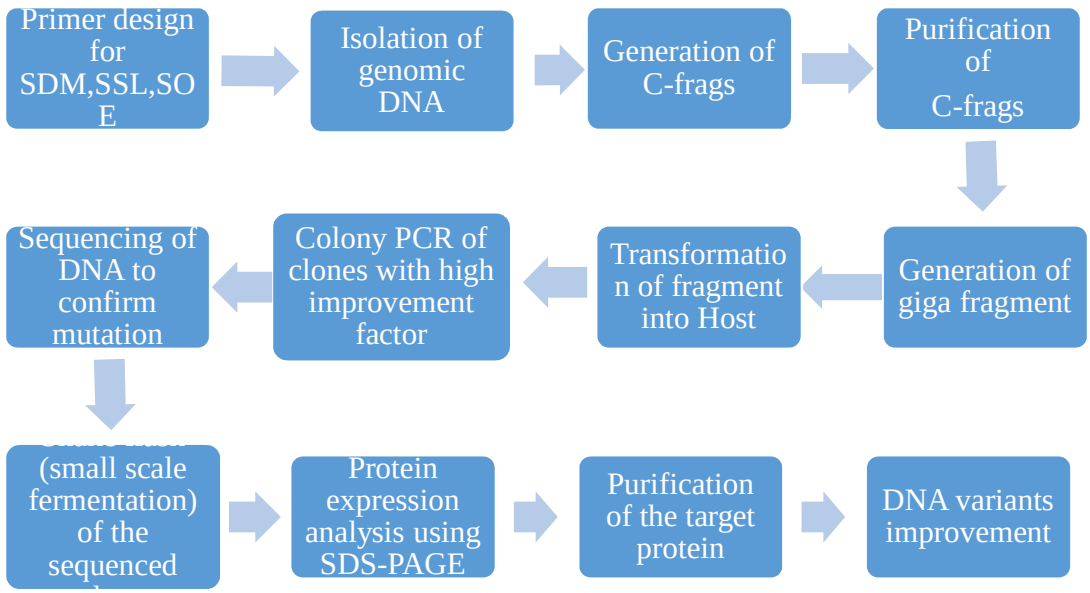
(3) Preparation of DNA to be cloned

(4) Creation of recombinant DNA

(5) Introduction of recombinant DNA into host organism

(6) Selection of organisms containing recombinant DNA

(7) Screening for clones with desired DNA inserts and biological properties.



Whole workflow that has been followed during the process

WORKFLOW

Creating a Gene insert using SOE/SDM/SSL's

↓

Competent cell Preparation *(E. coli* and *Bacillus subtilis)* and protoplast isolation *(Aspergillus oryzae)*

↓

Transformation of the GOI into the vector

↓

Selection of transformants in selective plates

↓

Analyzing the activity of the transformed cells (using substrate specific plates)

↓

Confirming the integration using colony PCR

↓

Small scale fermentation of positive transformants (expressed in *Bacillus* and A*spergillus*)

↓

Best expressing clone sequenced (sanger)

↓

Large scale fermentation of hit picks from the sequencing

↓

Enzyme Purification and Assay to analyse the change in activity and stability

3.1 Preparation of Competent Cells

Competence is defined as the capability of cell to take up foreign DNA from its environment. Transformation efficiency can be defined as the efficiency of the cells through which it can take up and incorporate extracellular DNA in the system and express the genes that are encoded by it**.**

3.1.1 *E. coli*

1. Inoculate 5mL of LB medium (without antibiotics) with *E. coli* strain of choice overnight at 37°C at 180 rpm.
2. Streak the culture to isolate single colony.
3. A single colony from the streaked plate is inoculated in 10mL Luria Broth using a 50ml test-tube and incubate overnight at 180 rpm (37 °C). Prepare TSS buffer (composition given below)

Components	**Volume**
1M $MgCl_2$	0.30g/ml
PEG 3350	0.1g/ml
DMSO	50mg/ml
	Make up the volume using LB

4. 1% of the overnight grown culture is inoculated in 500ml LB containing flask and incubated at 180 rpm (37 °C). The culture is maintained until the O D of the culture at 600nm reaches 0.3 to 0.4 ($0.3 \leq OD_{600} \leq 0.4$).
5. Once the O.D reaches 0.4 transfer the culture into 500mL PP centrifuge tubes and centrifuge under 3000g for 10 min at 4°C. (All the steps should be carried out on ice)
6. Discard the Supernatant and re-suspend the pellet in 5mL (approx.) of pre-chilled TSS buffer with gentle swirling.
7. Chill TSS suspended cells on ice for 15 min. Label 1.5mL eppendorf tubes (snap-cap) for aliquoting

8. Distribute 100 µL of TSS suspended cells to each 1.5mL PP tube while ensuring the cells remain well mixed.
9. Cells were used immediately, or stored at -80°C

Sterility test for competent cells

1. Plates were labelled as LB-Cam, LB-AMP, LB-Plain and LB-Kan.
2. Glycerol stock of the competent cell vial was thawed and 2 µL was streaked and incubated at 37°C for 24 hours.

3.1.2 *Bacillus subtilis*

1. The host for the competent cells are streaked or patched on an LB [containing 0.5% glucose] agar plate to isolate single colony
2. Incubate the single colony overnight in LB at 37℃ at 180 rpm.
3. Inoculate 1% overnight grown culture in a fresh, pre-warmed, SpC medium (200 ml) at 37℃ at 250 rpm.
4. The culture is maintained at 37℃, 250 rpm and take periodic OD_{600} readings to assess cell growth till the culture reaches a stationary phase.
5. When it reaches the exponential phase inoculate pre-warmed SpII medium (upto 200ml) with 1% of culture from the stationary phase. The incubation is monitored again at 37°C at 180-250 rpm
6. After Incubating for 1-2 hrs, the cells are transferred to a polypropylene tube and centrifuged at 8000 rpm for 5-10 min at RT.
7. The supernatant is discarded and pellet is gently re-suspended in 10-15ml of the supernatant with 2-3ml of absolute glycerol.
8. Competent cell (upto 1 ml) are aliquoted in autoclaved eppendorf tubes, and rapidly stored in either liquid nitrogen (N_2) or isopropanol bath followed by -80℃.

Sterility test for competent cells

1. Plates were labelled as LB-Cam, LB-AMP, LB-Plain and LB-Kan.
2. Glycerol stock of the competent cell vial was thawed and 2 µL was streaked and incubated at 37°C for 24 hours.

Table 2: Media used for *Bacillus* transformation

T Base	Components	Volume (g/l)
	$(NH_4)_2SO_4$ $K_2HPO_4 \cdot 3H_2O$ KH_2PO_4 Trisodium citrate$\cdot 2H_2O$	2 18.3 6 1 Autoclaved
SpC (always prepared fresh)		(ml)
	T base 50% glucose 1.2% $MgSO_4 \cdot 3H_2O$ 10% Bacto yeast extract 1% casamino acids	20 0.2 0.3 0.4 0.5 Filter sterilized
SpII (freshly prepared)		(ml)
	T base 50% glucose 1.2% $MgSO_4 \cdot 3H_2O$ 10% Bacto yeast extract 1% casamino acids 0.1 M $CaCl_2$	200 2 14 2 2 1 Filter Sterlized
SpII - EGTA		

	SpII upto200 ml with 4 ml of EGTA (0.1 M, pH 8.0) is used without the addition of $CaCl_2$. SpII-EGTA is stored at -20 in small aliquots. (frequent freeze thawing must be avoided)

3.1.3 *Aspergillus oryzae*

Day 1

1. 9 ml of Tween 20 solution (0.1%) or Asp media 1 was added to the slant, and the spores of the growing fungus were suspended manually and maintained at 37°C until it has been sporulated completely.
2. Spore suspension was transferred to shake flasks with baffles containing 100 ml of Asp media 1 containing 1% peptone, 0.5% glucose, 0.5% potassium dihydrogen phosphate, 0.1% sodium nitrate, magnesium sulfate 0.05% and flask was incubated overnight at 30°C at 180 RPM.
1. Mycelium was collected by filtration with a filter paper or a mira cloth with sterile autoclaved milliQ water.
2. The mycelia was digested with 200mg/50ml yatlase enzyme for 2-4 hours till the protoplast was out of the cells (visualized after every half an hour under a microscope). If clumps of mycelia are predominantly visible then further digestion is allowed until isolated protoplasts are visible.
3. Once the protoplast is out of mycelia the solution is filtered and centrifuged at 6500 RPM for 15 minutes.
4. The mycelia was resuspended in glycerol and stored at -80°C.

3.2 Isolation of Genomic DNA from *Bacillus subtilis* (GenElute™ Bacterial Genomic DNA Kit, Sigma)

The wild type genomic DNA was isolated from the host on which the mutations were created using Site Directed Mutagenesis and Site saturation Mutagenesis.

The Sigma GenElute™ Bacterial Genomic DNA set is a combination that involves the benefits of a silica based microspin format. It eliminates the necessity for affluent resins, alcohol precipitation and unsafe organic compounds like phenol and chloroform. The microbes are lysed through a chaotropic salt containing sol'n that confirms exhaustive denaturation of molecules. Ethanol addition causes the DNA to bind to the silica membrane. Subsequent to washing, the DNA is eluted with Tris-EDTA.

Reagents that are required but not provided in the kit:

1. Lysozyme Solution: 45 mg/ml working solution was prepared by dissolving in Gram positive lysis solution provided in the kit. The solution should be freshly prepared each time.

2. Proteinase K Solution: 20 mg/ml working solution was prepared by dissolving in autoclaved MilliQ water.

PROTOCOL

1. Pellet 2mL of an overnight bacterial broth culture by centrifuging for 2 minutes at 12000g. Discard the supernatant completely.

2. Resuspend the pellet thoroughly in 200µL lysozyme solution and incubate for 30 minutes at 37^0C.

3. Add 20 µL RNase A solution and incubate for 2 minutes at room temperature.

4. 20 µL of Proteinase K solution is added to the sample to which 200 µL of Lysis Solution is added. Vortexed and incubated at 55 ^{0}C for 10-15 minutes.

5. Column preparation: To every column, 500 µL of the column preparation solution is added and eluted. Centrifuge the column at 12000 rpm for 1 minute. Discard the eluate.

6. 200 µL of ethanol (95–100%) is added to lysate and mixed thoroughly through vortexing for 10-15 seconds.

7. The contents of the tube are transferred into the binding column which is then centrifuge at 6500 rpm for 1-2 min. The eluate is discarded and the column is transferred to a new collection tube.

8. 500 µL of Wash Solution 1 is added to the column and centrifuged for 1 min at 6500 rpm. The collection tube containing eluate is discarded and column is transferred into a new collection tube.

9. 500 µL of Wash Solution (containing ethanol) is added to the column and centrifuged for 2-3 minutes at 12000 rpm. The empty spin is given to dry the column and remove excess ethanol. The eluate is discarded and column is placed to a new collection tube.

10. 50-100µL of pre-warmed Elution Solution is directly added in the center of the column and centrifuged for 2 min at 6500 rpm.

11. The bacterial genomic DNA was isolated and analyzed on 0.8% agarose gel.

3.3 Plasmid Isolation

Plasmid is an extrachromosomal genetic element that occurs in many bacterial strains. Plasmids are circular deoxyribonucleic acid (DNA) molecules that replicate independently of the bacterial chromosome. Bacteria containing the plasmid of interest are first cultured followed by centrifugation. EDTA being a chelating agent chelates the divalent metal cations such as Mg^{2+} and $Ca^{2+,}$ that are required for the proper functioning of DNAses (degrade DNA). Glucose is mainly used to maintain the osmotic pressure in the buffer that prevents the cell from bursting. Tris will maintain the pH of the cell at 8.0. RNase will get rid of the RNA present in the solution that will interfere with plasmid isolation.

A resilient alkaline solution consisting of sodium dodecyl sulfate (SDS) and a strong base like sodium hydroxide (NaOH) is added to the solution

Harvest & lyse bacteria

1. E.coli culture (glycerol stock) was inoculated in sterilized test tubes containing 10ml LB media containing 10µl Ampicillin (100mg/ml).

2. Tubes were incubated overnight at 180 rpm at 37°C.

3. Next day, cells were pelleted in 2ml Eppendorf tubes at 12000 rpm for 3 minutes and supernatant was discarded (till entire volume was done).

4. 200µl Resuspension Buffer (RNAse added) was added to the pellet and vortexed for 5 minutes.

5. 200µl of Lysis solution was added and mixed gently by inverting (lysis reaction should be kept for 5 minutes to avoid prolonged lysis.)

6. 350µl of Neutralisation solution was added and mixed gently, then spun for 10 minutes at 12000 rpm in microcentrifuge.

Column Preparation

1. Meanwhile, readymade silica micro-columns from *Sigma-Aldrich* kit were placed in 1.8 ml Eppendorf tubes and 500µl column preparation solution was loaded on columns.

2. Spun for 1 minute at 13200 rpm.

3. Flowthrough was discarded.

4. Cleared lysate was loaded on columns and spun for 1 minute at 13200 rpm.

5. Flowthrough was discarded.

Optional Wash

1. 500µl Optional wash solution was loaded on columns.

2. Spun for 1 minute.

3. Flowthrough was discarded.

Washing

1. 750µl of wash solution was loaded on column

2. Spun for 1 minute.

3. Flowthrough was discarded.

4. Empty spin was given for 3 minutes at 13200 rpm.

Elution

1. Columns were transferred to 1.5ml Eppendorf tubes.
2. Elution buffer was loaded on the columns.
3. Incubation at room temperature for 30 seconds was given.
4. Tubes were spun for 1 minute.
5. Columns were discarded.
6. Tubes stored at -20°C for further use.

3.4 Primer Design

After thorough analysis of the enzyme structure and amino acid sequence primers are designed to include appropriate mutations in the gene of interest. Site Directed Mutagenesis approach was used to introduce mutations into the gene of interest. For the Mega-primer approach, only a single mutagenic primer is designed with the mutation lying in the center of the oligonucleotide sequence. The flanking primers used are common for the gene of interest and lie in the regions of homology for chromosomal integration during transformation.

3.5 Primer Reconstitution

- Lyophilised products of forward and reverse primers specific for the genes were reconstituted with requisite amounts of sterile deionized water to make final concentration of 100µM. Oligonucleotide data sheets provided by vendor mentioned the exact volumes of water required to achieve the 100µM concentration. The vials were then thoroughly vortexed to achieve a homogenous mixture.

3.6 Primer Dilutions

- Reconstituted primers were diluted with sterile MilliQ water to a total volume of 100µl and concentration of 10µM. The vials containing the primers were thoroughly vortexed to achieve a homogenous mixture.

3.7 Site-directed Mutagenesis by Megaprimer Method/ Giga primer method

3.7.1 Generation of C-fragments

In the first step for PCR, the mutagenic primer is used as a forward primer and a primer in the flanking region downstream of the gene of interest is used as a reverse primer to generate a megaprimer which is called C-fragment in this case. At the end of this PCR amplification, DNA fragments which are around 3 kb in size carrying the desired mutation are generated. The samples were then analyzed on a 0.8% agarose gel with Hyper ladder

PCR conditions

Step	**Temperature in ºC**	**Time**
1	98	5 minutes
2	98	15 seconds
3	60	30 seconds
4	72	5 minutes
Go to 2, repeat 29 cycles		
5	72	5 minutes
Hold at 15 ºC		

PCR Reaction Mixture

Reagent	**Volume**
Sterile MilliQ water	34.5 µL
5X HF Buffer	10 µL
2.5mM dNTP	2 µL
10 µM Mutagenic Forward primer	1 µL
10 µM Flanking Reverse primer	1 µL
Phusion enzyme	0.5 µL
Genomic DNA	1 µL
Total volume	**50 L**

3.7.2 Purification of c-fragments

After the preparation of c-fragment containing the required mutation, all the other remaining unused components are removed and the product from first round of PCR is purified. Purification is done using filtration using Millipore MultiScreen 96-well plates. The MultiScreen 96-well plates plate is used for the purification of PCR products. It enables the purification upto 20-100µl volume of PCR reactions with high and concentrated recovery status. This involves the vacuum based size elimination that effectively removes contaminating salts, unused dNTPs and primers from PCR reactions. The DNA thus purified is appropriate for downstream applications.

1. **Binding**: The PCR product was diluted with 100 µl sterile MilliQ water and mixed thoroughly. The contents were transferred to Millpore purification plates and kept in vacuum. The vacuum based exclusion effectively removes all salts, unused primers and dNTPs

2. **Wash**: 100 µl sterile MilliQ water was added to each well in the plate and kept in vacuum until the water was completely drained.

3. **Elution**: 30-40 µL of elution sterile milli Q water was added to the respective wells and spun at 1100 rpm for 8 minutes.

4. The purified products were transferred to a fresh 96 well PCR plate and analyzed on 0.8% agarose gel for required intensity of the band. The products were stored at -20 °C for the next round of PCR.

3.7.3 Generation of 6 kb fragments (Megaprimer/Giga PCR)

In the second round of PCR, the c-fragment is used as a reverse primer and a primer in flanking region upstream of the gene of interest is used as a forward primer to generate a full length product, 6 kb in size containing the gene of interest with flanking regions on either side that is required for homologous recombination into *B.subtilis* host.

PCR Conditions

Step	**Temperature in ºC**	**Time**
1	98	5 minutes
2	98	15 seconds
3	60	30 seconds
4	72	5 minutes
Go to 2, repeat 29 cycles		
5	72	5 minutes
Hold at 15 ºC		

PCR Reaction Mixture

Reagent	**Volume**
Sterile MilliQ water	13.5 µL
5X HF Buffer	5 µL
2.5mM dNTP	3 µL
10 µM Flanking Forward primer	1 µL
c- fragment (Reverse primer)	1 µL
Phusion enzyme	0.5 µL
Genomic DNA	1 µL
Total volume	**25 µL**

3.8 Site-Directed Mutagenesis by Splice-Overlap Extension PCR

SOE PCR involves generation of fragments with overlapping point mutations using internal primers that generate overlapping, complementary 3’ ends, gel elution of the PCR product to obtain the desired fragment and hybridization of overlapping strands and extension of the PCR product. SOE is a simple method for fusing two gene fragments together. It is ideal for introducing point mutations.

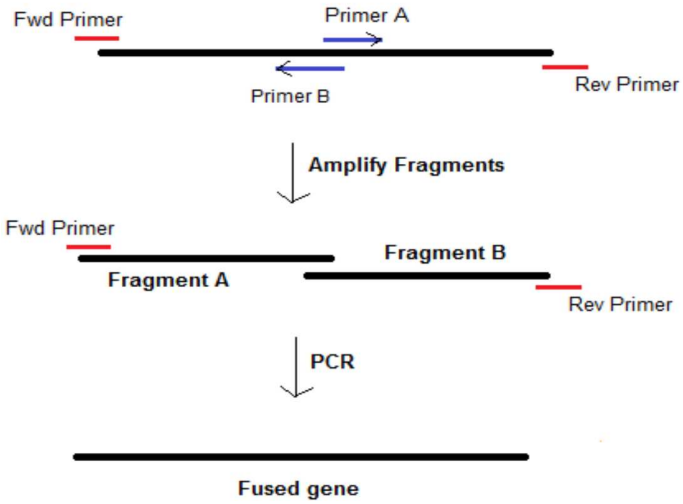


Figure 3: Inserting single mutations by SOE

3.8.1 Fragment generation

Reagent	**Volume (µL)**
5X HF buffer	10
dNTPs (2.5 mM)	3
Forward primer (2 pmol/ µL)	2
Reverse primer	2
Water	31.5
Template (50-100 ng)	1
Phusion polymerase	0.5

First Denaturation ⟶ Final Denaturation ⟶ Primer annealing ⟶ First Extension ⟶ Final extension

98°C, 2 min 95°C, 1min 60°C, 1 min 72°C, 45 sec 72°C, 5min

|---------- 32 cycles----------|

3.8.2 SOE PCR

Reagent	**Volume (µL)**
Water	12.5
5X HF buffer	5
dNTPs (2.5 mM)	3
Forward primer (10 pmol/ µL)	1
Reverse primer (10 pmol/ µL)	1
Template (50-100 ng) Fragment 1 Fragment 2	1 1
Phusion polymerase	0.5
Total	**25**

First Denaturation ⟶ Final Denaturation ⟶ Primer annealing ⟶ First Extension ⟶ Final extension

98°C, 2 min 95°C, 1min 60°C, 1 min 72°C, 45 sec 72°C, 5min

|---------- 32 cycles----------|

The quality of Splice Overlap Extension PCR product was checked on 0.8-1% agarose gel. The product thus obtained were transformed to a suitable *Bacillus subtilis* host.

3.9 Site Saturation Mutagenesis/ Site Saturated Libraries

3.9.1 Generation of c-fragments

In the first step for PCR, the mutagenic primers containing all 19 amino acid substitutions is used as a forward primer and a primer in the flanking region downstream of the gene of interest is used as a reverse primer to generate a megaprimer which is called C-fragment in this case. At the end of this PCR amplification, DNA fragments which are around 3 kb in size carrying the mutation are generated. The samples were then analyzed on a 0.8% agarose gel with Hyper ladder

PCR conditions

Step	Temperature in ºC	Time
1	98	5 minutes
2	98	15 seconds
3	60	30 seconds
4	72	5 minutes
5	Go to 2, repeat 29 cycles	
6	72	5 minutes
7	Hold at 15 ºC	

PCR Reaction Mixture

Reagent	Volume (µL)
Sterile MilliQ water	34.5
5X HF Buffer	10
2.5mM dNTP	2
10 µM Mutagenic Forward primer	1
10 µM Flanking Reverse primer	1
Phusion enzyme	0.5
Genomic DNA	1
Total volume	**50**

3.9.2 Purification of c-fragments

After the preparation of c-fragment containing the required mutation, all the other remaining unused components are removed and the product from first round of PCR is purified. Purification is done using filtration using Millipore MultiScreen 96-well plates. The purified products were transferred to a fresh 96 well PCR plate and analyzed on 0.8% agarose gel for required intensity of the band. The products were stored at -20 °C for the next round of PCR.

3.9.3 Generation of 8 kb fragments (Megaprimer/Giga PCR)

In the second round of PCR, the c-fragment is used as a reverse primer and a primer in flanking region upstream of the gene of interest is used as a forward primer to generate a full length product, 8 kb in size containing the gene of interest with flanking regions on either side that is required for homologous recombination into B.subtilis host.

PCR Conditions

Step	**Temperature in ºC**	**Time**
1	98	5 minutes
2	98	15 seconds
3	60	30 seconds
4	72	5 minutes
Go to 2, repeat 29 cycles		
5	72	5 minutes
Hold at 15 ºC		

PCR Reaction Mixture

Reagent	**Volume**
Sterile MilliQ water	13.5 µL
5X HF Buffer	5 µL
2.5mM dNTP	3 µL
10 µM Flanking Forward primer	1 µL
c- fragment (Reverse primer)	1 µL
Phusion enzyme	0.5 µL
Genomic DNA	1 µL
Total volume	**25 µL**

3.10 Transformation into competent cells

B.subtilis

Linear DNA molecules partially homologous with the *B. subtilis* chromosome, but unable to replicate in this bacterium, can integrate into the chromosome, provided that the region of non-homology they contain is flanked on both sides by regions homologous with the chromosome. Two crossing over events, one within each homologous region, have been suggested to be necessary for integration. As a result of this process, chromosomal sequences were replaced by heterologous sequences and were maintained stably in the cell if no gene essential for cell survival was carried within the replaced region. The 6 kb product was transformed into competent *B.subtilis* by direct integration into host chromosome. The competent cells are stored in -80 ºC.

1. The competent cells were thawed using equal volume of SPII + EGTA (pre-warmed at 37 ºC) and mixed thoroughly.

2. To 2 µL of the 6 kb product, 100 µL of the competent cells were added.

3. The mixture was incubated at 37°C with shaking at 180 rpm for 35 minutes to enable the uptake of DNA into the host.

4. 100 µL of the mixture was then plated onto 90mm plates containing LB CAM (6µg/mL).

5. The plates are incubated at 37 ºC overnight.

Inoculation for culture supernatant assay

1. A single colony was picked from the each of the transformation positive plates and inoculated into 96 well plate containing the appropriate expression media.

2. Each plate has adequate media and wild-type controls.

3. This plate was delivered for supernatant assay to check for activity and stability.

Transformation in competent *E. coli* cells

1. Thaw approximately 300µl of *E coli* com cells on ice (ensure that the cells are completely mixed)
2. Add 1 µL of plasmid DNA to each 100 µL transformation reaction.
3. Add 900 µL of room temperature LB to each transformation vial and incubate at 37°C in a water bath for 2 min.
4. Transfer the cells on ice for 30-60 minutes.
5. Using pre-warmed solid selective medium (LB- Amp), plate out 100 µL of cells and incubate at 37°C overnight (~18-24 h).

Transformation efficiency

TE = Colonies/µg * Dilution

Colonies = the total number of colonies on the plate

µg = the amount of DNA transformed (µg)

Dilution = the total dilution of DNA before plating

Transformation in competent *Aspergillus oryzae* cells

1. Thaw approximately 100µl of *Aspergillus oryzae* com cells. (ensure that the cells are completely mixed)
2. Addition of 1 µL plasmid DNA to every 100 µL transformation reaction.
3. Addition of 900 µL media to the transformation vials and incubate at 37°C in ice for 30min.
4. Transfer the cells to water bath 20 minutes.

Using pre-warmed solid selective medium (acetaamide), plate out 100 µL of cells and incubate at 37°C for 4-7 days.

3.11 Colony PCR

The clones which show significant improvement factor (IF value) in comparison to the wildtype were picked as hits. These hits have to be sequenced to check for the correct gene

sequences. This includes confirmation of desired mutation and eliminating the possibility of undesired ones. The culture is diluted with sterile MilliQ water. This diluted culture is used directly for the PCR reaction.

1. Isolated single colonies that grew on selection plates were picked, streaked on gridded LB-Amp/ CAM plate (mother plate) and inoculated in 10µl MilliQ water (autoclaved and filter sterilized) in PCR strips, in sterile conditions.
2. The PCR strips are then placed for lysis at 55°C for 15 minutes.
3. Spin for 40 seconds to pellet out cell debris and 1µl of samples are transferred to fresh PCR strips and PCR mix aliquoted in the vials.
4. PCR was done using primers specific for conserved region in vector.
5. The composition of PCR mix is given below

Constituent	Volume(µl)
Milli-Q water	39.8
10X Taq PCR Buffer	5
dNTPs (2.5mM)	2
Forward Primer (FP)	1
Reverse Primer (RP)	1
Taq DNA Polymerase	0.2
Template	1
Total volume	50

First Denaturation ⟶ Final Denaturation ⟶ Primer annealing ⟶ 1st Extension ⟶ Final extension

98°C, 4 min | 95°C, 1min | 60°C, 1 min | 72°C, 1 min | 72°C, 3 min (hold)

|---------- 32 cycles----------|

Purification of Colony PCR

1. **Binding**: The PCR product was diluted with 100 µl sterile MilliQ water and mixed thoroughly. The contents were transferred to Millpore purification plates and kept in vacuum.

The vacuum based separation is done for 15-20 minutes that effectively removes salts, unused dNTPs and primers from PCR reactions.

2. **Wash**: 100 µl sterile MilliQ water was added to every well in a plate and kept for vacuum until water was completely drained.

3. **Elution**: 30-40 µL of elution buffer was added to the respective wells and spun at 1100 rpm for 8 minutes.

4. The purified products were transferred to a fresh 96 well PCR plate and analyzed on 0.8% agarose gel for required intensity of the band and sent for sequencing**.**

3.12 Shake-flask Fermentation/ Large Scale Fermentation

The sequence confirmed clones were revived from the hit plates stored at -80 ºC by streaking them onto fresh plates containing LB agar + Chloramphenicol, incubated at 37 ºC overnight.

2. Inoculum preparation: The colonies were inoculated into LB media containing Chloramphenicol antibiotic (6µg/mL), that were incubated at 37 °C with continuous rotations at 180 rpm for 12-18 hours.

3. 2% of the inoculum was used to inoculate 300 mL of the expression media in a 1 liter baffled flask.

4. The flasks were incubated at different temperatures in different media till the optimization was complete.

A typical expression media contains the following components:

- Buffering agent to maintain the pH (Phosphates)
- Carbon source (glycerol / starch – moving from simple to complex, increasing viscosity)
- Nitrogen source (hydrolysed casein / yeast extract)
- Trace elements (Ca^{2+}, Zn^{2+}, Na^{+}, Cu^{2+} etc. – some are useful for the stability of the overexpressed protein, some help *Bacillus subtilis* against lysis)

These components are often interchangeable. Additional factors that influence expression and stability of a protein are: pH, temperature and duration, scale of fermentation (media: air ratio) and amount of seed culture added.

3.13 Protein expression analysis

1. The culture was taken in 1.5 mL centrifuge tube that were centrifuged at 12000 rpm for 3-5 minutes.

3. To 40 µL of the supernatant, 10 µL of SDS dye was added and mixed thoroughly.

4. The mixture was heated at 99ºC for 10 minutes.

5. 15 µL of the mixture was loaded onto 12% normal polyacrylamide gels.

6. With appropriate amount of buffer the gels were run at 180V for approximately for 50 minutes.

7. The gels were later stained with a staining solution for 20 minutes. This was followed by de-staining using a de-staining solution to visualize the protein bands.

8. The gels were then a viewed on a Trans-Illuminator for bands of precise proteins.

3.14 Purification

The variants expressing the desired protein was taken forward to purification using FPLC. The purified protein was confirmed for its performance/stability through Secondary Screening. The hits which have shown a significant increase in the improvement factor (IF value) were selected for further study.

CHAPTER 4: RESULTS AND DISCUSS

Table 3: Overall procedures and their results

PROCEDURE	COMMENTS
PCR amplification	In some cases, analysis of PCR amplified products showed nonspecific bands; band of interest had to be eluted out. In cases where PCR was unsuccessful, successive repetitions where performed along with minor variations in reaction conditions. A gradient PCR- set over a range of annealing temperature was also carried out in a few cases to achieve ideal amplification.
Gel Elution and PCR purification	Elution is done to isolate specific band of interest corresponding to each gene. Purification is done to remove contaminants such as unused dNTPs and primer dimers and to obtain a pure amplified product.
Genes cloned successfully into *Bacillus subtilis* and *A. oryzae*	Cloning by SOE was used to ensure efficient insert-vector ligation. Transformed *Bacillus subtilis* colonies were selected on Chloramphenicol (cam) containing agar plates. Isolated single colonies were identified after an overnight incubation at 37°C. A small number of such colonies were randomly selected for further screening.
Colony PCR	Done to check whether transformed colonies actually contained the insert. It is important to identify the false positives at this step.

Sequencing

Positives identified in colony PCR were inoculated in culture, grown and their plasmids were isolated which were sent for sequencing to get further verification.

Transformation into *Aspergillus oryzae* and *Bacillus subtilis*

Transformation was achieved after digesting the fungal mycelia to obtain isolated naked protoplasts. The protoplasts were plated onto a solid media containing acetamide as a selectable marker. The plates were incubated at 30°C for ~1 week.
Bacillus subtilis was transformed by homologous recombination and screened using LB-Cam selection plates

Small scale analysis: checking for recombinant protein expression

The media from each flask containing the cultured *Aspergillus oryzae* was used to run an SDS-PAGE.
Controls included: positive control showing expression of a protein and host control for checking background protein expression.

Fig 4: Bioline hyperladderTM

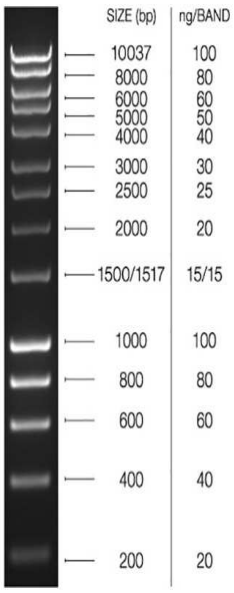


Fig 5: 1 Kb DNA Ladder

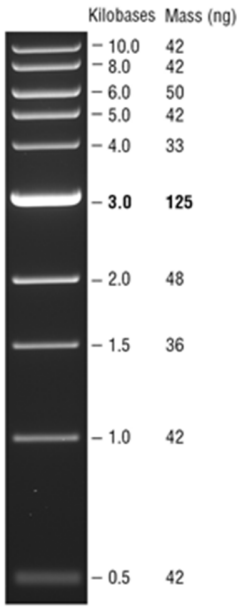


Figure 4 and 5 represent the commercially used ladders used by Bioline

4.1 Competent Cell Preparation

The competent cell preparation require the constant management of growth of the cells with increase forming a pattern of S-phase curve. The table 4 represents the absorbance recorded after different time and incubation periods for competent cell.

Table 4: Cell density of competent cells in nm

Time in minutes	**Cell Density (OD in nm)**
0	0.083
60	0.149
120	0.459
180	1.488
200	1.772
210	1.796

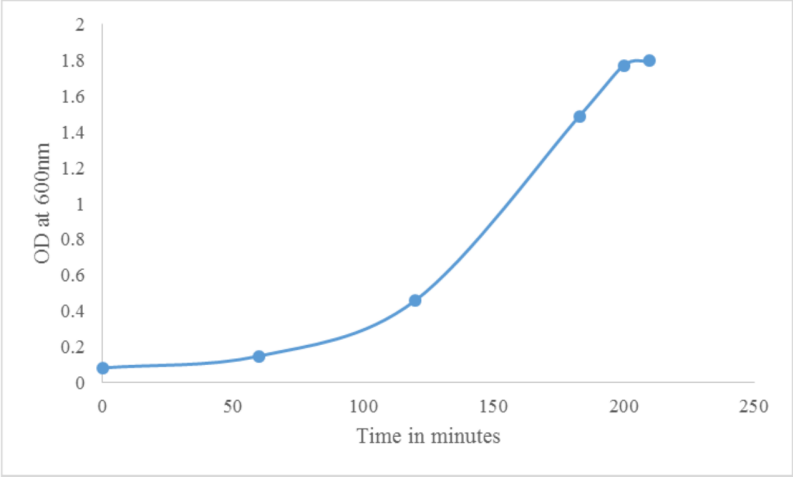


Fig 6: Graph representing growth curve during competent cell preparation

The graph (Fig. 6) represents the growth pattern of the host cells when incubated in SP11 media. According to the research done by Y Sadaie and T Kada the competent cells follow a trend that shows an

increase in the growth that resembles an S shaped curve whether supplemented or not with the antibiotics. In our study the pattern was recorded without addition of antibiotics and additional amino acids.

4.2 Sterility Test for Competent cells:

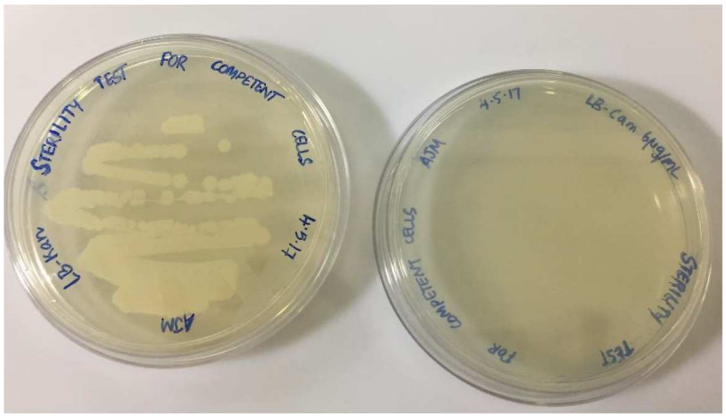


Fig 7: Sterility test for competent cells

Sterility test was performed to check whether the competent cells were devoid of other contaminating cultures. The competent cells will grow on the same media as the host. The growth of com cells on any different media suggests that the com cells have been contaminated or either mutation has occurred. Growth of Bacillus com cells on LB-Cam plate indicated that the competent cells were not contaminated and absence of growth on LB-Cam proved that the strain is resistant to chloramphenicol as that of host.

4.3 Protolast Isolation

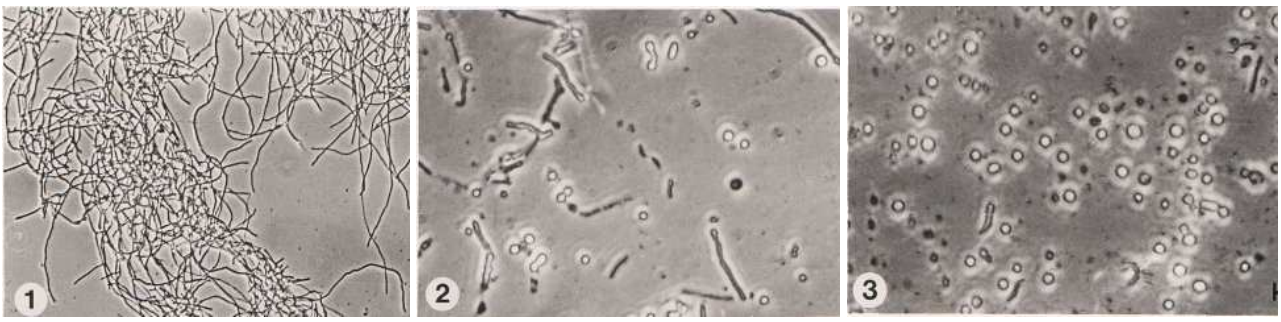


Figure 8: Formation of protoplasts from mycelium of *Aspergillus oryzae* after treatment with lytic enzyme.

(1) Mycelium before lytic digestion.

(2) After 2 h of incubation with the enzyme showing fragmented mycelium and protoplasts.

(3) Suspension of protoplasts after 4-5 h followed by slow centrifugation to remove the mycelial debris.

Figure 8 represents the formation of proroplast after 5 hrs of lytic digestion with enzyme. According to the study done by Paul F.Hamlyn *et al.,* shows that high yields of protoplasts can be prepared from a variety of fungi using relatively cheap commercial enzymes. The yields obtained were normally as good as or better than those previously produced. The enzyme volume used was 200mg/50ml that reduced mycelium in 4-5 hrs forming good amount of protoplast to be used and stored for further use. According to Hayat, S. *et al.,* all lytic enzymes did not produce protoplasts from fungal spores but lysing enzyme at the concentration rate of 15mg/ml was found optimum for the isolation of protoplasts in strains of *sclerotium rolfsii.* According to the study done by Yun Cai *etal.,* the amount of protoplast obtained is always proportion to the activity, weight and concentration of enzyme used.

4.4 Genomic DNA isolation

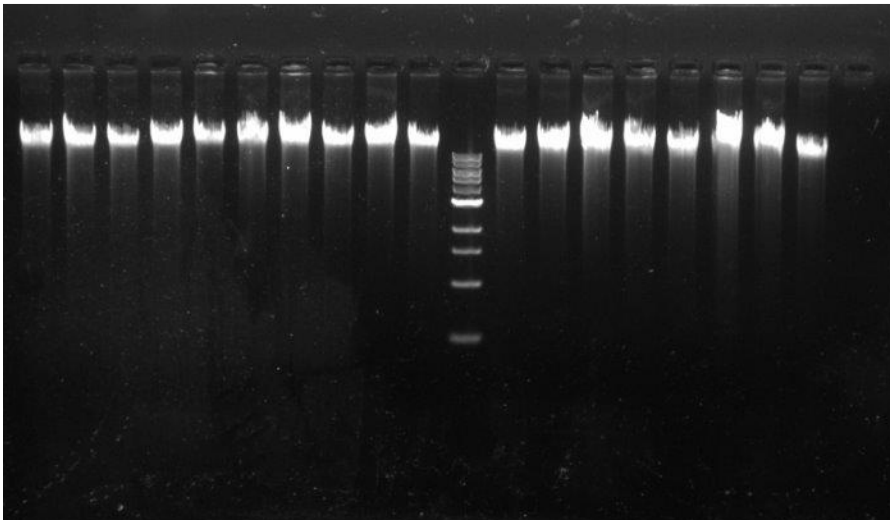


Figure 9: M: Bioline Hyperladder™ (1 kb) Lane 1-4: Genomic DNA isolated from various backbones.

The quality of genomic DNA isolated was observed on a 0.8-1% agarose gel. The bands were found to be intact and intense bands indicated good concentration of DNA. The genomic DNA was further used as template to generate C-fragment. The genomic DNA isolated is generally in megabase pairs. According to the study done by Mitchell Henry Wright *etal.,* the DNA isolated was around 23,130 bp (23kb) in size and is generally identified as the top most band in the agarose gel.

4.5 Site Directed Mutagenesis

4.5.1 Generation of C-fragments

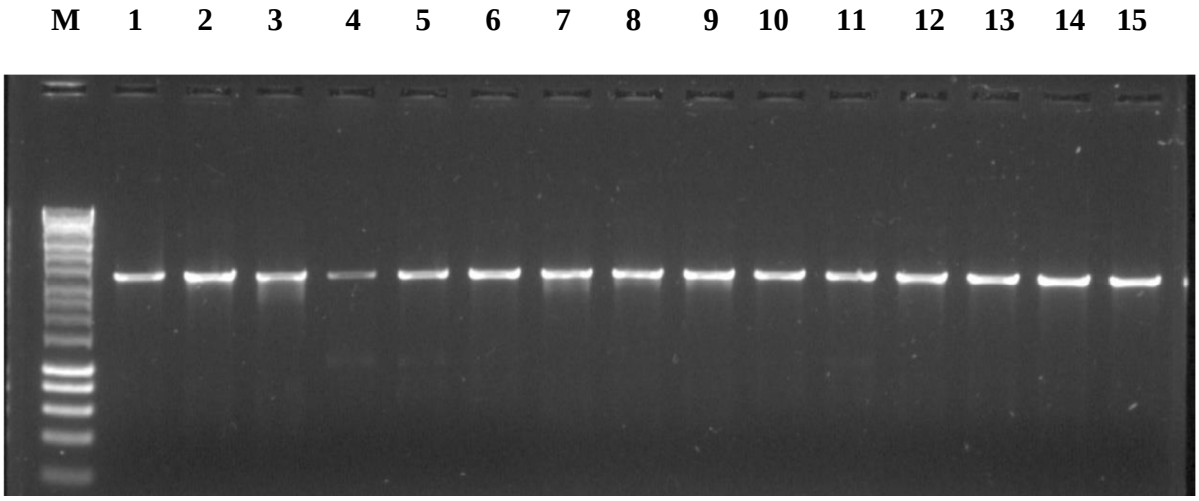


Fig 10: Agarose Gel electrophoresis of C-fragments.

M: Bioline HyperladderTM (1 kb); Lane 1-15: c-fragments generated from wildtype genomic DNA using a corresponding mutagenic forward primer and universal flanking reverse primer.

At the end of PCR amplification, DNA C-fragments which are around 3 kb in size carrying the desired mutation are generated. These C-fragments band were observed without any primer dimer or non-specific bands which are depicted in the above figure.

4.5.2 C-fragments after purification

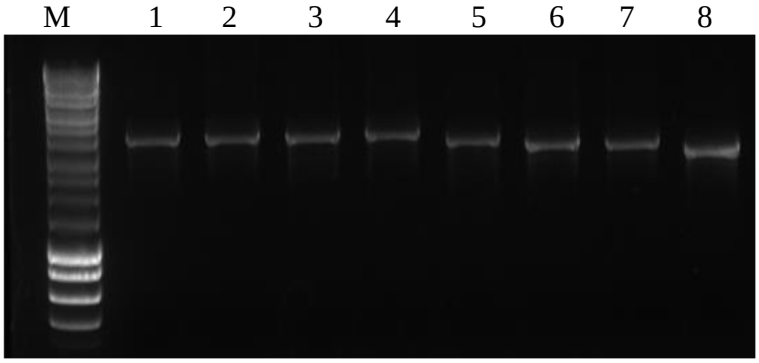


Fig 11: Agarose Gel electrophoresis of C-fragments after purifcation.

M: Bioline HyperladderTM (1 kb); Lane 1-4: C-fragments purified using Millipore purification plate.

C-fragment was purified by using Millipore Multiscreen PCR purification plates. The vacuum based size exclusion separation effectively removes contaminating salts, unincorporated dNTPs and primers from PCR reactions and purified product is obtained. The C-fragments obtained are further used as a template for generation of giga (6 KB) product.

4.5.3 Generation of 6 kb Product

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

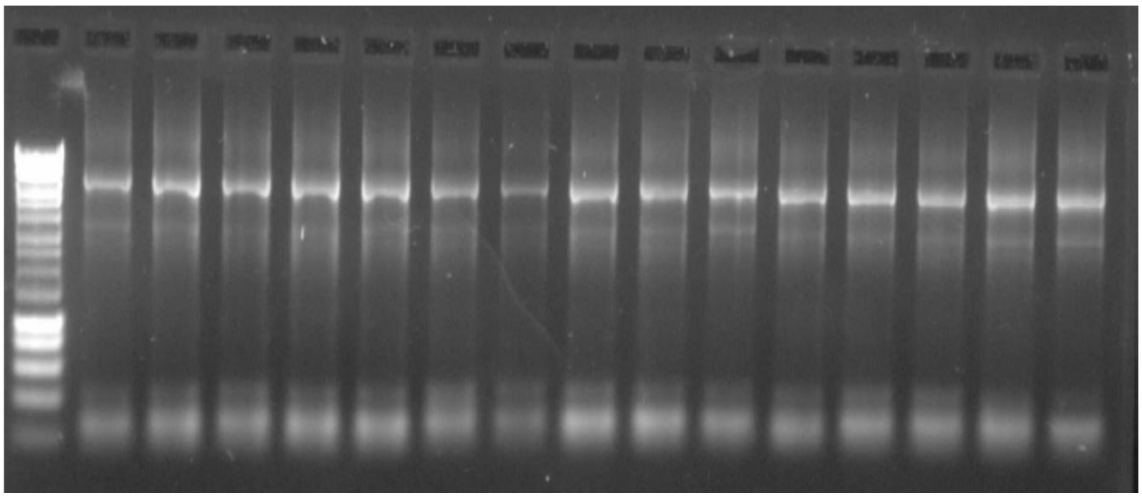


Figure 12: Agarose Gel electrophoresis of 6 kb products after purification.

M: Bioline HyperladderTM (1 kb); Lane 1-15: 6 kb products generated from wildtype genomic DNA using corresponding c-fragment as reverse primer and universal flanking forward primer.

Amplification of 6 kb band was observed with the unused primer dimer at the base of the agarose gel . Non-specific bands observed here are quite common when large fragments are amplified. However, they do not generally interfere in subsequent transformation, as only the larger fragment has the homology to undergo homologous recombination.

4.6 Fragment Generation

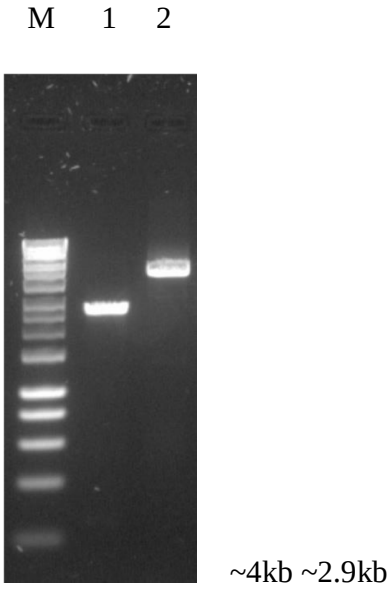


Fig 13: Products of Fragment PCR

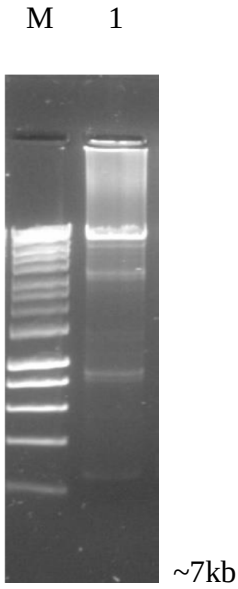


Fig 14: SOE product

13: M: Bioline Hyperladder™ (1 kb), Lane 1-2: Fragment generation

14: M: Bioline Hyperladder™ (1 kb), Lane 1: SOE product.

A 7kb product was generated by Splice overlap extension by stitching two fragments of sizes approximately 2.9kb and 4kb and amplified. This product was transformed into the *Bacillus subtilis* host. In a study by Waneskog M *etal.*, they used OE-PCR that generated large DNA fragments (upto 7.4 kb), where they included maximum of 13 changes. Their study concluded that SDM can be introduced anywhere between 50-1800 bp from each other.

4.7 Transformation into Bacillus Subtilis

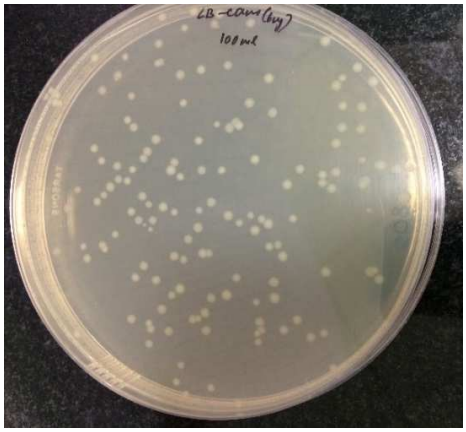


Figure 15: Growth of transformed *Bacillus* Colonies on LA-CAM plates (6µg/ml)

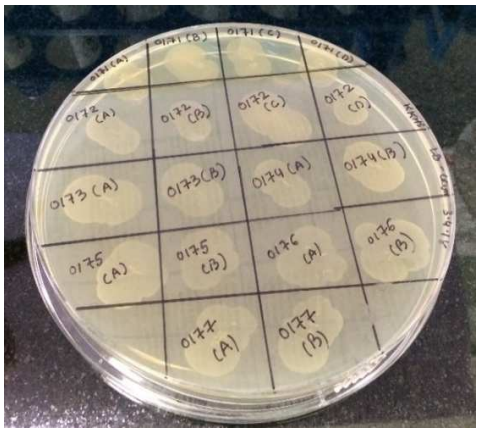


Figure 16: Streaked grid master plate for colony PCR (*Bacillus subtilis*)

Giga product of ~8kb was transformed into a *Bacillus subtilis* host. Each colony has single amino acid substitution. Around 84 colonies per SSM is picked and sequenced and the unique substitutions are taken ahead for assays. During homologous recombination, Kanamycin resistance of the host is replaced with Chloramphenicol resistance along with the mutated DNA observed on a 6 µg/ml LB-Chloramphenicol plate. This plate represents one of the 8kb product successfully transformed onto the *Bacillus subtilis* host.

The transformed *Bacillus subtilis* colonies that were screened on a selective plate was further used as a template for colony PCR and also streaked on a Grid master plate. Single colonies were selected for colony PCR.

4.8 Transformation into *E.coli*

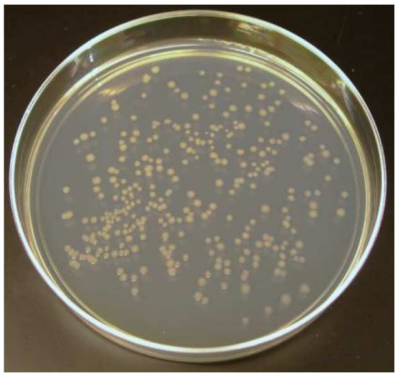


Figure 17: Growth of transformed *E.coli* colonies on LA-Amp plates

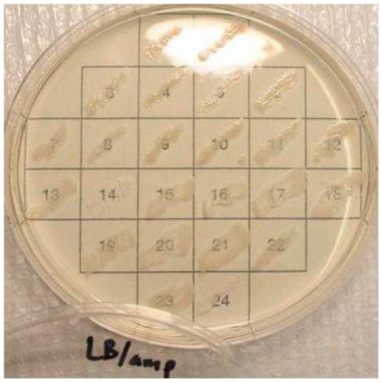


Figure 18: Streaked *E.coli* grid master plate for colony PCR

The transformed *E Coli* colonies that were screened on a selective plate was further used as a template for colony PCR and also streaked on a Grid master plate. Single colonies were selected for colony PCR.

4.9 Transformation into *Aspergillus oryzae*

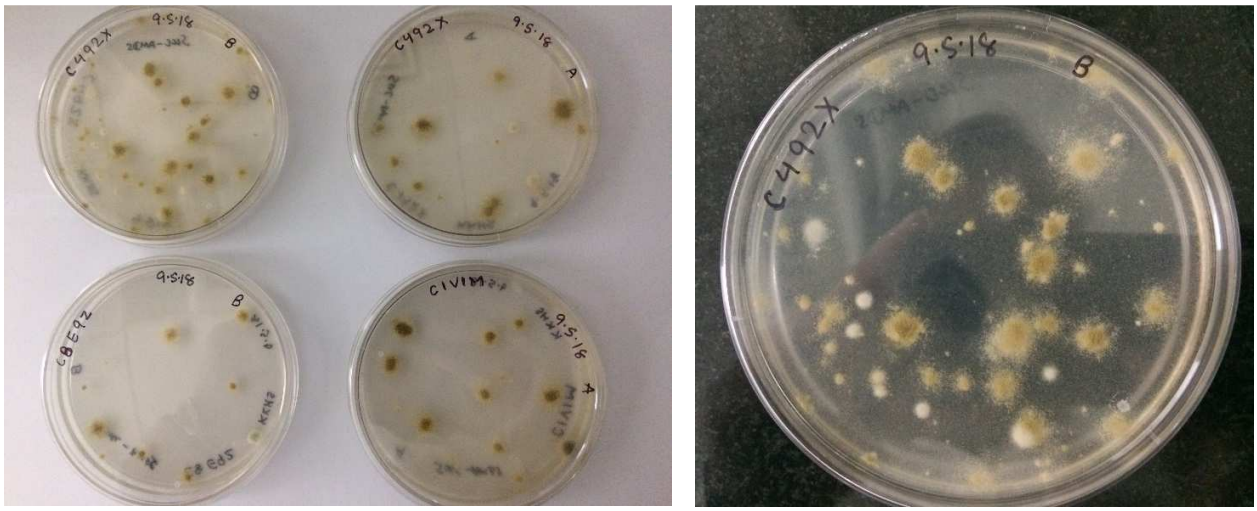


Figure 19: Growth of transformed *Aspergillus oryzae* on acetaamide plates

Giga product of ~8kb was transformed into the *Aspergillus oryzae* host. Each colony has single amino acid substitution. The transformed *Aspergillus oryzae* colonies that were screened on a selective plate and also streaked on a PDA slants (containing glycerol) **(FIG 19)**

In a research done by Katsuya gomi *etal.*, they used amdS gene (acetamide-encoding gene) as a dominant selectable marker. Although the transformation efficiency is low, the transformants generally contain high number of copy number of plasmids integrated into the system.

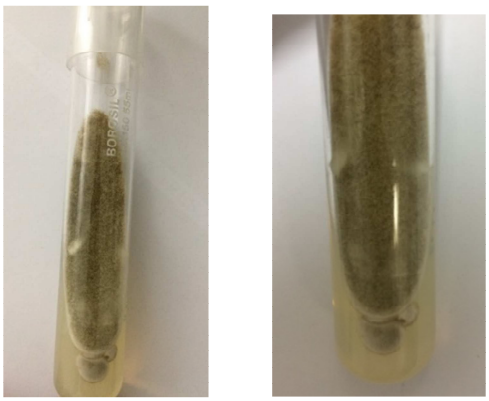


Figure 20: Growth of transformed *Aspergillus oryzae* on PDA slants

Aspergillus oryzae single colonies after transformation are streaked on PDA (containing glycerol) slants for fermentation and glycerol stocks. It was maintained until it sporulated completely (surface changing from white to green indicate generation of spores)

4.10 Colony PCR:

1 2 3 4 5 6 M

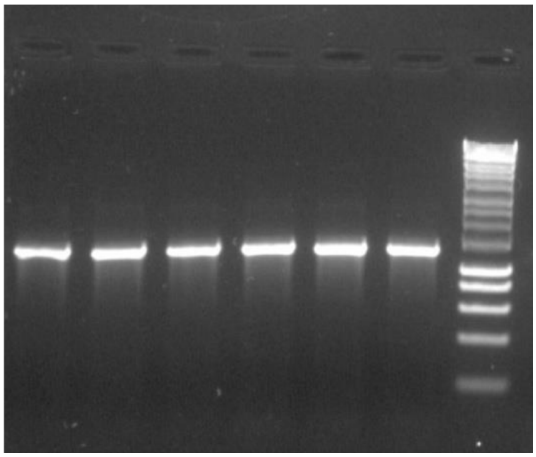


Fig 21: Colony PCR before purification. Lane 1-6: Colony PCR amplified products of the Hits (1kb size), M: Bioline Hyperladder™ (1 kb)

M 1 2 3 4 5 6

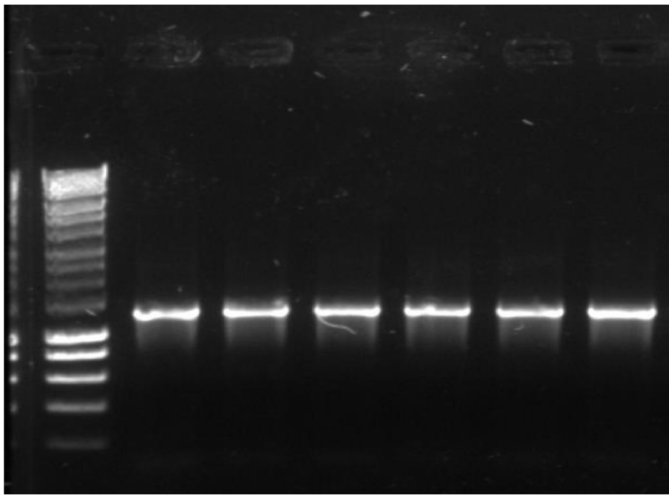
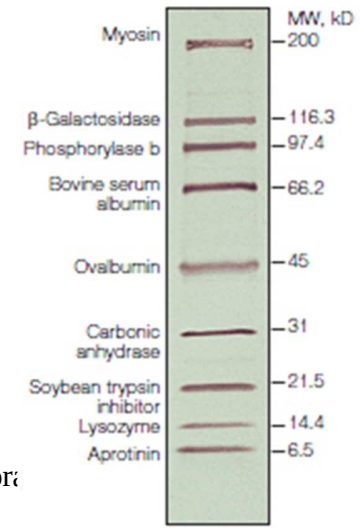


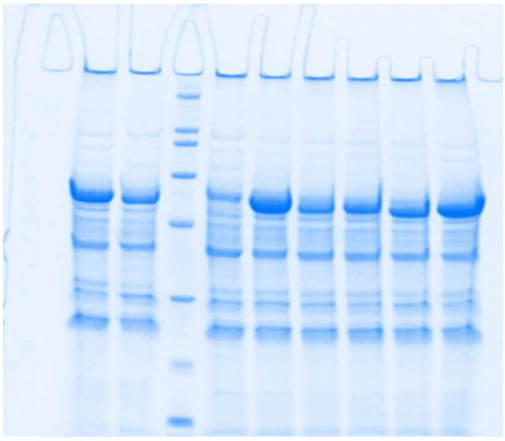
Fig 22: Colony PCR after purification. Lane 1-6 Purified colony PCR samples, M: Bioline hyperladder™ (1 kb)

Amplification of ~ 1 kb band was observed which indicates colony has the insert positive. In a study done by Packeiser H *etal.*, it was observed that the colony PCR worked efficiently with Tris, 0.2%SDS and 10Mm EDTA in extraction of PCR quality genomic DNA and incubation was done only for bacteria having rigid cell wall eg: *Chlorella vulgaris.* It still needs to be sequenced to confirm the desired mutation and to eliminate the possibility of undesired ones. The sequenced confirmed are further processed for large scale fermentation. The colony PCR products are purified using Millipore's multiscreen PCR 96 plates which removes unwanted dNTPs, unused primers and primer-dimers leaving behind the required DNA on the membrane. DNA is eluted using Tris-EDTA (10mM Tris+0.5mM EDTA) buffer.

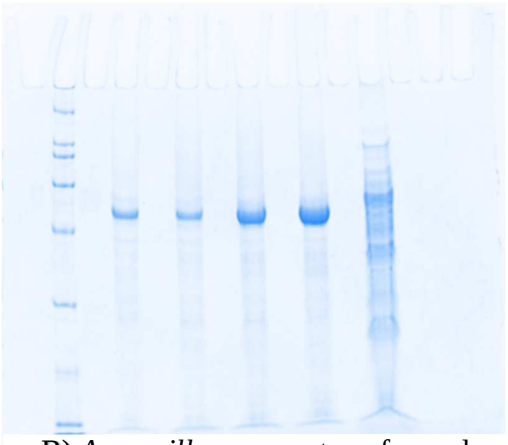
4.11 Large Scale Harvest: Protein Expression



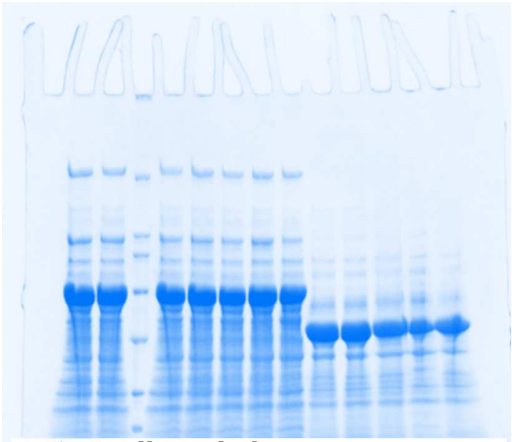
Biora



A) *Bacillus subtilis* transformed colonies expressing amylase enzyme



B) *Aspergillus oryzae* transformed colonies expressing amylase enzyme



C) *Bacillus subtilis* Hits expressing amylase enzyme (large Scale)

Fig 23: SDS-PAGE of fermented variants.

M- Biorad SDS-PAGE Marker;

A) *Bacillus subtilis* transformed colonies expressing amylase enzyme (12% gel)

B) *Aspergillus oryzae* transformed colonies expressing amylase enzyme (12% gel)

C) Bacillus subtilis Hits expressing amylase enzyme (large Scale) (4-20% gel)

The samples harvested after fermentation were checked on a 4-20% (gradient gel) and 12% (native gel) gel and a band at approximately 45-65 kDa corresponds to amylase (figure 23). The transformed colonies were screened as shown in figure A and B as expression level varies in different colonies and species. After screening the hits are selected and given for large scale fermentation (figure C). According to the research done by Dibyanganga Raul *etal.*, the amylase expression was finest at a temperature of 37°C kept for 60-72 hrs. According to their study the band of α-amylase was observed between 50-60 Kda which was same as our band with exception a *B. licheniformis* at 31 Kda. In a research done by Katsuya gomi *etal.*, the promoter of the amdS gene was replaced by the expression promoter of the α-amylase encoding gene (amyB) from *A. oryzae*, thereby improving the selection of transformants.

CHAPTER 5: CONCLUSION

Enzymes that are found in nature have been exploited in industry mainly due to the intrinsic catalytic properties. They catalyze complex chemical processes under mild experimental and environmental conditions. It is often difficult to achieve the desired industrial goal using the native form of the enzyme

Hence, it is important to alter the properties of enzyme such as thermostability, stability in organic solvents, changes in Vmax, Km, optimum pH and temperature and also their specificity. Rather than playing off one approach against the others, we use a combinatorial approach which includes Site Directed Mutagenesis. Splice Overlap Extension and Site Saturation Mutagenesis to obtain enzyme with Molecular Diversity.

Methods to generate variants depends on the number of mutations to be incorporated. Megaprimer PCR was efficient in incorporating single mutations, but the background work is arduous and time consuming. Splice-Overlap Extension PCR on the other hand allows incorporating several mutations, however the quality of the template DNA and the accurate design of primers contribute to the success of this method. Generating DNA variants using Site Saturation Mutagenesis has an advantage over Site Directed Mutagenesis because all potential substitutions can be attained with similar effort, allowing a wide-ranging analysis of the functions of the novel amino acid in the targeted position.

The cumulative results thus far provide an idea of important positions that if mutated leads to increase in property of the engineered protein when compared to the wildtype protein. This study focused on generating DNA variants using Site Directed Mutagenesis and Site saturation Mutagenesis. The variants generated were cloned into a Bacillus subtilis expression host. The hits thus obtained from the high throughput assay were subjected for further assays until the enzyme with desired enhanced properties is achieved.

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