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CONSTRUCTION OF PARTIAL GENOMIC LIBRARY OF *Bacillus subtilis* AND SCREENING FOR RECOMBINANTS PRODUCING αAMYLASE, PROTEASE AND LIPASE

ABSTRACT

Genomic libraries contain a clone of every gene and extragenic sequences found in the cells of any organism and screening of the library is done to isolate clones that contain our gene of interest. Genomic libraries can be retained for many years and propagated and therefore it is a popular method of choice for maintaining every gene sequence of the organism whose gene library is being created. Since microbial production of enzymes is preferred due to easy genetic manipulation and availability, our aim was to construct a genomic library of *Bacillus subtilis* and then screening the recombinants that produced amylase (starch-hydrolyzing), protease (protein-hydrolyzing) enzymes and lipase (lipid hydrolyzing) which have many industrial applications. The vector used for cloning was pUC 18 and E.coliDH5α served as host cells. The recombinant cells were identified by biochemical characterization using starch agar plate, gelatine plate and lipid agar plate. The growth of colonies on plates of M9 minimal media together with the respective compound and the zone of clearing around the colony served as confirmatory test to detect the presence of recombinant organisms.

INTRODUCTION

Life as we know it is specified by the genomes of the myriad organisms with which we share the planet. Every organism possesses a genome that contains the biological information needed to construct and maintain a living example of that organism. The genome of an organism is the entire nucleotide sequence present in each of its cells and its knowledge is of utmost importance to study the molecular mechanisms of its biological functions. This information specified by nucleotides (DNA for all other cellular life forms and RNA for viruses) then serves as a basis for determination and analysis of relevant features of an organism. The first step in obtaining the sequence requires the availability of libraries of cloned segments encompassing the whole genome of an organism. These are termed as genomic libraries.

A genomic library is a set of recombinant clones that contains the entire DNA present in an individual organism. Genomic libraries are formed from genomic DNA with insert size varying over several orders of magnitude (100's of bp to Mb) and they are maintained in host cells like yeast or, more commonly, *E. coli*. Genomic libraries can be retained for many years, and propagated so that copies can be sent from one research group to another.

In a genomic library, the DNA is stored in a population of identical vectors, each containing a different insert of DNA. Construction of a genomic library begins with the extraction of the organism's DNA from cells and then partial digestion with a restriction enzyme to cut the DNA into fragments of a specific size. The fragments are then inserted into the vector using the enzyme, DNA ligase. Next, the vector DNA can be taken up by a host organism- commonly a population of *Escherichia coli* or yeast with each cell containing only one vector molecule. Using a host cell to carry the vector allows for easy amplification and retrieval of specific clones from the library for analysis. Individual clones each containing a specific gene of an organism can be propagated in

isolation, with the gene present in a clone identified with the help of PCR amplification using gene specific primers or biochemical assays.
Genomic libraries are commonly used for sequencing applications that aim to sequence the complete genome, not just the expressed sequences [1]. They have played an important role in the whole genome sequencing of several organisms, including the human genome and several model organisms. Successful genome projects have relied on these libraries to gather the bulk of the sequence data required (shotgun sequencing). Genomic libraries are also utilized for genome wide association studies where genetic sequences of different individuals or organisms are compared to elucidate similarities and comparison within chromosomal regions. 11

GENOMIC LIBRARIES

A library in the field of genetic engineering is a collection of clones sufficient in number to be likely to contain every single gene present in a particular organism. Libraries are of two kinds- genomic library and complementary DNA (cDNA) library. If the goal of the library is to represent the entire genome, DNA is extracted from somatic tissue. If the library is to represent only the coding region of the genome that is expressed in a particular tissue, organ, or stage of life, mRNA is extracted and converted into cDNA with the help of the enzyme reverse transcriptase. The major difference between the two libraries is in its ability to express proteins. cDNA library is capable of expressing proteins while genomic library is unable to do so. Also, the size of insert in a genomic library varies considerably depending on the choice of vector and is usually larger whereas in a cDNA library, the maximum size of insert is 5kb.

FACTORS TO BE CONSIDERED BEFORE CONSTRUCTING A GENOMIC LIBRARY

- Organism whose genomic library to be constructed
- The choice of vector for carrying the insert in the host cell
- The choice of host cells for stable maintenance of the insert

Essentially any source of DNA can be used as starting material for plasmid library construction (genomic, organellar etc). The genome of the organism whose genomic library is to be constructed must be extracted with precision following the proper DNA isolation protocol. Care must be taken to ensure that the DNA prep is free of any contaminating DNA source. Once the target DNA has been purified, it must be fragmented to the desired size before cloning into the chosen plasmid vector. There are several kinds of vectors available with various insert capacities [1]. Generally, libraries made from organisms with larger genomes require vectors featuring larger inserts, thereby fewer vector molecules are needed to make the library. Researchers can choose a vector also considering the ideal insert size to find a desired number of clones necessary for full genome coverage.
The choice of vector depends on the specific needs. Considerations include the ideal fragment size, the desire for screening out "empty" clones (*i.e.*, clones without inserts), compatibility with other cloning systems, downstream uses of the sequencing clones, etc. In general, all-purpose cloning vectors such as pUC18/pUC19 provide a simple, inexpensive vehicle. A variety of commercial cloning systems are available, some of them with clever features to speed up construction, to minimize empties, etc. Also, low and intermediate copy number vectors are available (such as pSMART, Lucigen); these are useful in instances where libraries in high copy number vectors are biased. 12

Plasmids, cosmids, λ phage, PACs, BACs, and YACs etc are all examples of vectors for cloning foreign DNA into host cells. They mainly differ in capacity to hold insert sizes ranging from small to very large. Vectors such as plasmids, cosmids, λ phage are mainly used for the construction of

genomic libraries of organisms with small size genome like bacteria, virus etc while bacterial artificial chromosomes (BACs), yeast artificial chromosomes(YACs) are preferred for construction of insect or mammalian genomic libraries.

Vector type	**Insert size**
Plasmids	upto 12 kb
Phage lambda (λ)	upto 25 kb
Cosmids	upto 45 kb
Bacteriophage P1	70 to 100 kb
P1 artificial chromosomes (PACs)	130 to 150 kb
Bacterial artificial chromosomes (BACs)	120 to 300 kb
Yeast artificial chromosomes (YACs)	250 to 2000 kb

Below is a table of several kinds of vectors commonly used for genomic libraries and the insert size that each generally holds.

ISOLATION OF GENOMIC DNA

A routine procedure in molecular biology and forensic analysis, DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods. To isolate genomic DNA some basic steps are involved, and these are the following.

1. Breaking open (lysis of) the cells or virus containing the DNA of interest- This is often done by sonicating or homogenizing the sample with glass beads. Vortexing with phenol (sometimes heated) is often effective for removing proteinaceous cellular walls or viral capsids. The addition of a detergent such as SDS is necessary to remove lipid membranes.

2. DNA associated proteins, as well as other cellular proteins, may be degraded with the addition of a protease. RNA is removed by the addition of RNAse. When the sample is vortexed with phenol-chloroform and centrifuged, the denatured proteins remain in the organic phase while the upper aqueous layer contain nucleic acid (DNA) mixed with chloroform. The correct salt concentration and pH must be used during extraction to ensure that that DNA remains in the aqueous phase without any contaminants.

3. DNA is then precipitated by mixing with ice-cold ethanol or isopropanol in presence of Na or K-acetate and then centrifuging. The DNA is insoluble in the alcohol and will come out of solution, and then 70% alcohol serves as a wash to remove the salt previously added.

4. After pouring the alcohol off the pellet and drying, the DNA can be re-suspended in a buffer such as Tris-EDTA of pH 8.0.

5. Presence of DNA can be confirmed by electrophoresing on an agarose gel containing ethidium bromide, or another fluorescent dye that reacts with the DNA, and visualizing under UV light.

The purpose of using various chemical compounds are following:-

1. **Role of SDS (Sodium Dodecyl Sulfate):** It acts as an anioinic detergent, causing lysis of cell membrane by solubilising the lipids in the membrane. Thus the proteins constituting the cell membrane are denatured and the cells get broken. The membrane as well as the nuclear envelope surrounding the chromosomes is removed and genetic material exposed.

2. **Role of Lysozyme:** Lysozyme hydrolyses the glycosidic bonds present in the peptidoglycan cell wall of bacterial cells and thus aids in breaking open the cells, facilitating access to DNA.

3. **Role of TE Buffer:** Purpose of TE is to protect DNA or RNA from degradation. EDTA in TE buffer chelates any Mg2+ ions present, which is essential for any DNase or RNase activities. It is a buffer for storage of DNA or RNA.

4. **Role of Phenol-Chloroform:** The standard way to deproteinize a cell extract is to add phenol or a 1:1 mixture of phenol and chloroform. These organic solvents precipitate proteins but leave the nucleic acids in aqueous solution. The result is that if the cell extract is mixed gently with the solvent, and the layers then separated by centrifugation, precipitated protein molecules are left as a white coagulated mass at the interface between the aqueous and organic layers. The aqueous solution of nucleic acids can then be removed with a pipette. The function of chloroform is to remove phenol from the nucleic acid mixture.

5. **Role of potassium/sodium acetate and ethanol:** The most frequently used method of concentration is ethanol precipitation. In the presence of salt i.e sodium/potassium acetate, and at a temperature of −20°C or less, absolute ethanol efficiently precipitates polymeric nucleic acids. With a thick solution of DNA the ethanol can be layered on top of the sample, causing molecules to precipitate at the interface.

19

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, and clinical chemistry to separate a mixed population of DNA or proteins in a matrix of agarose [5]. Biomolecules like nucleic acids are separated on the basis of their size as they move through the gel matrix under the influence of applied electric field.

Agarose, derived from sea weed forms a solid gel when dissolved in aqueous solution at a concentration between 0.5 and 2.0% (w/v). The gel is a three-dimensional matrix formed of helical agarose molecules in supercoiled bundles that are aggregated into three-dimensional structures with channels and pores through which biomolecules can pass. The 3-D structure is held together with hydrogen bonds and can therefore be disrupted by heating back to a liquid state. Agarose gel has a gelling temperature of 35-42°C and a melting temperature of 85-95°C.

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. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease.

GENERAL PROCEDURE

Casting of gel - The gel is prepared by dissolving the agarose powder in an appropriate buffer, such as Tris acetic acid EDTA (TAE) or TBE, to be used in electrophoresis. The agarose is dispersed in the buffer before heating it to near-boiling point. The melted agarose is allowed to cool sufficiently before pouring the solution into a cast. A comb is placed in the cast to create wells for loading sample, and the gel should be completely set before use.

The concentration of gel affects the resolution of DNA separation. For a standard agarose gel electrophoresis, a 0.8% gives good separation or resolution of large 5–10kb DNA fragments, while 2% gel gives good resolution for small 0.2–1kb fragments.

Loading of samples - Once the gel has set, the comb is removed, leaving wells where DNA samples can be loaded. Loading buffer is mixed with the DNA sample before the mixture is loaded into the wells. The loading buffer contains a dense compound, which may be glycerol or sucrose that raises the density of the sample so that the DNA sample may sink to the bottom of the well. The loading buffer also include colored dyes such as xylene cyanol and bromophenol blue used to monitor the progress of the electrophoresis. The DNA samples are loaded using a pipette. 20

Electrophoresis - Agarose gel electrophoresis is most commonly done horizontally in a submarine mode whereby the slab gel is completely submerged in buffer during electrophoresis. The DNA samples are placed in the wells of the gel surface and the power supply is switched on. For optimal resolution of DNA > 2kb in size in standard gel electrophoresis, 5 to 8 V/cm is recommended. As the DNA is negatively charged, DNA fragments move through the gel towards the positive electrode. The rate of migration of DNA is dependent on the size and shape. In general smaller linear fragments move faster than the larger ones and in this way, separation of the mixture of DNA fragments of varying size occurs.

Staining and visualization - DNA as well as RNA are normally visualized by staining with ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light. The ethidium bromide may be added to the agarose solution before it gels, or the DNA gel may be stained later after electrophoresis. The gel is viewed with an ultraviolet (UV) transilluminator where DNA with intercalated EtBr appears as bright bands against a dark background.

RESTRICTION DIGESTION

Gene cloning requires that DNA molecules be cut in a very precise and reproducible fashion. Cutting of genomic DNA at specific locations is done using enzymes known as restriction endonucleases. These are also called as molecular scissors [6]. Restriction endonucleases also called restriction enzymes recognize and cleave DNA at specific sequences (recognition sequences or restriction sites) to generate a set of smaller fragments. Restriction enzymes are found in a wide range of bacterial species. Werner Arber discovered in the early 1960s that their biological role is to recognize and cleave foreign DNA (the DNA of an infecting virus, for example): such DNA is said to be restricted. In the host cells' DNA the sequence that would be recognize by its own restriction endonuclease is protected from digestion by methylation of DNA, catalyzed by a specific DNA methylase. The restriction endonuclease and the corresponding methylase are sometimes referred to as restriction modification system.

There are three types of restriction endonucleases designated as I, II and III. Types I and III are generally large, multisubunit complexes containing both the endonucleases and methylase activity. Type I cleave DNA at random sites that can be more than 1000 base pairs from the recognition sequence. Type III cleaves the DNA about 25 bp from the recognition sequence. Both types move along the DNA in a reaction that requires the energy of ATP.

The central feature of type II restriction is that each enzyme has a specific recognition sequence at which it cuts a DNA molecule. It requires only Mg2+ ion as cofactor. They are a homodimer, with recognition sites are usually undivided and palindromic and 4–8 nucleotides in length. These features make them key tools in molecular biology and recombinant DNA techniques, including genome mapping, RFLP analysis, DNA sequencing, and cloning.

Some restriction endonucleases make staggered cuts on the two DNA strands, leaving two to four nucleotide of one strand unpaired at each resulting end. The unpaired strand are referred to as 'sticky-ends'. They can base pair with each other or with complementary stick ends of other DNA fragments. Other restriction endonucleases cleave both strands of DNA at the opposing phosphodiester bonds leaving no unpaired bases on the ends often called 'blunt-ends'. 22

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EcoR1 RESTRICTION ENZYME

***Eco*RI** is an endonuclease enzyme isolated from strains of *E. coli*, and is part of the restriction modification system [7]. In molecular biology it is used as a restriction enzyme. The nucleic acid sequence where the enzyme cuts is GAATTC, which, has the complementary sequence is CTTAAG, has rotational symmetry. Digestion of DNA by EcoR1 at its recognition site generates sticky ends with 5' end overhangs.

Restriction enzymes such as EcoRI are used in a wide variety of molecular genetics techniques including cloning, DNA screening and deleting sections of DNA *in vitro*. Restriction enzymes like EcoRI that generate sticky ends of DNA are often used to cut DNA prior to ligation, as the sticky ends make the ligation reaction more efficient. EcoRI can exhibit non site-specific cutting, known as star activity, depending on the conditions present in the reaction. Conditions that can induce star activity when using EcoRI include low salt concentration, high glycerol concentration, excessive amounts of enzyme present in the reaction, high pH and contamination with certain organic solvents.

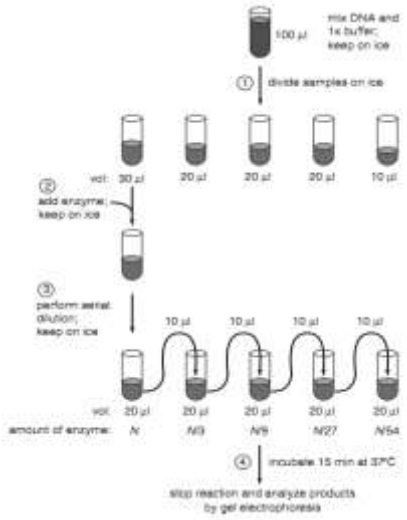
PARTIAL RESTRICTION DIGESTION

Restriction digestion conditions should be optimized on a small scale before performing large-scale digestion of genomic DNA for preparation of a genomic library. For construction of genomic library, partial restriction digestion is employed. Partial restriction digestion means that the restriction enzyme would not be able to cut at all the restriction sites present in the genome thereby generating fragments of longer sizes. If full restriction digestion is carried out, then RE may cut within a gene segment if its restriction site is present within that gene. In that case, the gene would become non-functional. In a genomic library, the size of the insert is required to be 23

around 2Kb for pUC18 vector and so partial restriction digestion is the method of choice.

There are two methods for partial digestion of genomic DNA-

 Serial dilution – The enzyme concentration taken for full restriction digestion of genomic DNA by a particular restriction enzyme is gradually reduced by means of serial dilution. Partial restriction digestion with varying concentration of enzyme in different tubes is done and the results are checked by agarose gel electrophoresis. The concentration of the RE which corresponds to insert of optimal size is then taken for further experiments.



Varying the time of incubation- After mixing all the materials required for restriction digestion, the mixture is incubated for some time at 37°C. If the time of incubation is reduced to 45minutes, then the RE will not have sufficient time to cut at all the restriction sites, thereby fragments of larger size will be obtained. Also in most cases, aliquots of the mixture are taken out at varying time interval and then the reaction is quenched using EDTA. All the aliquots are then loaded on an agarose gel to determine the optimal time for partial digestion.

24

LIGATION

Joining linear DNA fragments together with covalent bonds is called ligation. More specifically, DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another.

The enzyme used to ligate DNA fragments is DNA ligase. The DNA ligase catalyzes the formation of covalent phosphodiester linkages, which permanently join the nucleotides together. After ligation, the insert DNA is physically attached to the backbone and the complete plasmid can be transformed into bacterial cells for propagation.

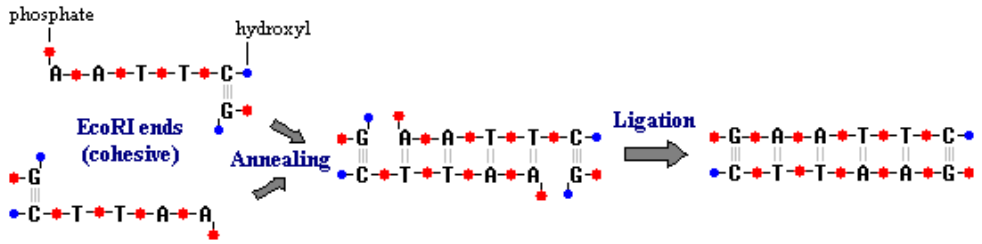
DNA fragments with either sticky ends or blunt ends can be inserted into vector DNA with the aid of DNA ligase. During normal DNA replication, DNA ligase catalyzes the end-to-end joining (ligation) of short fragments of DNA, called Okazaki fragments. For purposes of DNA cloning, purified DNA ligase is given to covalently join the ends of a restriction fragment and vector DNA that have complementary ends. The vector DNA and restriction fragment are covalently ligated together through the 3’ → 5’ phosphodiester bonds of DNA. When termini created by a restriction endonuclease that creates cohesive ends associate, the nicks in the joints has few base pairs apart in opposite strands. DNA ligase can then repair these nicks to form an intact duplex.

A ligation reaction requires three ingredients in addition to water [8]:

1. Two or more fragments of DNA that have either blunt or compatible cohesive ("sticky") ends.

2. A buffer which contains ATP. The buffer is usually provided or prepared as a 10X concentrate which, after dilution, yields an ATP concentration of roughly 0.25 to 1 mM. Most restriction enzyme buffers will work if supplemented with ATP.

3. T4 DNA ligase. A typical reaction for inserting a fragment into a plasmid vector (subcloning) would utilize about 0.01 (sticky ends) to 1 (blunt ends) units of ligase.



Bacteriophage T4 DNA ligase is a single polypeptide with a M.W of 68,000 Dalton requiring ATP as energy source. The maximal activity pH range is 7.5-8.0. The enzyme exhibits 40% of its activity at pH 6.9 and 65% at pH 8.3. The presence of Mg++ ion is required and the optimal concentration is 10mM.

T4 DNA ligase has the unique ability to join sticky and blunt ended fragments. Cohesive end ligation

is carried out at 12°C to 16°C to maintain a good balance between annealing of ends and activity of the enzyme. If reaction is set at higher temperatures annealing of the ends become difficult, while lower temperatures diminishes the ligase activity. All T4 DNA ligase is inactivated by heating at 65°C for 10 minutes. T4 ligase can also ligate any two blunt DNA ends. Lack of cohesive termini makes blunt end ligation more complex and significantly slower. Since annealing of ends is not a factor, the reaction is done at 24˚C. However, 10 - 100 times more enzyme is required to achieve similar ligation efficiency as that of cohesive end ligation.

PREPARATION OF COMPETENT CELLS

Competent cells are ready to use bacterial cells that possess more easily altered cell walls by which foreign DNA can be passed through easily. Most types of cells cannot take up DNA efficiently unless they have been exposed to special chemical or electrical treatments to make them competent. However, some types of bacteria are naturally transformable, which means they can take up DNA from their environment without requiring special treatment.

Competence is distinguished into natural competence and induced or artificial competence [9,10].

Natural Competence: Bacteria are able to take up DNA from their environment by three ways; conjugation, transformation, and transduction. In transformation the DNA directly enters the cell. Uptake of transforming DNA requires the recipient cells to be in a specialized physiological state called competent state.

Artificial Competence: It is a laboratory procedure by which cells are made permeable to DNA, with conditions that do not normally occur in nature. This procedure is comparatively easy and simple, and can be used in the genetic engineering of bacteria but in general transformation efficiency is low. There are two main methods for the preparation of competent cells. They are Calcium chloride method and Electroporation. In $CaCl_2$ method, the competency can be obtained by creating pores in bacterial cells by suspending them in a solution of ice cold $CaCl_2$ of 0.1M strength. Brief exposure of cells to an electric field also allows the bacteria to take up DNA and this process is called as electroporation

Rapidly growing cells are made competent more easily than cells in other growth stages. So it is necessary to bring cells into log phase before the procedure is begun.

Since *E.coli* is not naturally transformable, the ability to take up DNA or competency must be induced by chemical methods using divalent and multivalent cations (calcium, magnesium, and manganese, rubidium, or hexamine cobalt). Alteration in the permeability of the membranes allows DNA to cross the cell envelope of *E.coli.* The negative charges of the incoming DNA, however, are repelled by the negatively charged portions of the macromolecules on the bacterium's outer surface. The addition of $CaCl_2$ serves to neutralize the unfavourable interactions between the DNA and the polyanions of the outer layer. The DNA and competent cells are further incubated on ice for thirty minutes to stabilize the lipid membrane and allow for increased interactions between calcium ions and the negative components of the cell. 27

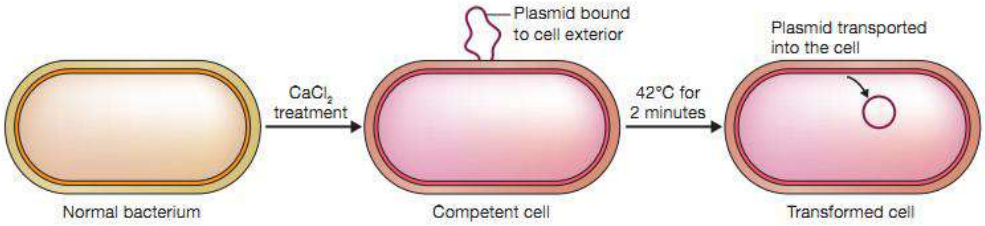
After incubation, transformation of the competent cells with foreign DNA is performed.

TRANSFORMATION:

DNA can be exchanged among bacteria by three methods: transformation, transduction and conjugation. Transformation is one of the most popular techniques of molecular genetics because it is often the best way to reintroduce experimentally altered DNA into cells. Transformation was the first mechanism of bacterial gene exchange to be discovered by Frederick Griffith in England in 1928 during his work with *Staphylococcus aureus* [11]. During the process of transformation, genes are transferred from one bacterium to another as 'naked' DNA solution.

The competent cells are subjected to transformation to facilitate the actual uptake into the cell. When DNA is added to $CaCl_2$ treated cells, it remains attached to the cell exterior, and is not at this stage

transported into the cytoplasm. The actual movement of DNA into competent cells is stimulated by applying heat shock to the competent cells for 45 seconds-1min by raising the temperature to 42°C. In molecular cloning, transformation is employed to facilitate the entry of recombinant vector (i.e vector containing the gene insert) in the host cells. After giving heat shock, the competent cells are immediately placed on ice for a brief period for the pores generated in the plasma membrane to seal, thereby re-establishing the integrity of the plasma membrane.



SELECTION OF TRANSFORMED CELLS

Most plasmid cloning vectors carry at least one gene that confers antibiotic resistance on the host cells, with selection of transformants being achieved by plating onto an agar medium that contains the relevant antibiotic. It should be noted that resistance to the antibiotic is not due merely to the presence of the plasmid in the transformed cells. The resistance gene on the plasmid must also be expressed, so that the enzyme that detoxifies the antibiotic is synthesized. Expression of the resistance gene begins immediately after transformation, but it will be a few minutes before the cell contains enough of the enzyme to be able to withstand the toxic effects of the antibiotic. For this reason the transformed bacteria should not be plated onto the selective medium immediately after the heat shock treatment, but first placed in a small volume of liquid medium, in the absence of antibiotic, and incubated for a short time. Plasmid replication and expression can then get started, so that when the cells are plated out and encounter the antibiotic, they will already have synthesized sufficient resistance enzymes to be able to survive [24].

Confirmation of the transformants is done by selection on LB plate containing ampicillin. E.coli DH5α is naturally sensitive to ampicillin. Since pUC18 contains a gene encoding a protein that confers resistance to ampicillin, only those cells in which pUC 18 has entered during heat shock (successful transformants) will be able to form colonies on LB Amp plates while the non-transformants will die in the presence of ampicillin.

BLUE-WHITE SCREENING

The **blue-white screen** is a screening technique that allows for the rapid and convenient detection of recombinant bacteria in vector-based molecular cloning experiments. DNA of interest is ligated into a vector. The vector is then transformed into competent cell (bacteria), and the competent cells are grown in the presence of X-gal. Cells transformed with vectors containing recombinant DNA will produce white colonies; cells transformed with non-recombinant plasmids (i.e. only the vector) grow into blue colonies.

In Molecular Biology a mutant β-galactosidase with its N-terminal residues 11—41 deleted (termed the ω-peptide) is unable to form a tetramer and is inactive. This mutant form of protein however may return fully to its active tetrameric state in the presence of an N-terminal fragment of the protein, the α-peptide [12].

In this method of screening, the host E. coli strain carries the lacZ deletion mutant (lacZΔM15} which contains the ω-peptide, while the vectors used carry the lacZα sequence which encodes the first 59 residues of β-galactosidase, the α-peptide. Neither are functional by themselves. However, when the two peptides are expressed together, as when a vector containing the lacZα sequence is transformed into a lacZΔM15 cells, they form a functional β-galactosidase enzyme.

The blue/white screening method works by disrupting this α-complementation process. The vector

carries within the lacZα sequence has an internal multiple cloning site (MCS). This MCS within the lacZα sequence can be cut by restriction enzymes so that the foreign DNA may be inserted within the lacZα gene, thereby disrupting the gene and thus production of α-peptide. Consequently, in cells containing the vector with an insert, no functional β-galactosidase may be formed.
The presence of an active β-galactosidase can be detected by X-gal within the agar plate. X-gal is cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo. This results in a characteristic blue colour in cells containing a functional β-galactosidase. Blue colonies therefore show that they may contain a vector with an uninterrupted lacZα (therefore no insert), while white colonies, where X-gal is not hydrolyzed, indicate the presence of an insert

BIOCHEMICAL CHARACTERIZATION

E.coli DH5α doesn't possess gene encoding α- amylase, protease (gelatinase) or lipase whereas B.subtilis is capable of producing all the three enzymes. It is only when recombinant pUC18 carrying insert of these genes enter competent E.coli DH5α as a result of transformation, then the individual enzymes get expressed in E.coli host cells and they are capable of hydrolyzing starch, gelatin or lipid. It should be however noted that each vector contains only one insert and therefore each recombinant E.coli DH5α clone is capable of producing only one enzyme characteristic of B. subtilis. Therefore biochemical characterization of clones representing a genomic library serves as a tool to identify the desired clone with our gene of interest in absence of gene specific primers. Biochemical characterization is a relatively easy and reliable method of choice for screening of genomic library. 31

STARCH HYDROLYSIS TEST

This test is used to differentiate bacteria based on their ability to hydrolyze starch with the enzyme a-amylase or oligo-l,6-glucosidase. It aids in the differentiation of species from the genera *Corynebacterium*, *Clostridium*, *Bacillus*, *Bacteroides*, *Fusobacterium*, and members of *Enterococcus* [13].

PRINCIPLE

Starch is a polysaccharide made up of a-D-glucose subunits. It exists as a mixture of two forms, linear (amylose) and branched (amylopectin), with the branched configuration being the predominant form. The a-D-glucose molecules in both amylose and amylopectin are bonded by 1, 4-a-glycosidic (acetal) linkages. The two forms differ in that the amylopectin contains polysaccharide side chains connected to approximately every 30th glucose in the main chain. These side chains are identical to the main chain except that the number 1 carbon of the first glucose in the side chain is bonded to carbon number 6 of the main chain glucose. The bond is, therefore, a 1, 6-a-glycosidic linkage.
Starch is too large to pass through the bacterial cell membrane. Therefore, to be of metabolic value to the bacteria it must first be split into smaller fragments or individual glucose molecules. Organisms that produce and secrete the extracellular enzymes a-amylase and oligo-l,6-glucosidase are able to hydrolyze starch by breaking the glycosidic linkages between the sugar subunits. Although there usually are intermediate steps and additional enzymes utilized, the overall reaction is the complete hydrolysis of the polysaccharide to its individual a-glucose subunits.
Starch agar is a simple plated medium of minimal media, soluble starch and agar. Since minimal media contains no carbon source, only organisms capable of hydrolyzing starch into simpler sugars will be able to grow on it. When organisms that produce a-amylase and oligo-1,6-glucosidase are grown on starch agar they hydrolyze the starch in the medium surrounding the bacterial growth. Because both the starch and its sugar subunits are soluble (Clear) in the medium, the reagent iodine is used to detect the presence or absence of starch in the vicinity around the bacterial growth.

Iodine reacts with starch and produces a blue or dark brown colour; therefore, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth. *Bacillus subtilis* gives a positive result while *Escherichia coli* gives a negative result.

GELATINASE TEST

PRINCIPLE

Gelatin is a protein derived from collagen -a component of vertebrate connective tissue. Gelatinases comprise a family of extracellular enzymes produced and secreted by some microorganisms to hydrolyze gelatin. Subsequently, the individual amino acids can be taken up by the cell and used for metabolic purposes. Bacterial hydrolysis of gelatin occurs in two sequential reactions [14,15].

For the gelatin hydrolysis test, a minimal medium containing nutrient gelatin is used. Minimal media contains only essential salts that support bacterial growth. In the medium it serves as the solidifying agent (substituting in place of agar) in addition to its role as a source for carbon and energy. Consequently, when a plate of Nutrient Gelatin with minimal media is inoculated with a gelatinase-positive organism, secreted gelatinase will liquefy the gelatin. Gelatinase negative organisms do not secrete the enzyme and do not liquefy the medium. It 33

should be noted that gelatinase activity is sometimes very slow, producing only partial liquefaction after a 7-day incubation period.

A small disadvantage of nutrient gelatin is that it melts at 28°C. Therefore, it is typically incubated at 25°C along with an uninoculated Nutrient Gelatin control to verify any liquefaction is not temperature related.

LIPASE TEST

Lipase test is performed to determine whether the given organism is capable of utilising lipids from a given medium. The organisms harbouring genes encoding for enzyme lipase can successfully utilise the lipid source from a given medium. Lipase causes break down of lipids into smaller fragments. Triglycerides (lipids) are composed of glycerol and three fatty acids. These get broken apart and may be converted into a variety of end-products that can be used by the cell in energy production or other processes [16,17]. Other lipase tests use different fat sources such as corn oil, olive oil, peanut oil, egg yolk, and soybean oil. Use of the lipid can be observed as a zone of clearing around areas of growth

LITERATURE REVIEW

A genomic library consists of cloned DNA fragments representing the entire genome of an organism or we can say that it is a collection of recombinant clones representing total genomic DNA of an organism. Previously many works have been done with the genomic library. One such work focused on developing an ordered collection of the DNA segment of *Bacillus subtilis* clones in a YAC vector (yeast artificial chromosome). This work was done in the early 1993. A collection of 772 *Bacillus subtilis* DNA segments were obtained by cloning. This ordered collection of cloned segments constituting a library was now available for the determination of the sequence. Apart from that it was also used in many other physical and genetic analysis of *Bacillus subtilis* genome. As mentioned in the published article that such libraries were previously obtained using cosmid and phage lambda vectors, but the cloning system of yeast artificial chromosomes (YACs) offers the possibility to clone DNA segments >50 kb thereby making the ordering of the segments along the chromosome(s) easier. Thus YAC was preferred. The yeast strain they used were *Saccharomyces cerevisiae* SX4-6A (his3-532 ade2-1 ura3-1 322 trpl-289 inos- canR a p+ w+;). The bacterial strains used was *B. subtilis* BS168. Normal cloning procedure was carried out. The size of the YACs varied between 30 and 250 kb, and all YACs hybridized with a total *B. subtilis* DNA probe. The B. subtilis inserts of 288 clones

were mapped by hybridization using as probes 65 cloned genes and 188 isolated insert ends. In this way, 59 inserts were ordered in four contigs that cover >98% of the *B. subtilis* chromosome [19]. Another work was done in 1986 by British researchers using bacteriophage (phi 105J27) cloning vector which was used for shot gun cloning of sporulation genes in *B.subtilis*. Various genomic libraries had already been constructed and screened for the presence of recombinant phages capable of transducing strains containing sporulation *(spo)* mutations to Spo+. Of a total of 30 *spo* loci tested, transducing phages was isolated for 23, more than half of the known *spo* loci. Chromosomal DNA from *B. subtilis* strain 168 was partially digested with *MboI* and the fragments were size fractionated to the range of 3 to 4 kbp. About 200 ng of this DNA was ligated to about 500 ng of the vector 4105J27, that had been cleaved at its unique BamHI site. Here, instead of normal transformation, transfection methos was used to transfer the desired gene in host. After transfection of strain CU267 protoplasts, about lo5 p.f.u. were obtained and the progeny phage were recovered as four independent pools of 35

roughly equal size. A series of sporulation mutants were screened for transduction by the library of recombinant phages. Spo+ survivors were pooled, temperature induced and the sterilized lysates were tested for a second round of Spo+ transduction. Successful cloning was indicated by high frequency transduction of the Spo+ phenotype. Most strains were tested for transduction by a mixture of phage particles from all four pools. Several strains were tested against each individual pool and in such cases more than one independent transducing phage was sometimes isolated [20].
Another very interesting work done in 2013 focused on *Bacillus subtilis* genome (BGM) vector, which is a novel technique in cloning for large DNA fragments. In this research, the entire 4.2Mb of *B.Subtilis* genome served as a vector. The researchers aimed to reconstruct the genomic DNA for transgenic mice. The BGM vector system has several attractive properties, such as a large cloning capacity of over 3 Mb, stable propagation of cloned DNA and various modification strategies using RecA-mediated homologous recombination. However, genetic modifications using the BGM vector system had not been fully established and therefore this system had not been applied to transgenesis. Thus the researchers developed important additions to the genetic modification methods of the BGM vector system. Using two contiguous bacterial artificial chromosome clones containing several class I OR genes, two transgenes in the BGM vector were constructed by inserting a reporter gene cassette into one class I OR gene. They were oriented in opposite directions. An inversion modification was performed to align their orientation and then fused them to enlarge the genomic structure. DNA sequencing revealed that no mutations occurred during gene manipulations with the BGM vector. A further demonstration that the modified, reconstructed genomic DNA fragments could be used to generate transgenic mice was done. Thus, the BGM vector system can be an alternative platform for engineering large DNA fragments in addition to conventional systems such as bacterial and yeast artificial chromosomes [21]

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