Transposon mutagenesis of gene involved in limonin degradation in *Pseudomonas putida*

A
DISSERTATION

Submitted in partial fulfillment of the requirements for the award of the degree of

Masters of Science
(Biotechnology)

By
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Certificate

This is to certify that the dissertation entitled **'Transposon mutagenesis of gene involved in limonin degradation in *Pseudomonas putida*'** submitted by Mirnalini Sharma (Reg. no 3030109) in partial fulfillment of the requirements for the award of Degree of Master of Sciences in biotechnology, to Thapar Institute of Engineering and Technology (Deemed university), Patiala, is an authentic record of Student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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Abstract

The metabolic capability of *Pseudomonas putida* to degrade complex organic and inorganic compounds had been exploited in the present study to degrade limonin, a complex phytochemical that causes delayed bitterness in citrus fruit juices. *P.putida* had been observed to reduce limonin to 64% when limonin was provided as sole carbon source while 36% of total limonin contributed to residual limonin.

The study employed transposon mutagenesis as a genetic approach in order to characterize the gene(s) implicated in limonin degradation in *P.putida*. Biparental Conjugation had been successfully performed to mobilize Tn5 transposon into *P.putida* from *E.ColiS17* 1 with the help of pGS9 suicide vector. Insertional inactivation of the gene(s) was carried out by Tn5 transposon to produce 1 putative Tn5 mutant. PCR reaction supported the stable integration of Tn5 in genome of mutants of *P.putida* as evident from 625 bp amplification of Tn5 region in genomic DNA of mutant.

Tn5 fragment had been used for physical analysis of the disrupted gene. Using Tn5 as probe Southern Hybridization would enable us to localize and determine the size of gene of interest. For this pGS9 plasmid was digested with *Hpa* I to release Tn5 element of size 5.2 Kb. The *EcoR* I digested product of genomic DNA of wild type and Tn5 mutant were compared to *Hpa* I digested product of pGS9 plasmid to localize and approximate size of the disrupted gene. The amplified product of Tn5 element had also been cloned into PCR product cloning pGEM-T vector.

List Of Abbreviations

$CaCl_2$	Calcium chloride
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamminetetraacetic acid
EtBr	Ethidium bromide
µg	microgram
µl	microliter
IPTG	Isopropyl-β-D- thioglactopyranoside
LA	Luria Agar
LB	Luria Broth
mg	milligram
ml	milliliter
NaCl	Sodium chloride
SDS	Sodium Dodecyl Sulphate
TBE	Tris Borate EDTA
TE	Tris EDTA
TTC	2,3,5- Triphenyl Tetrazolium Chloride
X-gal	5- Bromo-4- Chloro-3- indolyl- β-D- galactoside

Contents

Introduction

Microorganisms are metabolically versatile as a function of wide range of catalyzing enzymes and can degrade variety of complex organic and inorganic compounds. Some of the predominant microorganisms that possess such capabilities are *Pseudomonas*, *Arthrobacter, Rhodococcus, Bacillus, Aspergillus, and E.coli etc.* Among these *Pseudomonas* is ubiquitous and possesses diverse metabolic capability for utilizing different complex aliphatic and aromatic compounds.

Limonin, a phytochemical belongs to the class of limonoids, which are one of the bitter principles in citrus juices. It is a highly oxygenated triterpenoid compound, which comprises of a furan ring and an epoxide group. Limonin is chemically related to triterpene derivatives found in Rutaceae and Malvacae families and accounts for most of the delayed bitterness in citrus fruit juices. This delayed bitterness due to limonin is a serious economic problem of citrus juice industry worldwide and attempts are being made to solve the problem by either preventing limonin formation or removal after it is formed. But the existing methods have severe economic and technical limitations since they impair the quality and stability of the juice by affecting the components other than limonin.

The successful use of microorganisms depends on development of better understanding of molecular mechanisms adapted to degrade limonin. A few microorganisms that have been studied to degrade limonin include *Arthrobacter globiformis, Pseudomonas 321-18*, *Corynebacterium facians, Rhodococcus* sp. However no molecular characterization at genetic level of any microorganism has been done with respect to limonin degradation or metabolism.

One of the putative microorganisms in response to limonin metabolism is *Pseudomonas* due to its diverse metabolic capabilities to degrade large variety of substrates as carbon source. The ability to utilize limonin is characterized in *Pseudomonas putida*, by virtue of its metabolism. It is already well established that *Pseudomonas putida* is a nutritional opportunist par excellence and

paradigm of metabolically versatile microorganism. An arsenal degradative function presumably reflects its existence spectrum of housekeeping catabolic pathways and enzymes. However so far no known strains of *P.putida* has been studied from the perspective of limonin degradation.

The sequence of complete genome of many bacteria has been identified but the role of various putative genes is yet to be established. Recently transposon based strategies have been developed for molecular characterization of the gene in addition to mutagenesis by chemicals or UV radiation. Transposon mutagenesis is a powerful tool for identifying genes as transposon has capacity to move to new site within genome of host organism thereby causing null mutations, chromosome rearrangements, introduction of physical and genetic markers and novel patterns of gene expression. Transposons are most versatile tools for the genetic analysis of bacteria. Out of the different classes of transposons Tn5 is one of the better-characterized transposon and has been extensively used in bacterial molecular genetics. Thus present study aims at performing Tn5 mutagenesis in *Pseudomonas putida* to identify and localize gene(s) and establish the role of strain in limonin degradation under the framework of following objectives.

Objectives

1. Insertional inactivation of gene involved in limonin degradation by transposon mutagenesis.

2. Genetic studies for molecular characterization of gene involved in limonin degradation in *P. putida*.

Review of literature

Before undertaking the present study in order to update the knowledge and develop insights pertaining to research and development made so far an extensive survey of chronicles was carried out. The study includes application of microorganisms with respect to their metabolic versatility with special reference to *Pseudomonas putida* in biotransformation of Limonin a triterpenoid compound, which is the major cause of delayed bitterness in citrus fruit juices, mechanism of delayed bitterness, pathways for degradation of limonin, various debittering approaches employed, various genetic approaches adopted to characterize gene(s) and use of Tn5 transposon mutagenesis for molecular characterization of gene(s) encoding enzymes for limonin degradation along with the suicide vectors for mobilization of transposon. A brief recapitulation of all these are presented here.

1. Microbial biotransformation

Microorganisms have been exploited for their specific biochemical and physiological properties from the earliest times for baking, brewing, and food preservation and more recently they have an important application in bioremediation and environmental problems, also for producing antibiotics, solvents, amino acids, feed supplements, and chemical feedstuffs. Rapid growth, unrivaled enzymatic and nutritional versatility of microorganisms cast them in role of biotransforming, degrading, catalyzing and recycling agents for the biosphere. Over years microbes have evolved an extensive range of enzymes, pathways and control mechanisms in order to degrade an array of organic and inorganic compounds. This nutritional versatility helps them to metabolize different complex organic and inorganic compounds as sole source of carbon and energy. These capabilities are mediated by mechanisms like biotransformation, biocatalysis, mineralization or volatilization etc.

2. *Pseudomonas*

Pseudomonas is a gram negative, rod shaped, non-spore forming, and motile, aerobic bacteria. It can grow on a wide variety of organic substrates. Most *Pseudomonas* are free living saprophytic organism in soil or water where they play an important role in decomposition, biodegradation and carbon and nitrogen cycles.

Because of their lifestyle, *Pseudomonas* are characterized by great range of metabolic diversity and are able to utilize a wide range of carbon sources, including molecules which few other organism can break. Consequently they are important organisms in bioremediation, recycling of biogenic and xenobiotic organic compounds, food spoilage, growth promotion and protection from pathogens of plants, parasitism of other bacteria and parasitism and disease production in plants and animals, etc.

2.1. *Pseudomonas putida*: an opportunist par excellence

Pseudomonas putida is a nutritional opportunist par excellence and paradigm of metabolically versatile microorganisms that recycle waste in aerobic compartments of the environment, thus playing a key role in the maintenance of fascinating biochemistry and physiology. Its robustness, rapid growth, easy handling and manipulation have made *P. putida* a lab workhouse for research.

It is ubiquitous and predominant in selective medium containing exotic compound as carbon source under copiotrophic condition i.e. relatively high concentration of substrates, non-limiting minerals, high aeration and incubation temperature of 20-30^{0}C. *P. putida* has chromosomally encoded pathways for catabolism of various organic and inorganic compounds. It also harbors catabolic plasmids such as NAH plasmid (Dunn and Gunsalus, 1973), TOL plasmid (Williams and Murray, 1974) and CAM plasmids (Rheinwald et.al, 1973), encoding catabolism of naphthalene, toluene and camphor respectively. This metabolic versatility reflects its extensive spectrum of house keeping catabolic pathways and enzymes, its tendency to freely acquire plasmids from other bacteria and it's relaxed specificity gene expression system, allowing expression of genes derived from other bacteria.

P.putida strains have been described that catabolize natural products like vanillin, limonene, mandelate, camphor etc. and industrial compounds (styrene, bromoxynin, methyl-tert-butyl ether (MTBE), trichloro ethylene, nitroglycerine). Genes encoding metabolic pathways for proto-catechuate, catechol, *p*-hydroxy benzoate, vanillin have been identified in *P.putida* K2440 genome. Enzymes for the metabolism of toluene, xylene and methyl benzoates were once encoded by complete genetic material of original *P.putida* K2440 strain, known as strain mt-2 that contain pWWO (TOL) plasmid. The complete sequence of this has also been identified. (Wackett, 2003)

2.2. *Pseudomonas putida* PpG7

Pseudomonas putida PpG7 is known organism for naphthalene degradation because it contains plasmid NAH7 that regulates the mineralization of naphthalene. The biochemistry and genetics of naphthalene degradation pathway contained on plasmid NAH7 have been well characterized and thoroughly reviewed by Yen and Serder (1988). The gene that codes naphthalene degradation has been shown to be plasmid encoded and transferable by conjugation (Dunn and Gunsalus, 1973). *nah* gene have been further localized to a 25-Kb fragment of an NAH7:: Tn5 mutant (Grund and Gunsalus,1983; Schell,1983). It has been shown that same genes are responsible for partial degradation of phenanthrecene and anthracene as well as naphthalene.

3. Limonin: major cause of Delayed bitterness

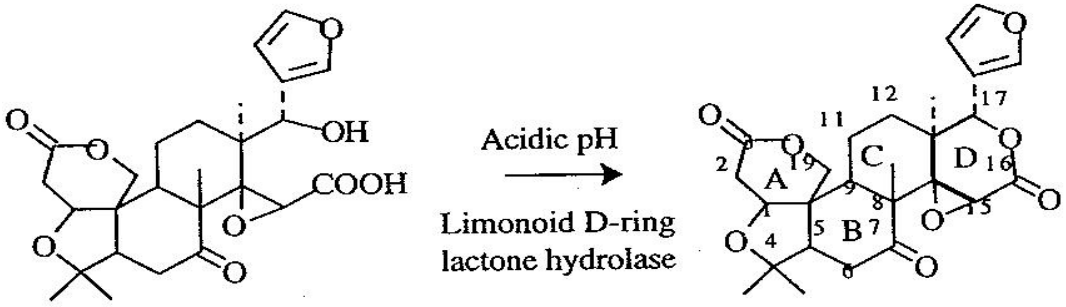
Limonin is a triterpenoid compound comprising of a furan ring and an epoxide group. Limonin belongs to the class of limonoids, which are chemically related to triterpene derivatives found in Rutaceae and Malvacae families isolated from Washington naval orange juice. Twenty-nine limonoids, 18 neutral and 11 acidic, have been isolated from citrus hybrids. Four of them, Limonin, nomilin, ichangin and nomilinic acid are bitter.(Hasegawa et.al, 1983) However limonin is intensely bitter and major limonoid in citrus fruit juices and processed

products and thus major cause of delayed bitterness. The taste threshold of limonin bitterness in commercial orange juice is 6ppm. (Guadagni et.al, 1973)

Most citrus fruits and their squeezed juice do not taste bitter if they are consumed fresh. However after sometime at room temperature or if stored overnight in refrigerator juice becomes bitter. This gradual development of bitterness or delayed bitterness is caused by formation of limonin.

Mechanism of delayed bitterness

Limonin is not present as such in citrus fruit but its non-bitter precursor, limonoate-A-ring lactone (LARL) is found to be endogenously present in the cell cytoplasm of the membranous sacs at neutral or alkaline pH. During fruit processing when these sacs get ruptured the precursor (LARL) encounters acidic pH and enzyme limonin-D-ring lactone hydrolase catalyzes closure of ring to form limonin. (Hasegawa et.al, 1996 and Puri et. al, 1996)



Limonoate A-ring lactone **2**
(Nonbitter)

Limonin **1**
(Bitter)

Figure 1. Mechanism of delayed bitterness

This delayed bitterness continues to be an economic impediment in citrus fruit juice industry since it lowers the quality and value of commercial juices.

4. Debittering approaches

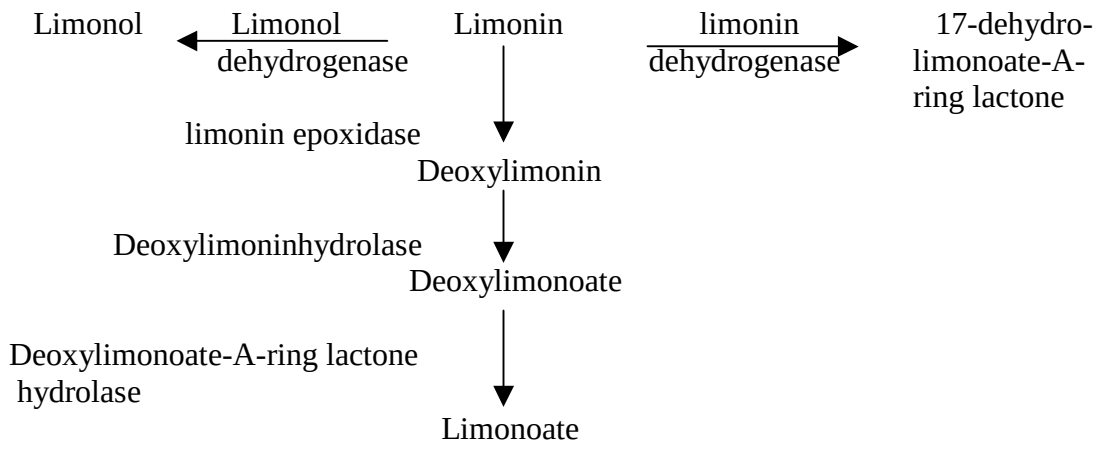
In order to solve the above problem of delayed bitterness in citrus fruit juice for consumer acceptability number of physicochemical and enzymatic methods have been devised. These approaches involve (1) removal of limonin by use of variety of adsorbents such as cellulose acetate, nylon membrane and ion exchangers which have been explored to reduce bitterness in grapefruit juice (Johnson and Chandler, 1988) (2) conversion of bitter limonoids to non bitter metabolites by immobilizing microbial mass that possess enzymes that can degrade terpenoids or enzymes on suitable matrix like application of *Pseudomonas 321-18* was extended to reduce limonin from grapefruit juice (Hasegawa et.al,1992) (3) preharvest treatment with ethylene that induces ripening in fruit with concomitant reduction in bitterness. (Maier et. al, 1973)

But all these methods have severe economic and technical limitations since these are non-specific in nature, alter the chemical composition of juice and affect the nutritional quality, texture, flavor, odor and stability of juices. (Puri et.al, 1996)

4.1. Limonin metabolism

The enzymatic reduction of limonin to non-bitter compounds occurs via three pathways. (Puri et.al, 1996)

Several investigators have reported studies directed at using biotransformation as a means to avert bitterness by converting bitter limonoids to non-bitter metabolites.



The microbial transformation processes have therefore been explored for terpenoids with a view of achieving desired conversion, optical resolution of product as well as understanding of the metabolic pathway for degradation of terpenoids. (Chatterjee and Bhattacharya, 2000)

Some microorganisms have been reported to have pathway for the debittering of limonin. The applications of microorganisms as a tool for debittering that have been studied so far are –

Microorganisms	**Pathways**	**References**
Arthrobacter globiformis	17-Dehydrolimonoids	Hasegawa et.al, 1972
Pseudomonas 321-18	Deoxylimonoids, 17-Dehydrolimonoids	Hasegawa et.al, 1972, 1974
Acinetobacter sp.	Deoxylimonoids	Vaks and Lifshitz, 1981
Corynebacterium fascians	17-Dehydrolimonoids, trans-19-HBA	Hasegawa et.al, 1983, 1985
Rhodococcus fascians	-	Manjon et.al, 1991

4.2. Role of *Pseudomonas putida* in limonin degradation

Pseudomonas putida PpG7 contains NAH7 plasmid that encodes for naphthalene degradation pathway. The same gene has been found to degrade phenethrene and anthracene. This information was implied in metabolism of llimonin, a compex aromatic triterpenoid compound. Also Hasegawa et.al, (1974) isolated and characterized limonoid-metabolizing enzymes, Deoxylimonin hydrolase and limonoate dehydrogenase from cell free extract of new bacteria named *Pseudomonas 321-18.* Chatterjee and Bhattacharaya (2000) also reported that *Pseudomonas putida* MTCC1072 could successfully metabolize limonene, a monoterpenoid, to perillyl alcohol and *p*-menth-1-ene-6, 8-diol.

So, this strain of *Pseudomonas* was used for the present study in order to establish its role in limonin degradation.

5. Genetic approaches for molecular characterization

In this era of genomics, biologists aim to understand the function of all genes. The function of a particular gene can be determined when the gene of interest is mutated and the resulting mutant is analyzed for differences compared to its wild type.

There are basically two ways to mutate a gene: reverse and forward genetics. In reverse genetic approaches, one start with a (sequenced) gene of interest selects a mutation in that gene, and then tries to identify a phenotypic change. In forward genetic approaches, one predicts the specific effect of a mutation for a process under research and then tries to isolate mutants with the predicted phenotype or one try to isolate the genetic sequence that underlies any phenotype of interest. Thus, contrary to reverse genetics, forward genetics starts with a phenotypic mutant and tries to identify the gene responsible for the altered phenotype. Both approaches are valuable and complementary. (Peters et.al, 2003)

Definitive proof of function in biological systems requires genetic analysis. Only when a specific function is lost by the loss of a particular protein one can be sure that the protein truly affects the function. Changing the pattern of gene expression through forward genetics i.e. random mutagenesis, followed by selection of mutant or variant cells and identification of a missing or overexpressed protein, one is able to confirm the roles of specific protein to be

used in defining detailed mechanisms through structure-function analyses. (Stark et.al, 1999). Forward mutagenesis is also helpful or can be extensively used when the little information is available for the genetic locus of the gene involved i.e. only few intermediary metabolites, metabolic steps and enzymes have been identified. Mutations, when they occur specifically in the pathways of concern can be identified by the loss of metabolic phenotype, i.e. the ability to catabolize a specific compound. Also, as a result of this block, intermediary metabolites may accumulate and further study may be conducted.

Transposon mutagenesis

5.1.Advantages of transposon mutagenesis

Both insertional and chemical mutagenesis, extensively used approaches of forward genetics, can be used to perform mutant screens. When the gene that causes the mutation is tagged by a T-DNA or transposon insertion, a rapid identification of the gene of interest is possible, whereas identification of a gene affected by a chemically induced mutation requires a more time-consuming positional cloning approach. Chemical mutagenesis generates a greater diversity of mutations and thus allows identification of genes unlikely to be identified by insertional mutagenesis. It also results in more mutations per organism, which reduces the number of organism to be screened to find the phenotype of interest. Positional cloning approach is a very important tool to facilitate the identification of gene functions. However, it is a relatively time-consuming method. We therefore carried out transposon mutagenesis to identify the gene involved in limonin degradation in *Pseudomonas putida* PpG7. (Peters et.al, 2003)

5.2.Transposons as mutagens

Transposition is a recombination process in which DNA segment called transposable elements move from original position on a DNA molecule to the same or different DNA molecule. It is independent of the classical homologous recombination system of the organisms and also transposon insertion to new site does not require extensive homology between the ends of the element and target site.

Transposons are being used as efficient tool in bacterial genetics because insertion of transposon leads to complete inactivation of the gene resulting in null mutations and producing non-leaky phenotypes, which are relatively stable since the frequency of precise excision, or reversion is very low as compared to UV mutagenesis. Also, if it gets integrated in an operon it produces polar effect allowing genes downstream to express thus helping in determining the structure of operon. An important application of transposons in genetic engineering is that it introduces new genetic and physical markers into the target site like antibiotic resistance genes, new restriction sites and unique sequences, which can be identified by genetic means. Transposon can generate rearrangements such as deletions, inversions, translocation or duplication and can also insert specific gene into genome of specific bacterium. Unlike chemical mutagenesis all these characteristics have made transposon mutagenesis a valuable addition to more classical mutagenesis techniques. (de Bruijn and Rossbach, 1982)

Transposons are mobile genetic elements that can relocate from one genomic location to another. As well as modulating gene expression and contributing to genome plasticity and evolution, transposons are remarkably diverse molecular tools for both whole-genome and single-gene studies in bacteria, yeast, and other microorganisms. Efficient but simple in vitro transposition reactions now allow the mutational analysis of previously recalcitrant microorganisms. Transposon-based signature-tagged mutagenesis and genetic footprinting strategies have pinpointed essential genes and genes that are crucial for the infectivity of a variety of human and other pathogens. Individual proteins and protein complexes can be dissected by transposon-

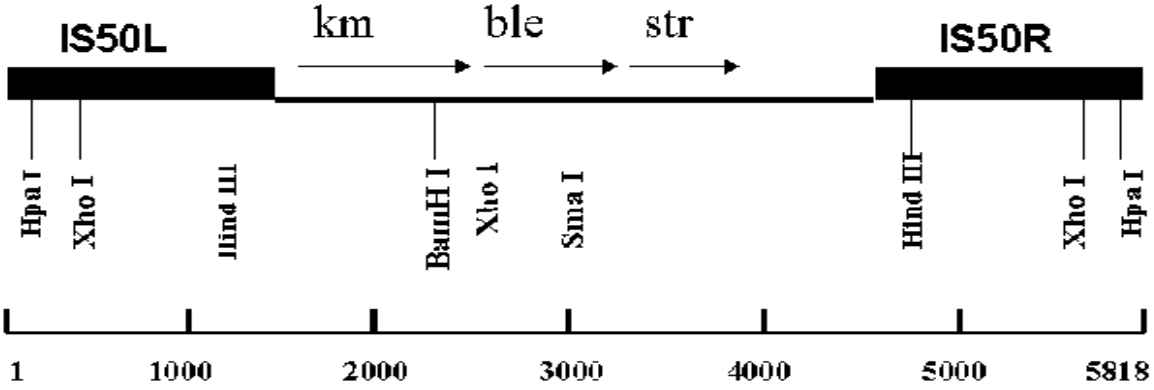
mediated scanning linker mutagenesis. These and other transposon-based approaches have reaffirmed the usefulness of these elements as simple yet highly effective mutagens for both functional genomic and proteomic studies of microorganisms. (Finbarr, 2003)

5.3. Tn5 as a model transposon

Tn5 is a 5.8-Kb composite structure containing two inverted versions of the transposable insertion sequence *IS50* bracketing three antibiotic resistance genes conferring resistance against kanamycin, bleomycin and streptomycin. The *IS50* elements are defined by two 19bp sequences termed the outside end (OE) and inside end (IE), which are found to be essential for Tn5 transposition. IS50R encodes transposase enzyme responsible for Tn5 transposition (*tnp* gene), as well as transposition inhibitor (*inh* gene). (Berg, 1989)

The frequency of Tn5 transposition is very high in *E.coli* (10^{-2} to 10-3) and it generates 9-bp target site duplication on insertion. It also has low insertional specificity and thus can insert in large number of sites in genome or at multiple positions in same gene. (Berg and Berg, 1983 and de Bruijn et.al, 1984) Mutation induced by Tn5 is very stable, as reversion (by process of precise excision) frequency is quite low.

Structure of Tn5



Physical map of Tn5

Tn5 mutagenesis can be random or region directed mutagenesis. Random mutagenesis involves the introduction of transposon in host bacteria by either transformation or conjugation or transduction, followed by selection for antibiotic resistance marker carried by Tn5 whereas in region directed mutagenesis Tn5 mutated gene cloned into plasmid in *E.coli* is isolated and then mutant phenotype is characterized in same host or other bacteria after reintroduction of Tn5 mutated loci by gene replacement. (de Bruijn, 1984).

5.4. Mechanism of Tn5 transposition

Tn5 transposition involves four steps namely synapsis, DNA cleavage, target capture and strand transfer and disengagement of strand transfer complex.

Synapsis

The first step is the formation of the synaptic complex in which two molecules of transposase enzyme are bound to 19bp end DNA sequences. This involves conformational change in Tnp followed by dimerization of Tnp-end DNA sequence complex to form synaptic complex.

DNA cleavage

This step involves 3 catalytic reactions; 3’-strand nicking, hairpin formation and hairpin cleavage. (Bhasin et.al, 1999; Reznikoff, 2002). First oxygen from water molecules acts as nucleophiles to nick the 3’ strand on both the ends, which is also catalyzed by transposase. Then 3’- OH groups of the transferred strand act as nucleophile and attack the 5’ strand to form hairpin structure. Formation of hairpin also requires denaturation of DNA near the nicked end and reorientation of one of the phosphodiester backbone to form intrastrand phosphodiester bond.

Target capture and strand transfer

The two 3'-OH groups farther than 9-bp from two target phosphodiester bonds are embedded in a cleft for holding the DNA helix. The target DNA is bound to cleft awaiting strand transfer. After target capture, 3'-OH groups attack the target DNA phosphates 9-bp apart leading to integration of transposon into target DNA.

Disengagement of the strand transfer complex

It involves the release of transposase from target site. For Tn5 it is hypothesized that a protease that cleaves the protein adjacent to residue R40 (part of transposase) might be involved. (W.S.Reznikoff, 2003)

After strand transfer complex disengagement, the host fills in the two 9bp gaps at either end of the integrated gaps.

6. Suicide vectors

Since transposons are themselves not autonomous replicons, so they have to be delivered efficiently into cell by means of a plasmid vehicle that itself is not maintained thus allowing transposon insertion and selection of mutants by using antibiotic markers contained in transposon.

The first class of suicide plasmids to be used for mutagenesis of λ-sensitive bacterial strains is IncP-type plasmid carrying a copy of Mu in addition to Tn5 like pJB4JI (Beringer et.al, 1978). Second class of Tn5 containing suicide plasmid is based on replicons carrying temperature sensitive origin of replication allowing Tn5 transposition at elevated temperatures. Third class of vectors are based on narrow host range IncW and IncN type plasmids. These have been successfully used for mutagenesis of different bacterial species like Rhizobium meliloti (pGS9) (Selvaraj and Iyer, 1983).

Once a satisfactory transposon plus delivery vector has been constructed there are number of techniques that have been developed to transfer the delivery plasmid from donor to recipient. These involve the use of conjugal mating techniques allowing sex pilus formation between bacterial cells. The donor cell

must possess genes that code for mobilization and transfer of DNA (mob and *tra* genes) by some means (Friefelder, 1987). These genes can also be provided by helper plasmid by using triparental mating technique such as pRK2013 or the host cell itself, which has its own fertility genes.

After the mobilization of Tn5 in host cell via suicide vector, subsequently it gets inserted into bacterial genome. Once it is inserted it is incapable of further transposition, since it lacks suicide vector borne *tnp* gene, resulting in stable insertion. (Herrero et.al, 1990)

Materials and Methods

Bacterial strain and plasmid

A bacterial strain of *Pseudomonas putida* (MTCC1072) was selected to carry out physiological and molecular studies in response to limonin. Freeze dried culture was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of microbial Technology (IMTECH), Chandigarh. The plasmid pGS9 in *E.coli* S171 was used to carry out transposon mutagenesis.

Culture media and growth conditions

The complete medium used for *Pseudomonas putida* was Luria-Bertani Agar/Broth M63 minimal medium was also used and culture was incubated at 30^{o}C overnight. A subculture was made at a 1:100 ratio into a flask of LB broth to final volume of 5ml and was grown to mid log phase. The culture was harvested and pellet was resuspended in 200µL of phosphate buffer and stored in cryovial containing 40% glycerol at -80^{o}C. *E.coli* was maintained in LB agar/broth with kanamycin antibiotic at a concentration of 80 µg/ml at growth temperature of 37^{o}C.

Transposon Mutagenesis

Generation of spontaneous mutants

Pseudomonas was tested to be sensitive to rifampicin. In order to obtain a selection marker in transposon mutagenesis spontaneous mutants were generated as follows: Thick log phase culture of *P.putida* was spread on Luria Agar plate containing rifampicin (80 µg/mL) and incubated overnight at 30^{o}C. The colonies obtained were transferred to fresh LA plates containing rifampicin. The frequency of spontaneous mutants was also recorded.

Biparental conjugal mating

A biparental conjugal mating between *E.coli S17*1 and *P.putida* was optimized. The donor (*E.coliS17*1 with pGS9) and the recipient cells (spontaneous mutants of *P.putida*) were grown till mid log phase and thick cultures were harvested by centrifugation (10,000 rpm, 3 min). The pellets were washed with 0.85% saline several times. Donor and recipient cells were mixed in different ratios and mixture was placed on to a (0.45 µ Millipore) which was placed on kanamycin and rifampicin double antibiotic plates and incubated at 30^{o}C for 24 hrs to allow conjugal mating. The two cultures were also diluted and spread on respective antibiotic plate i.e. *E.coli* on kanamycin containing LA plate and *P.putida* on rifampicin containing LA plate before mixing the two and incubated the plates at 30^{o}C overnight to calculate the frequency of conjugation.

Screening of transconjugants for inability to utilize limonin as carbon source

The transconjugants on the double antibiotic plate were lifted with sterile toothpicks and replica plated on luria agar double antibiotic plates (kanamycin 80 µg/ml and rifampicin 80µg/ml). From luria agar plates they were plated on minimal media containing glucose with double antibiotics followed by transfer to minimal media containing limonin with double antibiotics. Further the colonies were transferred to microtiter plates in which there were 96 U shaped wells each having 0.1 ml minimal media with limonin (40ppm) and TTC (1%). The plates were incubated at 30^{o}C for 3 days. Appropriate controls were taken. The colonies which did not show red color precipitate in the wells were selected from master plates and further retested as limonin mutants.

Isolation of Genomic DNA (Maniatis, 1982)

Centrifuged 1.5 ml of the culture in eppendroff tube for 2 min at 10,000rpm. Resuspended the cell pellet in 567µl of TE and 30 µl of 10%SDS and added 3 µl of 20mg/ml proteinase K. Mixed the tube gently by inversion and incubated at 37^{o}C for 1hr. During this time cells are usually lysed.

Added 5M NaCl 100 µl, and vortexed the tubes for few seconds. Added 80 µl of 10% CTAB mixed the contents well and incubated for 10 min at 65^{o}C. Added an equal volume of chloroform, vortexed and centrifuged for 5min at 11000rpm. Collected the upper aqueous layer into a fresh tube and added equal volume of phenol-chloroform, vortexed to mix and centrifuged for 5min at 11000rpm.

Recovered the upper phase, then added 2 µl of 10mg/ml RNAse and incubated for 30min at 37^{o}C. Added an equal volume of isopropanol to precipitate the DNA. Kept the tube at room temperature for 5 min, then centrifuged for 5min at 11000rpm. Discarded the supernatant and washed the pellets with 70% ethanol. Centrifuged again for 5 min at 11000rpm. Dried the pellet and dissolved in 10-30µl of sterile TE. DNA was visualized by agarose gel electrophoresis, at 60V in 0.8% concentration of agarose.

Agarose Gel Electrophoresis

Horizontal agarose gel electrophoresis was performed using standard method. 0.8% agarose gel was prepared in 1X TBE buffer to which Ethidium bromide was added and was cast in gel tray. The DNA samples were loaded after mixing well with gel loading buffer and electrophoresis was carried out at 60V till the tracking dye covered 2/3 rd of the gel length. Finally the DNA bands were analyzed under UV light.

Amplification of Tn5 by PCR

Based on IS50 region of Tn5 a set of two primers that was designed is as follows:

Forward primer: 5'-CCATTGACCACACCACCTCT 3'

Reverse primer: 5'-CAGCAACAACCATTTCAACG 3'

A 50ul reaction mixture included 5-10ng of bacterial DNA as template,

1uM of each primer, 100uM dNTPs 1unit of Taq polymerase, 10Xbuffer (diluted to 1 X), 2.5mM $MgCl_{2.}$

Thermal program used was: initial denaturation of 5min at 94^{o}Cfollowed by 35 cycles of 1min. at 94^{o}C(denaturation), 1min at 50^{o}C (annealing) and 1 min at 72^{o}C (extension) and final extension of 6min. at 72^{o}C. The amplified DNA was visualized in 1.4-% agarose gel after ethidium bromide staining.

Restriction digestion of Genomic DNA

Genomic DNA of wild type and Tn5 *P.putida* putative mutants were subjected to restriction digestion with *EcoR* 1 restriction enzyme. 10µl of genomic DNA was diluted with sterile water and added buffer and enzyme(s) (1U) and incubated at 37^{o}C for 3-4 hrs. Terminated the reaction by placing the tubes at –20^{o}C. Electrophoresed the digested sample and analyzed the bands under UV light.

Isolation of plasmid DNA (Kado and Liu, 1981)

Log phase culture was harvested at 10,000 rpm for 5 min and washed with 0.85% saline. The cell pellet was completely suspended in 100µl of E-buffer and then added 200µl of lysis buffer. The sample as then incubated at 65^{o}C for 30 min followed by addition of 300µl of phenol-chloroform-isoamylalcohol (25:24:1). Centrifuged the tubes at 10,000rpm, 4^{o}C for 25 min and the upper aqueous layer was transferred to fresh tube. 25µl of the sample was mixed with 5µl of loading dye and loaded in 0.8% agarose gel.

Restriction digestion of plasmid DNA

Buffer (1X) and *Hpa*1 restriction enzyme (1-10 units) were added to the plasmid DNA and reaction volume was made up to 20µl with sterile distilled water. The restriction digestion was carried out for 3-4 hrs at 37^{o}C. The digestion profile was then visualized in agarose gel at 60V.

Cloning of Tn5 amplicon

Ligation

Combined 50 ng of vector with insert in 1:1, 1:3 and 3:1 molar ratios and adjusted volume to 10 µl with dH_2O. Added 5µl of 2X Quick Ligation Buffer and mixed. Added 1µl of Quick T4 DNA Ligase and mixed thoroughly. Centrifuged briefly and incubated at room temperature (25° C) for 5 minutes. Then ligation mixture was incubated at 16^{o}C overnight.

Transformation

Distributed 3-ml log phase (0.5 O.D.) *E.coli DH5α* culture in each eppendroff tube and centrifuged at 8000rpm for 4 min at 4^{o}C. Resuspended the pellet in 1 ml, 100mM $CaCl_2$ solution and centrifuged at 7000rpm for 4 min at 4^{o}C. Again resuspended pellet in 100µl $CaCl_2$ solution and incubated the samples in ice for 30 min to prepare competent cells. Added 2µl DNA in to 50µl competent cells, mixed the DNA with cells by flicking carefully and incubated on ice for 20 min. Heat shock was given at 42^{o}C for 50 sec. Immediately then transferred samples on ice for 3 min, added 1ml SOC medium and incubated the cells for 1.5 hrs at 37^{o}C with shaking. Spread 100µl culture on ampicillin, X-gal and IPTG containing luria agar plate and incubated at 37^{o}C overnight.

Results and Discussion

1. Degradation of limonin by *Pseudomonas putida*

Pseudomonas putida PpG7 was known to metabolize naphthalene. It was speculated that this strain probably could also metabolize limonin which is a triterpenoid phytochemical predominantly found in citrus fruits. When limonin was provided as a sole source of carbon in the minimal mineral media it was observed that this strain could utilize limonin as sole source of carbon and energy. As indicated in fig.1 64% of the total limonin was reduced by *P.putida* within 48 hrs and 36% of limonin contributed to the residual limonin. It was observed that limonin reduction was proportionate with growth of *P.putida*. The maximum reduction of limonin occurred during the log phase of growth of the organism but no significant change in limonin amount occurred as it reached stationary phase.

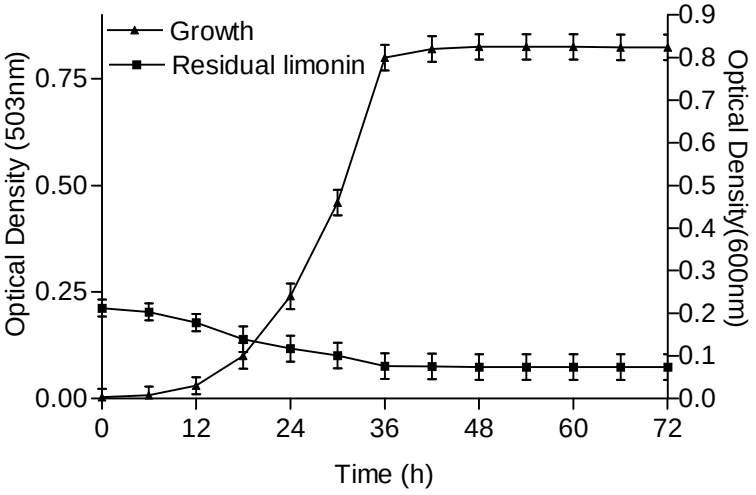


Figure-1: **Growth kinetics of *P.putida* in limonin as carbon source and limonin reduction in the medium with time.**

We assumed formation of other secondary metabolites might have some inhibitory effect on the cells, which might have rendered *P.putida* unable to utilize limonin further. Therefore complete removal of limonin was not achieved in the culture supernatant. It was established that this strain of *P.putida* could degrade limonin maximally during exponential phase.

2. Transposon Mutagenesis

In order to develop insights into the gene(s) responsible for regulation of limonin degradation pathway in gram-negative *Pseudomonas putida* PpG7 strain an approach of insertional inactivation of the gene was carried out by Tn5 transposon mutagenesis which is described in the present study. A random insertional inactivation of gene encoding for limonin degradation was carried out by mobilizing transposon element Tn5 in *P.putida* by biparental conjugation between host *P.putida* and donor *E.coli S17*1.

Biparental Conjugation

The donor strain of *E.coli* S171 harbored a broad host range plasmid (pGS9) that acts as a suicidal vector. The plasmid pGS9 possesses transposon (Tn5) and antibiotic selection markers. However prior to conjugation, spontaneous mutants of *P.putida* were generated against rifampicin (80 µg/ml) to obtain a selection marker for screening of exconjugants. Transposition of Tn5 element into the genome of *P.putida* strain conferred resistance against kanamycin. The Km^{r} Rif^{r} transconjugant derivatives of *P.putida* were selected for screening of the mutants of our interest. The frequency of transposition after 24 hrs of incubation as calculated from Table-I a & b was approximately 10^{-4}.

Table-I a: Cfu count of host *P.putida* and donor *E.coli S17* 1.

Table-I b: No. of exconjugants obtained from different donor: recipient ratios after biparental conjugation.

a.

Organism	Cfu/ml
P.putida (rifampicin mutant) (Recipient)	350 x 10^9
E.coli S171 (Donor)	1060 x 10^9

b.

Donor –Recipient Ratio	Exconjugants Cfu/ml
1:1	62 x 10^5
1:2	450 x 10^5
2:1	200 x 10^5
1:3	250 x 10^5
3:1	295 x 10^5

Screening of selective mutants for their inability to utilize limonin

Different ratios were plated in duplicate and about population of 1400 Tn5 containing Km^rRif^r colonies were screened successfully to achieve the mutant phenotype that was unable to utilize limonin as sole source of carbon. The studies were carried out on microtiter plates where a gradient of growth pattern was shown by the transconjugants. As shown in fig.2 and table II. a precipitate due to the formation of formazon (by oxidation of TTC added into the minimal medium) and turbidity as the result of growth in different wells of the microtiter plates indicated significant growth in limonin. There were three (shaded cells in Table II) such phenotypes in which growth was not observed and were selected for further study assuming them to be putative mutants. These three putative mutants were checked a number of times for lim^- phenotype and only one real mutant was selected as in other two it was difficult to comment whether they were really unable to utilize limonin.

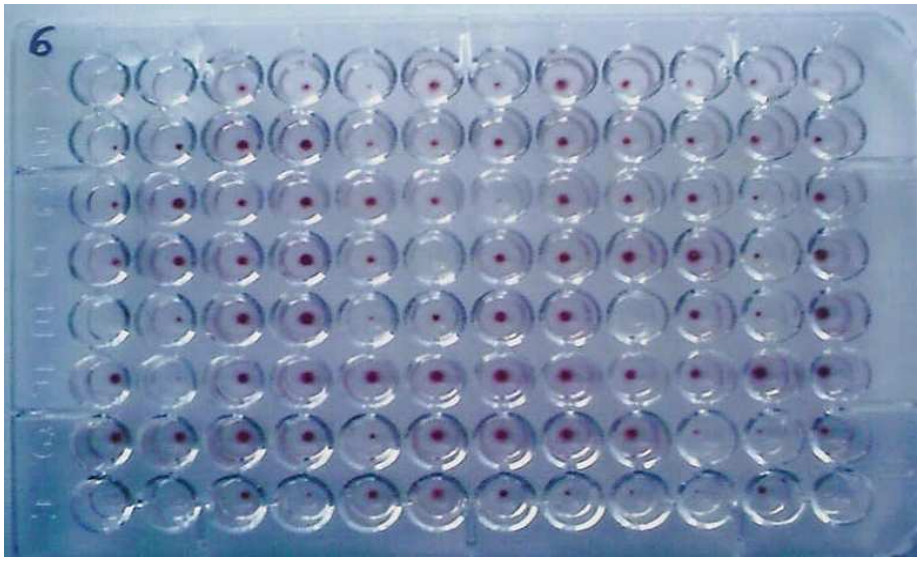


***Figure-2*: Transconjugants showing differential growth pattern in limonin.**

Table II: Score table for pattern of differential growth of transconjugants in limonin in microtiter indicator plate during screening of mutants.

	1	2	3	4	5	6	7	8	9	10	11	12
A	-vc	+vc	+++	+	+++	++++	+++	++	++	+	+++	+
B	+++	+++	++++	++++	++	+++	+++	+++	+++	++	+	+++
C	+++	+	+++	++++	++++	++++	+++	++++	+++	+++	++++	+++
D	0	+	++++	++++	+	+	+++	++++	0	+++	+	++++
E	++	+++	+++	+++++	+	0	+++	+++	++++	++++	++	+++
F	++	++++	+++	++++	+++	+++	+	++++	+++	+++	+	++++
G	+	++	++++	++++	++	++	+++	++++	+++	+++	+++	+++
H	+	+	++	++	+	+++	++	+++	++	+	++	+

-vc = negative control ; +vc = positive control ;
+++++ = excellent growth in glucose (standard) ;
++++ = very good growth;
+++ = average growth;
++ = less growth;
+ = poor growth;
0 = no growth

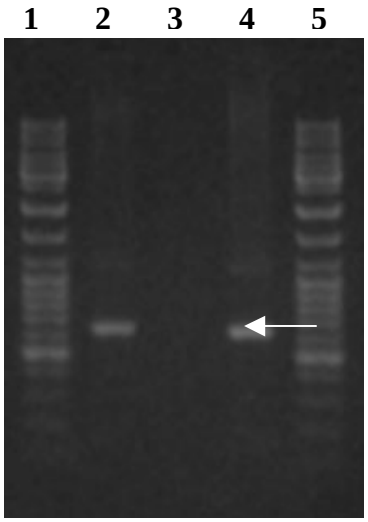
3. PCR amplification of Tn5

To confirm the mobilization and transposition of Tn5 in *P.putida,* genomic DNA of both the wild type and Tn5 putative mutant was isolated and then 625bp fragment, based on IS50 element of Tn5 was amplified by PCR. As shown in fig.3. no amplification was observed in the wild type *P.putida* strain (lane3). A 625bp fragment was amplified in genomic DNA of mutant (lane 4) as well as the Tn5 containing pGS9 plasmid DNA in lane 2. This indicated that Tn5 was transposed in genomic DNA of mutant with lim^- phenotype and caused insertional inactivation of the gene encoding the enzyme(s) for limonin degradation in *P.putida.*

Figure-3a: **Genomic DNA of *P.putida***

Lane-1: Genomic DNA of wild type P.putida

Lane-2: Genomic DNA of Tn5 putative mutant



625 bp amplification in Tn5 mutant

Figure-3b: **PCR amplification of Tn5**

Lane-1& 5: 100bp DNA Marker

Lane-2: Amplified product of plasmid pGS9

Lane-3: No amplification in wild copy of *P.putida*

Lane-4: Amplified product of mutant of *P.putida*

4. Cloning of Tn5 amplicon

PCR amplified 625bp element of Tn5 can be used to carry out Southern Hybridization in order to localize gene fragment flanking Tn5 in Tn5 putative mutant of *P.putida*. So, an attempt was made to clone amplified Tn5 fragment in pGEM-T for future genetic studies for molecular characterization of gene(s) implicated in limonin degradation.

pGEM-T vector facilitates the direct cloning of PCR product. These vectors have been linearized and have had 3'-terminal thymidine nucleotides added**.** pGEM®-T Easy Vector contains sequences on either side of the insert that are recognized by the restriction enzymes *Not* I and *Eco*R I. This allows the insert DNA to be removed with a single restriction digest using either of these enzymes.

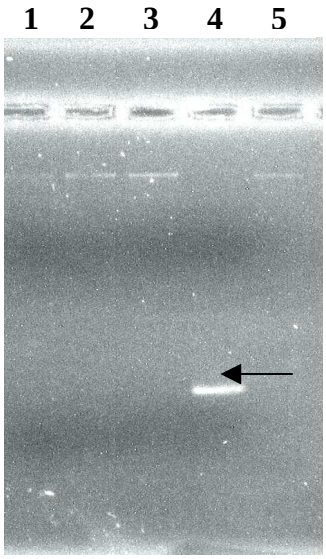


***Figure-4*: Optimization of concentration of Tn5 amplicon**

Lane-1: λ DNA of conc. 0.2µg/ml

Lane-2: λ DNA of conc. 0.4µg/ml

Lane-3: λ DNA of conc. 0.6µg/ml

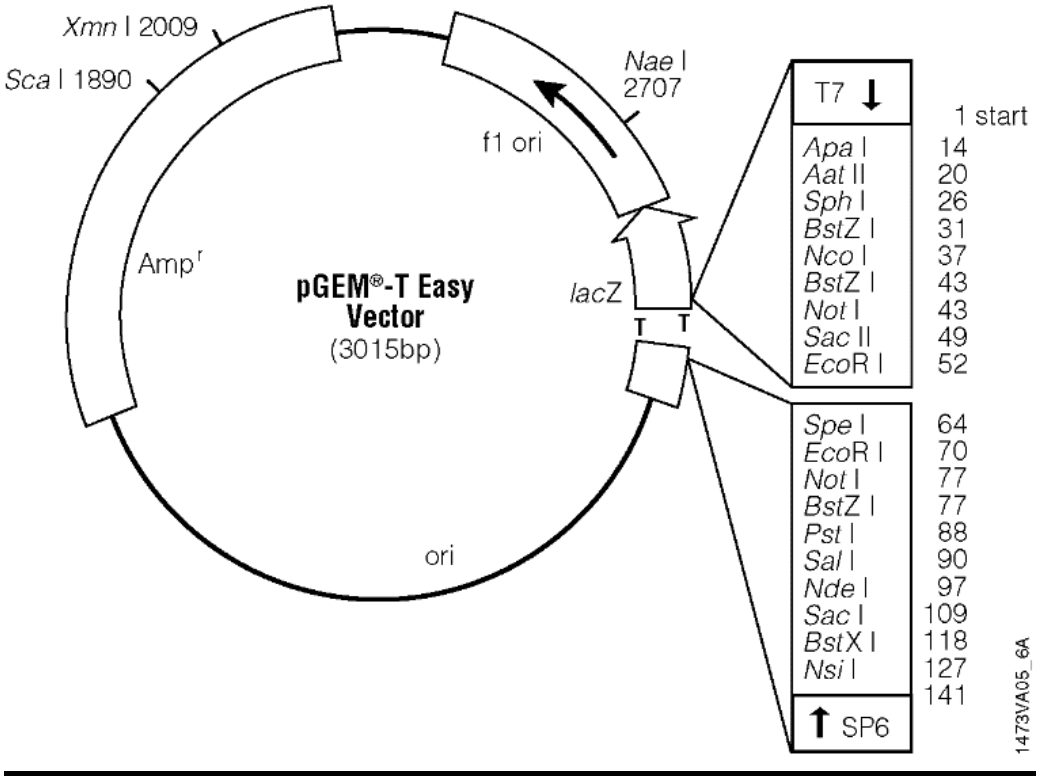
Lane-4: insert DNA of unknown conc.

Lane-5: λ DNA of conc. 0.8µg/ml

Prior to the ligation of insert i.e. amplified 625bp Tn5 fragment, to the pGEM-T vector the concentration of purified Tn5 insert DNA was determined by visual comparison of intensity of insert with standard λ-DNA of known concentration. As shown in fig.4 assuming concentration of insert DNA to be 1µg/ml and diluting it to 0.5µg/ml, the concentration of purified Tn5 DNA was approximated to be 750ng/ml after comparison of the intensities of λ-DNA and PCR product. As mentioned above the insert**:** vector ratio for the construct was determined empirically. Molar ratios ranging from 1:1 (insert: vector), 1:3, 3:1 had been employed. The insert DNA of optimized concentration along with the control DNA supplied by the manufacturer of pGEM-T was ligated to pGEM-T vector in insert**:** vector ratio of 1:1, 1:3, 3:1 under suitable conditions. pGEM®-T Easy Vector Systems take advantage of the template independent addition of a

single deoxyadenosine onto the 3'-end of PCR products by some thermostable DNA polymerases. These PCR fragments are ligated to linearized vector DNA that has been cleaved at an *Eco*R V site and had a single 3'-terminal thymidine added to both ends. By using these vectors, PCR products are directly cloned without further enzymatic manipulation while taking advantage of the high efficiency of a cohesive-end ligation.

pGEMT vector Map



The ligation mixture was then transformed in *E.coliDH5α* according to manufacturers instructions. Recombinants were selected by an initial blue/white colony screening followed by isolation and digestion of the plasmid vector to confirm the cloning of DNA (insert) of right size.

TableIII: No. of blue and white colonies obtained from different insert: vector ratio.

Ligation reaction	No. of blue colonies	No. of white colonies
Control: pGEMT vector (1:1)	7	1
Tn5 DNA: pGEMT vector (3:1)	5	1
Tn5 DNA: pGEMT vector (1:1)	6	0
Tn5 DNA: pGEMT vector (1:3)	5	0

The transformation efficiency can be calculated by dividing Total number of colonies counted by Total amount of DNA plated, where the total amount of DNA plated is the amount of DNA in the transformation reaction divided by appropriate dilution factors.

The transformation efficiency as calculated from the results was approximately 2 x 10^6 cfu/µg. Blue/white colony screening relies on disruption of the *lac*Z gene. Although the pGEM®-T Vector Control DNA will produce recombinants that generate white colonies, the insertion of other DNA fragments into the *lac*Z coding sequence may not result in white colonies unless the fragments disrupt the *lac*Z reading frame. Although this tends to occur most frequently with PCR products of 500bp or less, inserts of up to 2kb have been reported to result in blue colonies. Moreover, some insert DNAs can also result in pale blue colonies. For this reason, a control ligation without insert DNA was performed. The transformed white colonies were selected for the isolation of cloned plasmid vector with size of 3.6 Kb approximately.

5. Physical analysis of Tn5 to localize the disrupted gene

5.1. Restriction map of pGS9

Restriction map of Tn5 element shown in fig.5a reveals the presence of two restriction sites for the enzyme *Hpa* 1 at the extreme ends and cleavage of the 5.8kb Tn5 element yields a fragment of 5.2kb size. Other restriction sites that are present in Tn5 are *Xho* I, *Hind* III, *BamH* I and *Sma* I.

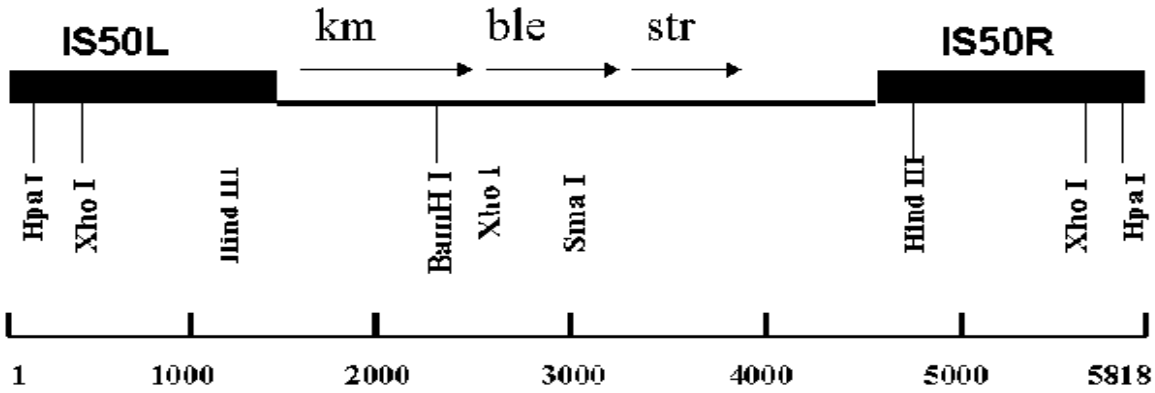
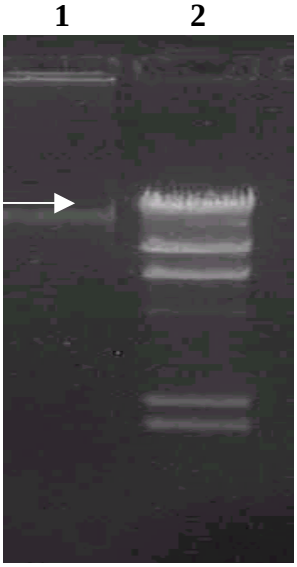


***Figure-5a*: Physical map of Tn5**

5.2. Restriction digestion of plasmid pGS9

Plasmid pGS9 harbored by *E.coli* S171 was isolated by method described by Kado and Liu. This method is usually employed for the isolation of large size plasmids. The size of the plasmid is approximately 36kb. (fig.5b)

Pasmid DNA digested with *Hpa* I as shown in fig.5c yield two fragments of size ~5.2Kb and ~31.Kb. The presence of two *Hpa* I sites at ends of Tn5 in plasmid cleaved the circular DNA to produce two fragments of Tn5 transposon and rest of the plasmid, while cleavage of plasmid with restriction enzyme other than *Hpa*I having restriction site within Tn5 would have cleaved Tn5 transposon from within.

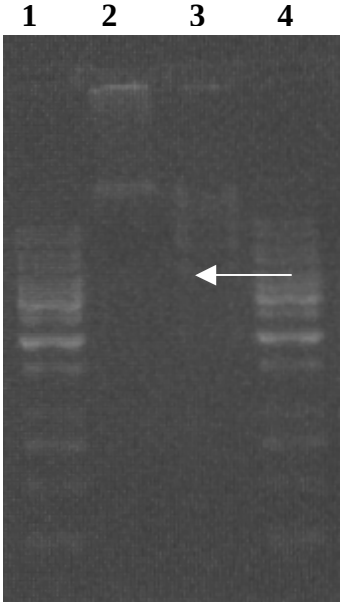


36 Kb pGS9 plasmid

Figure-5b: **pGS9 plasmid isolated by Kado & Liu method**

Lane-1: pGS9 plasmid

Lane-2: 23 Kb marker



5.2 Kb fragment of Tn5

Figure-5c**: Restriction digestion profile of pGS9**

Lane-1 and Lane- 4: IKb DNA marker

Lane-2: Plasmid DNA pGS9 without digestion

Lane-3: Plasmid DNA pGS9 digested with restriction enzyme *Hpa*I

5.3. *Restriction digestion of genomic DNA of P.putida*

To determine the approximate size and localize the gene responsible for limonin degradation in *P.putida* genomic DNA of both wild copy of the strain *P.putida* and its Tn5 mutant was digested with *EcoR* I. Since restriction site for *EcoR* I is absent in Tn5; restriction digest of lim^- mutant would yield DNA fragments where one of them might contain part of gene of interest flanking on both sides of Tn5.

Fig-5d. shows the restriction digestion profile of genomic DNA digested with *EcoR* I. After digestion the discrete bands appeared in the form of a smear indicating closely present multiple restriction sites of *EcoR* I. Here λ-DNA was used as reference. However the restriction enzymes such as *Kpn* I, *Cla* I, and *Sal* I. can also be used to digest genomic DNA as these enzymes do not have restriction site within Tn5 region and would also yield bands locating the flanking region of our gene of interest. Since these enzymes do not have restriction site within Tn5, fragment obtained would be the fragment of size greater than 5.8Kb, which would enable us to approximate of size of gene of interest.

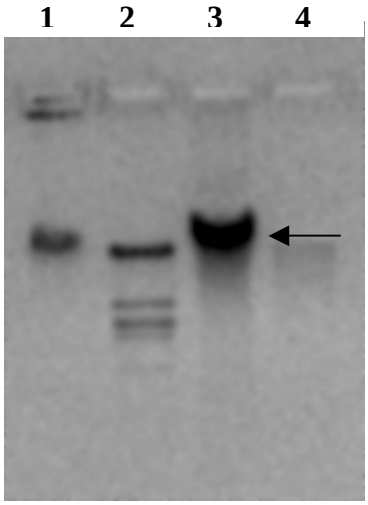


Figure-5d: **Restriction digestion profile of genomic DNA with *EcoR* I on agarose gel**

Lane1: λ DNA undigested

Lane2: λ DNA digested with *EcoR* I

Lane-3: Genomic DNA of Tn5 mutant of *P.putida*

Lane-4: Genomic DNA digested with *EcoR* I

5.4. Approximation of size of the gene

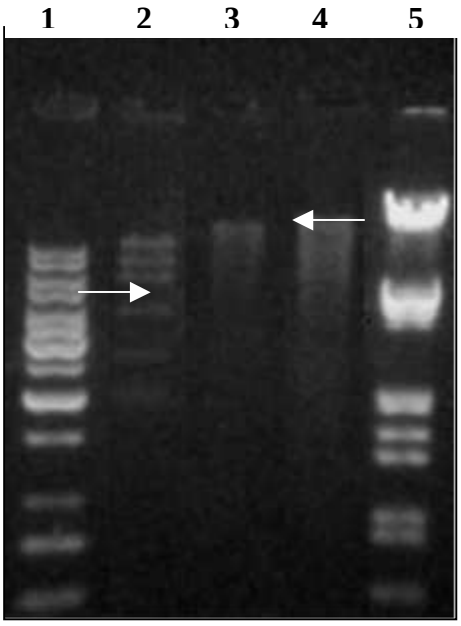


Figure-5e: Restriction digestion profile of different samples

Lane-1: 1Kb ladder
Lane-2: pGS9 digested with *Hpa* I
Lane-3: wild type P.putida digested with *EcoR* I
Lane-4: Tn5 mutant digested with *EcoR* I
Lane-5: 23 Kb marker

Fig. 5e reveals a comparison of digested product of wild type, mutant and pGS9 plasmid. Southern hybridization using Tn5 as probe would enable us to determine the size of the gene on the flanking sides of Tn5. Tn5 fragment of digested pGS9 would give a positive signal at approx. 5.8Kb which would be absent in wild type DNA digested with *EcoR* I while the *EcoR* I digested DNA of Tn5 putative mutant would generate a positive signal at higher molecular weight. Thus, it could be possible to locate and also determine size of limonin degrading gene in *P.putida*.

Conclusion

Molecular characterization studies carried on *Pseudomonas putida* for degradation of limonin addresses some of the central questions that were laid as objectives of the study. The observations are summarized as follows:

1. *Pseudomonas putida* PpG7 strain can utilize limonin, a highly oxygenated triterpenoid compound that is the major cause of delayed bitterness in citrus juice, as a sole source of carbon and energy. It is concluded that the microorganism possesses enzyme that acts on limonin to convert it into non-bitter metabolites.
2. It reduces 64% of total limonin provided in the medium within 48hrs.
3. Tn5 transposon mutagenesis was used as an approach to identify gene encoding for limonin degradation pathway in *P.putida*. pGS9 a suicide delivery vehicle, present in *E.Coli S17* 1 was used to successfully mobilize Tn5 into *P.putida.*
4. PCR amplification of 625bp, by primers designed from IS50 region of Tn5 in genomic DNA of transconjugants confirmed transposition and integration of Tn5 in their genome.
5. Approximately 1400 transconjugants were screened for their inability to utilize limonin as sole source of carbon out of which 1 mutant was found to have lost its ability to metabolize limonin.
6. 625bp amplified Tn5 fragment was cloned into PCR product cloning pGEMT vector.
7. To physically map Tn5 and localize the disrupted gene in the mutant of *Pseudomonas putida* pGS9 plasmid was isolated from *E.coli S17*1and digested with *Hpa*I to yield 5.2 Kb fragment of Tn5.
8. Using Tn5 as probe Southern hybridization would enable us to determine the size of the gene on the flanking sides of Tn5. For this genomic DNA of wild type and Tn5 mutant of *P.putida* was digested with *EcoR* I restriction enzyme

and compared to *Hpa* I digested pGS9 plasmid for approximation of the size of gene of interest.

Further study will lead to a deeper understanding of the complete set of catabolic activities of *P.putida* in response to limonin, which will pave the way for rational design of more efficient tools which can possibly solve the problem of delayed bitterness.

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Annexure

M63 MINIMAL MEDIA (1X)

KH_2PO_4	13g/L
$(NH_4)_2SO_4$	1g/L
$FeSO_4$	0.00005g/L
Adjust pH 7.2 by1N KOH.	
$MgSO_4$	1mM

TBE Buffer (5X)

Tris base	54g
Boric acid	28g
EDTA	3.8g
The pH of the buffer was adjusted to 8.0	

TE Buffer (1X)

EDTA	1.0mM(pH8.0)
Tris HCl	10.0 mM(pH 8.0)

Gel Loading Dye

Sucrose	35%(w/v)
EDTA	50.0mM(pH 8.0)
Bromophenol blue	0.2% (w/v)

Ribonuclease A

Stock solution	10mg/ml
Working solution	10-15g/ml

Ribonuclease A is prepared in a buffer containing100mM Tris (pH 8.0) and 15mM NaCl. To prepare DNAse free RNAse, the solution is boiled for 10 minutes, followed by the slow cooling, then dispensed into aliquots and is stored at -20° C for further use.

Lysozyme

Stock solution	10mg/ml
Working solution	300-400µg/ml

Lysozyme was prepared freshly in water.

E-buffer

Tris acetate	50mM
pH 8.0	

Lysis buffer

SDS	3%
Tris	50mM

pH was adjusted to 12.0 using 1M NaOH

SOC Medium (100 ml)

Solution A

Tryptone	2g
Yeast extract	0.5g
NaCl	1g
KCl (1M)	0.25ml

*2M Magnesium stock (*1ml)

$MgCl_2$	20.33gm
$MgSO_4$	24.65gm

2M glucose solution (1ml)