ISOLATION AND CHARACTERIZATION OF ENDOPHYTES FROM *Picrorhiza kurroa*

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CERTIFICATE

This is to certify that the work entitled, "ISOLATION AND CHARACTERIZATION OF ENDOPHYTES FROM *Picrorhiza kurroa*" submitted by Aayushi Mahajan and Neha Sharma in fulfillment for the award of degree of Bachelor of Technology in BIOTECHNOLGY of JayPee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Aayushi Mahajan **Neha Sharma**

CONTENTS

List of Abbreviations

1. Amp: Ampicillin
2. BLAST: Basic Alignment Local Search Tool
3. CaCl2: Calcium Chloride
4. DNA: Deoxy Ribo Nucleic Acid
5. dH20: Distilled Water
6. EDTA: Ethylene Diamine Tetra-acetic Acid
7. EtBr: Ethidium Bromide
8. LB: Luria broth
9. NCBI: National Centre for Biotechnology Information
10. PCR: Polymerase Chain Reaction
11. TE: Tris EDTA Buffer
12. TAE: Tris Acetic acid EDTA

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ABSTRACT

Endophytic bacteria reside within plant hosts without causing disease symptoms. In this study 14 bacterial strains, were isolated from the interior of healthy rhizome of Picrorhiza kurroa. According to the morphological and molecular analysis of 16s rRNA sequence the isolated strains were identified as Paenibacillus (6 strains), Acinetobacter , Serratia, Fillibacter, Pseudomonas and Bacillus subtilis. The strains were internal colonizer within the plant without any harmful side effects to the plant.

Aim

Isolation and Characterization of Endophytes from Picrorhiza kurroa.

Objectives

1. Isolation of Endophytes from P.kurroa.

2. Identification and Molecular Characterization of the Isolated endophytes.

Chapter I

INTRODUCTION

Endophytes may be defined as microbes that colonize living internal tissues of plants without causing any immediate, overt negative effects. They may originate from indigenous species that occur either naturally in soil or they may be introduced through agricultural practices. These are ubiquitous and have been found in all the species of plants studied to date. Endophyte is an all-encompassing topographical term that includes all organisms which during a variable period of their life-cycle colonize the living internal tissues of their hosts without producing symptoms of disease. Common endophytes include a variety of bacteria, fungi and actinomycetes, and they can be isolated from wild or cultivated plants.

Endophytes are microbial entities that live within living tissues of plants. In most cases their relationship with the host plant is symbiotic and probably mutualistic. Many are capable of synthesizing bio-active compounds that can be used by the plant for defense against pathogenic fungi and bacteria. Extensive colonization of the plant tissue by endophytes creates a "barrier effect", where the local endophytes outcompete and prevent pathogenic organisms from taking hold. Some of these compounds have proven useful for novel drug discovery. By encouraging the endophytes to grow outside the plant in nutrient rich media, it is possible to harvest the bio-active compounds that they produce.

Several classes of natural products have been reported to be produced by endophytes. These are antibacterial and antifungal antibiotics, antitumor agents, antiviral compounds, volatile insecticides, and industrial enzymes etc. Of late, even unusual molecules that act as anti-diabetic, immuno-modulatory, herbicidal, and plant growth promoting/plant protective agents have also been discovered from microbial endophytes.

Some prominent examples of biotech products from endophytes are novel antibiotics, from a Streptomyces sp. in snakevin e(Kennedia nigriscans) which have long been used as medicine by Australian aborigines, kakadumycins from another Streptomyces sp.

isolated from Grevillea pteridifolia, coronamycins from yet another Streptomyces sp. from Monstera sp., and arylcoumarins-novel anticancer compounds-from a Streptomyces aureofaciens strain isolated in Thailand from the plant, Gingiber officinale.

At present, much research has focused on isolation of endophytes from pharmaceutical plants, such as Camptotheca acuminate, pine and Taxus plants, discovering a vast number of undescribed endophytic species, some of which have potential to be used in the production of medicine. Picrorhiza kurroa is one such important medicinal plant whose endohytic study is un-explored.

Picrorhiza kurroa is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, and scorpion sting. It is a small perennial herb from the Scrophulariaceae family, found in the Himalayan region growing at elevations of 3,000-5,000 meters. The active constituents- Picroside I and Picroside II are obtained from the root and rhizomes. The plant is self-regenerating but unregulated over-harvesting has caused it to be threatened to near extinction.

Therefore the investigation of endophytes in P. kurroa is important as these could be a potential source of pharmacologically active secondary metabolites present in the plant and thus help in the conservation of the plant.

Chapter II

REVIEW OF LITERATURE

An endophyte is an endosymbiont, often a bacterium or fungus, which lives within a plant for at least part of its life without causing apparent disease. Endophytes are ubiquitous and have been found in all the species of plants studied to date. Endophytes are fungi or bacteria residing inside healthy plant tissues without any discernible Infectious symptoms (Wilson 1995). They could be a potential source of novel natural products for medicinal, agricultural, and industrial uses. Because they are relatively unstudied, much attention is now being paid to endophytic biodiversity, the chemistry and bioactivity of endophytic metabolites, and the relationships between endophytes and host plants (Schulz et al. 2002; Tan and Zou 2001). Endophytes provide a wide variety of structurally unique bioactive natural products, such as alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others (Tan and Zou 2001). Antibiotics, antiviral compounds, anticancer agents, insecticidal products, anti-diabetic agents, immunosuppressive compounds as well as antioxidants have been reported from endophytic metabolites (Strobel et al. 2004), and medicinal plants have been recognized as a repository of endophytes with novel metabolites of pharmaceutical importance (Strobel et al. 2004; Tan and Zou 2001; Wiyakrutta et al. 2004). Medicinal plants contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g., phenolic acids, flavonoids, quinones, coumarins, lignans, lignin, stilbenes, and tannins), nitrogen compounds (e.g., alkaloids and amines), vitamins, terpenoids, and other endogenous metabolites (Cai et al. 2004; Kahkonen et al. 1999; Zheng and Wang 2001).

The wide range of compounds produced by endophytes has been shown to combat pathogens and even cancers in animals including humans. One notable endophyte with medicinal benefits to humans was discovered by Dr. Gary Strobel, produces taxol, and is found within the Pacific Yew tree. Endophytes are also being investigated for roles in agriculture and biofuels production. Inoculating crop plants with certain endophytes may provide increased disease or parasite resistance while others may possess metabolic

processes that convert cellulose and other carbon sources into "myco-diesel" hydrocarbons and hydrocarbon derivatives.

It is speculated that there may be many thousands of endophytes useful to mankind but since there are few scientists working in this field, and since forests and areas of biodiversity are rapidly being destroyed, many useful endophytes for curing disease might be permanently lost for medicinal use before they are discovered. The effects of climate change on endophytes is being investigated. Studies of plants grown at different climates or at increased carbon dioxide levels have different distributions of endophytic species

While many endophytes are known to colonize multiple species of plants, some are host specific. Endophytic species are very diverse; it is thought that only a small minority of all existing endophytes have been characterized. A single leaf of a plant can harbor many different species of endophytes, both bacterial and fungal.

The endophytes have a tendency to grow in a mixed population when cultured in a nutrient media and thus need to be separated into pure cultures before proceeding with further characterization and identification.

Table2.1.1: Review on Work Done On Endophytes in various Plants

Author	Plant Source	Work Done	Isolated Endophytes
Araujo, W. L. et al (2004)	Sugar cane	Root endophytic and rhizoplane communities of transgenic sugarcane plants, resistant to imidazolina herbicides and insects, were evaluated. The isolates were identified by using 16s rRNA sequencing.	Bacillus cereus, Burkholderia sp., B. pseudomallei, Nocardiopsis sp., Paenibacillus illinoisensis, P. pabuli, Rhizobium sp. and Stenotrophomonas maltophilia
K.D. Lee et al (2005)	Bean Nodules	Isolation of Plant-Growth-Promoting Endophytic Bacteria from Bean Nodules	Bacillus subtilis
H. Lata et al. (2006)	Echinacea	Identification of IAA-producing endophytic bacteria from micropropagated Echinacea plants using 16S rRNA sequencing	Acinetobacter, Bacillus,Pseudomonas, Wautersia (Ralstonia) and Stenotrophomonas.
Mano, H.et al (2007)	Rice	Endophytic bacteria detected inside surface-sterilized plants or extracted from inside plants and having no visibly harmful effects on the plants.	Pantoea, Methylobacterium, Azospirillum, Herbaspirillum, Burkholderia and Rhizobium

Rijavec et al. (2007)	Maize Kernel	The germination of surface-sterilized maize kernels under aseptic conditions proved to be a suitable method for isolation of kernel-associated bacterial endophytes. Bacterial strains identified by partial 16S rRNA gene sequencing.	Pantoea sp., Microbacterium sp., Frigoribacterium sp., Bacillus sp., Paenibacillus sp., and Sphingomonas sp.
Tan, J. D. et al (2007)	Sweet potato	This study was an attempt to isolate endophytic microorganism with antimicrobial properties from sweetpotato that are grown in the Philippines. Endophytic microorganisms were isolated from surface-sterilized stem cuttings of selected Philippine sweet potato varieties such as BSP-SP-17, BSP-SP-22, and NSIC-SP-25. The isolates were purified and tested for antimicrobial activities using spot and streak inoculation methods against various diseases. Twenty-one isolates (12 bacteria and 9 fungi) exhibited antimicrobial activities against one or more indicator microorganisms tested. The bacteria were identified through 16S rDNA sequencing analyses. The fungi were also identified through their partial 26S rDNA and ITS region sequencing analyses	Bacillus megaterium, Bacillus pumilus, Microbacterium arborescens, Micrococcus luteus and Bacillus subtilis. The fungi were identified as Phomopsis sp., Aspergillus sp., Penicillium sp. and two belong to Mycosphaerellaceae and Polyporaceae family respectively

Thomas, P. et al (2008)	Banana	Identification of culturable and originally non-culturable endophytic bacteria isolated from shoot tip cultures of banana cv.	Enterobacter, Klebsiella, Ochrobactrum, Pantoea, Staphylococcus and Bacillus spp. constituted Brevundimonas, Methylobacterium, Alcaligenes, Ralstonia, Pseudomonas, Corynebacterium ,Microbacterium.
Zaiton, S. et al (2008)	Oil palm roots	Microbial endophytes were isolated from oil palm roots sampled from 12 sites in Perak, Malaysia, varying in soil type, age of palms, and severity of basal stem rot (BSR). Bacterial endophytes were found to be dominant in all areas sampled, followed by fungi, including actinomycetes. The incidence of bacterial and fungal isolates was highest in mature palms (>11 years old), followed by middle-aged (6-10 years old) and young (<5 years old) palms, in all sampling areas.	Pseudomonas, Burkholderia and Serratia
Tejesvi, M. V. et al	Terminalia arjuna, Terminalia	Thirty Pestalotiopsis species isolated from different parts of the medicinal plants were selected for the study. The	Pestalotiopsis species

(2008)	chebula, Azadirachta indica, and Holarrhena antidysenteri ca	antioxidant and antihypertensive properties of Pestalotiopsis isolates were determined by measuring 1,1-diphenyl-2-picrylhydrazyl inhibitory activity, lipid peroxidation, and angiotensin-converting enzyme inhibition activity.	
Rangeshwa ran, R et al (2008)	Chickpea (Cicer arietinum L.)	Five endophytic bacteria were isolated from healthy chickpea (Cicer arietinum L.) plants by surface-disinfestation method and identified based on morphological, physiological and biochemical tests. which had nitrate-solubilizing ability.	Erwinia herbicola and Enterobacter agglomerans, and Bacillus megaterium, B. circulons, E. agglomerans
Assumpca, L. de C. et al (2009)	Soyabean	The objectives of this work were to isolate, characterize and identify the endophytic bacterial community of soybean seeds, and to test the biotechnological potential of this community. Seeds from 12 soybean cultivars were used. The endophytic bacterial isolates were evaluated for in vitro antagonism against phytopathogenic fungi, synthesis of indoleacetic acid (IAA), and capacity to solubilize phosphate. Isolation	Acinetobacter, Bacillus, Brevibacterium, Chryseobacteriu m, Citrobacter, Curtobacterium, Enterobacter, Methylobacteriu m, Microbacterium, Micromonospora , Pantoea,
		techniques, amplified ribosomal DNA restriction analysis (ARDRA) grouping, and identification by means of partially sequencing the 16S rDNA	Paenibacillus, Pseudomonas, Ochrobactrum, Streptomyces,

		were used in community characterization.	Tsukamurella
Kaihui Liu et al (2009)	Taxus Chinensis	A total of 115 endophytic fungi isolates obtained from bark segments of T. chinensis were grouped into 23 genera based on the morphological traits and sequence analysis of the internal transcribed spacers (ITS1-5.8S-ITS2). The isolated fungi produced taxol as shown by HPLC analysis.	Diaporthe, Phomopsis, Acremonium, Pezicula
Barretti, P. B. et al (2009)	Tomato plant	Forty isolates of endophytic bacteria obtained from healthy tomatoes were tested for their potential as biological control agents of tomato diseases. A mass screening was performed in a greenhouse using Pseudomonas syringae pv. tomato and Alternaria solani as challenging pathogens. Based on the average number of lesions per plant, 4 isolates were selected as potential biological control agents of the tomato diseases caused by fungi and bacteria. These isolates were identified by 16S ribosomal DNA sequence analysis. The 4 endophytes selected for biological control were also evaluated for their	Acinetobacter johnsoni, Serratia marcescen, Sinorhizobium sp and Bacillus megaterium

		ability of promoting plant growth and only Serratia marcescens presented an increase in the height of the plants.	
Procopio, R. E. L. et al (2009)	Eucalyptus citriodora, E. grandis, E. urophylla, E. camaldulensis, E. torelliana, E. pellita, and a hybrid of E. grandis and E. urophylla	Endophytic bacteria were isolated from stems of Eucalyptus spp (Eucalyptus citriodora, E. grandis, E. urophylla, E. camaldulensis, E. torelliana, E. pellita, and a hybrid of E. grandis and E. urophylla) cultivated at two sites; they were characterized by RAPD and amplified rDNA restriction analysis (ARDRA). Endophytic bacteria were more frequently isolated from E. grandis and E. pellita. The 76 isolates were identified by 16S rDNA sequencing.	ErwiniaPantoea, Agrobacterium sp, Curtobacterium sp , Brevibacillus sp, Pseudomonas sp, Acinetobacter, Burkholderia cepacia,Lactococcus lactis
Liu Jie Feng et al (2009)	Solanaceae	Endophytic bacteria were isolated and tested for their activity against bacterial wilt in solanaceae.	Bacillus, Escherichia, Klebsiella, Agromonas, Erwinia and Curto Bacterium
Shi Ying Wu et al (2009)	Sugar beet	Three plant-growth-promoting isolates of endophytic bacteria from sugar beet roots produced indole-3-acetic acid (IAA) in vitro in a chemically defined medium. The three isolates were selected from 221 endophytic bacteria isolated from surface-disinfected beet roots and evaluated for potential to	

		produce IAA and to promote beet growth under gnotobiotic and glasshouse conditions.	
Kharwar, R. N. et al (2009)	Neem	The endophytic fungus Chloridium sp. produces javanicin under liquid and solid media culture conditions. This highly functionalized naphthaquinone exhibits strong antibacterial activity against Pseudomonas spp., representing pathogens to both humans and plants.	Chloridium sp
Dias, A. C. F.et al (2009)	Strawberry	Twenty endophytic bacteria were isolated from the meristematic tissues of three varieties of strawberry cultivated in vitro, and further identified, by FAME profile, into the genera Bacillus and Sphingopyxis. The strains were also characterized according to indole acetic acid production, phosphate solubilization and potential for plant growth promotion.	Bacillus and Sphingopyxis
Aravind, R et al (2009)	Piper nigrum L.	To isolate and identify black pepper (Piper nigrum L) associated endophytic bacteria antagonistic to Phytophthora capsici causing foot rot disease Three isolates, were found	Pseudomonas spp., Serratia, Bacillus spp., Arthrobacter spp.,
		effective for Phytophthora suppression in multilevel screening assays which recorded over 70% disease	Micrococcus spp., Curtobacterium

		suppression in green house trials.	sp.
Kuffner, M. et al (2010)	Salix caprea	To characterize bacteria associated with Zn/Cd-accumulating Salix caprea regarding their potential to support heavy metal phytoextraction.	Burkholderia sp., Actinobacterium
Rashmi Tiwari et al (2010)	Ocimum sanctum	Four endophytic bacteria OS-9, OS-10, OS-11, and OS-12 were isolated from healthy leaves of Ocimum sanctum. These isolated microbes were screened in dual culture against various phytopathogenic fungi viz. Rhizoctonia solani, Sclerotium rolfsii, Fusarium solani, Alternaria solani, and Colletotrichum lindemuthianum. Of these, strain OS-9 was found to be antagonistic to R. solani, A. solani, F. solani, and C. lindemuthianum while OS-11 was found antagonistic against A. solani only. The growth-promoting benefits of the endophytes were initially evaluated in the glasshouse by inoculated seeds of O. sanctum. Treatment with endophytes OS-10 and OS-11 resulted in significant enhancement of growth as revealed by increase in fresh as well as dry weight.	OS-11 was highly related to Bacillus subtilis.

2.1 Examples of genera of nonpathogenic bacteria that have been isolated from internal plant tissues (Chris P. Chanway):

Acetobacter,Clavibacter,Pasteurella,Achromobacter,Comamonas,Photobacterium, Acidovorax,Corynebacterium,Phyllobacterium,Acinetobacter,Curtobacterium, Providencia,Actinomyces,Deleya,Pseudomonas,Agrobacterium,Enterobacter, Psychrobacter,Alcaligenes,Erwinia,Rahnella,Arthrobacter,Esherichia,Rhizobium, Azoarcus,Flavobacterium,Rhodococcus,Azorhizobium,Herbaspirillum,Rickettsia, Azospirillum,Kingella,Serratia,Bacillus,Klebsiella,Shewanella,Bordetella,Lactobacillus, Sphingomonas,Burkholderia,Leuconostc,Staphylococcus,Capnocytophaga,Methylobacterium,Stenotrophomonas,Cellulomonas,Micrococcus,Variovorax,Chryseobacterium, Moraxella,Vibrio,Citrobacter,Pantoea,Xanthomonas ,Yersinia.

Picrorhiza kurroa:

It is a small perennial herb from the Scrophulariaceae family. The pharmacologically active components of P.kurroa i.e. Picroside I and Picroside II are found in the roots and rhiozomes. So the rhizome of Picrorhiza has been traditionally used to treat worms, constipation, low fever, scorpion sting, asthma and ailments affecting the liver. Current research on Picrorhiza kurroa has focused on its hepatoprotective, anticholestatic, antioxidant, and immune-modulating activity. (Shivkumar Chauhan et al.)

ChapterIII

MATERIAL AND METHODS

3.1 Materials:

3.1.1 Plant Sample: The plant sample was rhizome of Picrorhiza kurroa and was obtained from HFRI Shimla.

Samples were from:

Pattal forest, Near Chisna Pass

Code No. HFRI-PK-1 (JUIT)

Fig 3.1.1 Rhizome Sample PK-1(1)

Karu (near Jogni) Dhel Under Tungal Tree Moist shady places GHNP.

Code No. HFRI- PK-2(12)

Fig 3.1.2 Rhizome Sample PK-2(12)

3.1.2 Chemicals: The chemicals used in the present study were obtained from Merk Limited and Qualigens fine chemicals Limited. The various media used in the study namely Nutrient Agar, Nutrient Broth and Luria Broth were manufactured by HiMedia Laboratories Pvt. Ltd.

3.2 Methods:

3.2.1 Media preparation

Nutrient agar and Luria Broth were used as solid or liquid media for growth of endophytes which were prepared by mixing a desired concentration of media in distilled water which was necessarily followed by autoclaving in order to avoid any contamination.

3.2.2 Streak- plate technique:

By means of a transfer loop, a portion of the mixed culture is placed on the surface of an agar medium and streaked across the surface. This manipulation thins out the bacteria on the agar surface so that some individual bacteria are separated from each other.

3.2.3 Spread Plate technique:

In the spread plate method the mixed culture is not diluted in the culture medium; instead it is diluted in a series of tubes containing sterile liquid which is usually water.

3.3 Isolation of endophytes from Picrorhiza kurroa:

3.3.1 Surface Sterilization:

The samples were surface-sterilized by washing with labolene and then with tap water till the detergent were completely removed. Then the samples were given 2-3 washings with distilled water followed by washings with 70% ethanol for 1 minute .This was followed by washing with 0.1% mercuric chloride (v/v) for 2 min followed by 4-5 washings with autoclaved distilled water in the laminar air hood.

3.3.2 Isolation:

The sterilized rhizome was then placed on an autoclaved filter paper in a Petri-plate and the outer layer of the rhizome was removed using autoclaved and incinerated scalpel blade and forceps in the laminar air hood. It was then cut into 1 cm^2 pieces and the pieces were then pressed onto the nutrient agar and PDA plates. The plates were then sealed with parafilm and kept for incubation at 25^0C for 1 week. The plates were then daily checked for any contamination by comparing with the control plates.

Control plates were made by spreading 10 µl of water that was left after surface sterilization of the sample onto the plate and this plate was incubated under the same conditions. Another control was made by incubating the Nutrient Agar and PDA plate along with the other cultured plates.

The growth was observed in the inoculated plates after incubation of 6-7 days.

The cultures were then made pure using serial dilution at 10^{-6} and streak plate technique. 14 isolates were recovered and re-isolated and subjected to gram staining and catalase test.

3.4 Bacterial DNA Isolation:

Endophyte strains were grown in Luria broth culture overnight.4 ml of each of these cultures were taken up and centrifuged at 8000 rpm for 5 minutes. Supernatant was decanted. Pallete was further re-suspended in 1ml TE buffer by repeated pipetting. Again centrifuged at 6000 rpm for 9 minutes. Again supernatant was decanted and pallet was re-suspended in 400 µl of TE buffer and 40 µl of 10 % SDS. It was incubated at 37°C for 30 minutes. Equal volume of phenol and chloroform were added and mixed well but very gently to avoid shearing DNA by inverting the tubes until the phases were completely mixed. It was again centrifuged at 10,000 rpm for 10 minutes. The upper layer was carefully transferred to a new tube and $1/10^{th}$ volume of sodium acetate and 0.6 volume of iso-propanol were added and mixed gently. Again centrifuged at 8000 rpm for 3 minutes. The supernatant was discarded and the pallete was washed with 70% ethanol and air dried for 5 minutes. 50 µl of TE buffer was added to make up the volume.

3.4.1 Agarose gel electrophoresis of isolated DNA:

The isolated DNA (3 µl) mixed with 2µl of gel loading dye was loaded on 0.8% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5 µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

3.5 PCR amplification using 16s rRNA primers:

The sequence of primer U1 is 5'-CCAGCAGCCGCGGTAATACG-3'

sequence of primer of U2 is 5'-ATCGG(C/T)TACCTTGTTACGACTTC-3'

DNA amplification was carried out in a 15 µl reaction volume containing 0.5 µl DNA template, 0.6 µl of forward primer, 0.6 µl of reverse primer, 1.5 µl of 10X PCR Buffer, 0.3 µl of dNTP mix, 11.35 µl of autoclaved dH_2O and 0.15 µl of Taq Polymerase (Sigma).

PCR amplification was carried out in a thermocycler (Applied Biosystems) using the following conditions:

4-min denaturation at 95°C, then the reaction mixture was run through 30 cycles of denaturation for 30 sec at 94°C, annealing for 1 min at 52°C and extension for 1 min at 72°C, followed by an incubation for 7 min at 72°C.

3.5.1 Agarose gel electrophoresis of PCR Product:

The amplified PCR product 8 µl mixed with 2µl of gel loading dye was loaded on 1.2 % agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5 µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

3.6 Cloning Of the PCR Product:

The amplified PCR product was cloned using Linearized T-vector and T4 DNA Ligase supplied by Banglore GeneI.

3.6.1 Ligation:

The ligation reaction was set up in a total volume of 5 µl. the ligation mixture was prepared by adding 3.5 µl of PCR product, 0.5 µl of Ligation Buffer, 0.5 µl of T-vector and 0.5 µl of T4 DNA Ligase. The ligation mix was incubated at 4°C for 16 hours. Then store at -20°C.

3.7 Preparation of Competent cells by $CaCl_2$ Method:

Competent cells are those that possess more altered cell walls through which DNA can be passed easily. These cells readily incorporate foreign DNA. For the transformation experiments DH5α strains of E. coli was used. Single colony of the appropriate strain of the bacteria was streaked on to LB agar plate with ampicillin (1µ/ml). This was incubated at 37^0C for 12-16 hrs. These freshly grown colonies of E. coli cells were used for preparation of competent cells.

3.7.1 Procedure:

Culture of DH5α strain of E.coli was grown in LB overnight at 37°C. 2 ml of overnight culture of E. coli was inoculated in 200ml medium and grown at 37°C for 1-2 hr with continuous shaking. When the OD of the culture was 0.5 at 600 nm the culture containing cells was splitted into four tubes (50 ml tube). Then the tubes were placed on ice for 10 min. the culture was spinned at 3000rpm for 10 minutes at 4°C.Supernatant was discarded and the pallete was re-suspended in 10 ml ice cold filter sterilized 0.1 M $CaCl_2$. Again centrifuged at 3000rpm for 10 minutes at 4°C and cells were again re-suspended in 1ml of 0.1 M $CaCl_2$ as before. The chilled cell suspension was mixed with 5 ml of autoclaved glycerol. 0.2 ml of aliquots were made into sterilized 1.5 ml of micro centrifuge tubes. The tubes were freezed rapidly in liquid nitrogen and stored at -80°C for further use.

3.8 Transformation of Competent cells:

The competent cells which were stored at -80^{0}C (vol. 200 µl) were thawed on ice for 15 minutes. The ligation mixture (5 µl) was simultaneously thawed on ice for 15 minutes and added to the thawed competent cells in laminar air flow condition. The 2 components were mixed by gentle flicking and incubated on ice for 45 minutes. Heat shock was given to the cells for 90 seconds in a water bath at 42^{0}C (Heat shock converts the bacterial cell membrane in to gel state from solid state. This lead to formation of gaps in membrane which are sufficient for the entry of ligated plasmid cells in to bacteria).The cells were cooled immediately on ice for 5 min. (Due to cooling the membrane gets repaired and the gaps were closed after entry of ligated plasmid cells in to bacteria).900 µl of Luria broth (previously autoclaved) was added to the tubes containing transformed cells in laminar air flow condition and the tubes were incubated at 37^{0}C for 3 hr with moderate shaking (~150 rpm).During the mean time LB plates were prepared Containing ampicillin (1µl /1ml).40 µl of X-gal,20 µl of IPTG and 200 µl of transformed product were spread on LB amp plates and plates were incubated overnight at 37^{0}C.Next day after 12-16 hrs, the plates were observed for checking of development of transformed colonies.

3.8.1 Checking of transformed colonies:

After incubation the plates were checked for blue white screening if the ligation was successful, the bacterial colony will be white; if not, the colony will be blue. About 8 white colonies, growing on the LB agar plates were picked up, cultured separately on fresh LB agar plates as well as in tubes in laminar air flow condition and incubated at 37^o C for overnight to recheck the development of transformed colonies .

3.9 Plasmid isolation from transformed cells:

Plasmid isolation was done using AxyPrep Plasmid Miniprep Spin Protocol:

1-4 ml of overnight LB culture was collected. Centrifuged at 12,000×g for 1 minute to pellet the bacteria. The supernatant was decanted. The bacterial pallete was re-suspended in 250 µl of Buffer S1 by vortexing. 250 µl of Buffer S2 was added, and mixed gently by

inverting the tubes.350 µl of Buffer S3 was added and mixed gently by inverting. Again centrifuged at 12,000×g for 10 minutes to clarify the lysate. Miniprep column was palced into an uncapped 2 ml Microfuge tube. Clarified supernatant was transferred into the Miniprep column. The Miniprep column was transferred into 2 ml microfuge tube and spinned at 12,000×g for 1 minute.500 µl of Buffer W1 was pipetted into each Miniprep column and centrifuged at 12,000×g for 1 minute. 700 µl of Buffer W2 was pipette into each Miniprep column. And centrifuged at 12,000×g for 1 minute. Filtrate was discarded from the 2 ml Microfuge tube and the Miniprep column was placed back into the 2 ml Microfuge tube.Again centrifuged at 12,000×g for 1 minute. The Miniprep column was transferred into a clean 1.5 ml Microfuge tube. The plasmid DNA was eluted by adding 60~80 µl of Eluent (or deionized water) to the center of the membrane. It was left to stand for 1 min at room temperature and was then centrifuged at 12,000×g for 1 minute. The plasmid DNA was stored in -20°C.

3.9.1 Agarose gel electrophoresis of isolated plasmid:

The isolated plasmid (3 µl) mixed with 2µl of gel loading dye was loaded on 0.8% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in BioRad GelDoc.

3.9.2 Checking the insert in plasmid using T7 & SP6 primers:

The sequence of primer of T7 5' GTAATACGACTCACTATAGGGC 3'

sequence of primer of SP6 5' ATTTAGGTGACACTATAGAATAC 3'

DNA amplification was carried out in a 15 µl reaction volume containing 0.5 µl DNA template, 0.6 µl of forward primer, 0.6 µl of reverse primer, 1.5 µl of 10X PCR Buffer, 0.3 µl of dNTP mix, 11.35 µl of autoclaved dH2O and 0.15 µl of Taq Polymerase (Sigma).

PCR amplification was carried out in a thermocycler (Applied Biosystems) using the following conditions:

4-min denaturation at 95°C, then the reaction mixture was run through 30 cycles of denaturation for 30 sec at 94°C, annealing for 1 min at 52°C and extension for 1 min at 72°C, followed by an incubation for 7 min at 72°C.

3.9.3 Agarose gel electrophoresis of PCR Product:

The amplified PCR product 8 µl mixed with 2µl of gel loading dye was loaded on 1.2 % agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

3.10 Sequence Analysis:

The two samples that were sequenced after cloning their sequences were first analyzed for the presence of the vector sequence using VecScreen. The vector sequences were then removed and the remaining sequence was made to BLAST with the nucleotide database in the NCBI. The strain that was showing the maximum query coverage and maximum identity and was matching with our gram biochemical analysis was picked up as the strain we had isolated.

The PCR product were then sent for sequencing. The sequences that were obtained after sequencing were analyzed further for the identification of the strains. The noise was removed from the chromatogram peaks and the sequence obtained after that was made to BLAST with the nucleotide database in the NCBI. As two primers are used in PCR and so the sequencing is done with two primers U1 and U2 in this case. The sequence obtained with both the primers were first checked for any overlapping regions by doing a BLAST with both the sequences. The overlapping region was then deleted from either one of the two sequences by using EditSeq of DNA Star. The two sequences were then combined and were made to BLAST with the nucleotide database in the NCBI. The strain that was showing the maximum query coverage and maximum identity and was matching with our gram staining was picked up as the strain we had isolated.

Chapter IV

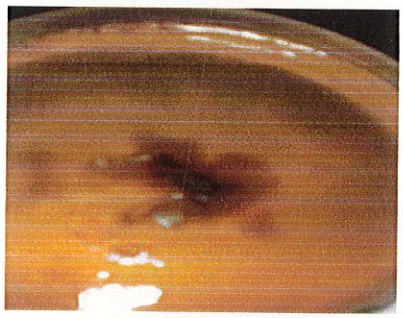
RESULTS AND DISCUSSIONS

4.1 Isolation of Endophytes:

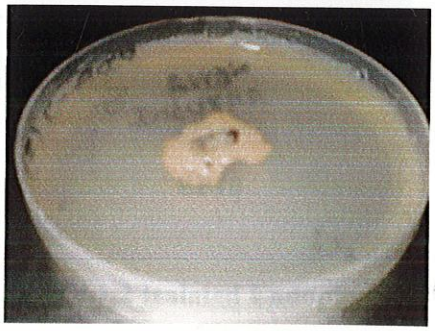
To isolate the endophytes the cultured plates were incubated at 25°C for 1 week and after incubation of 6-7 days following strains which we named as EP1, EP2, EP3, EP4, EP5, EP6, EP10, EP11 were obtained.

These cultures showed a mat like growth with variations in their opacity and texture. These were referred as master cultures.

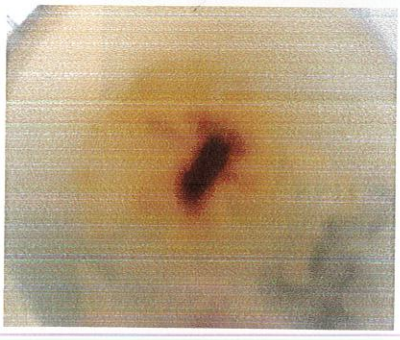
EP1



EP2

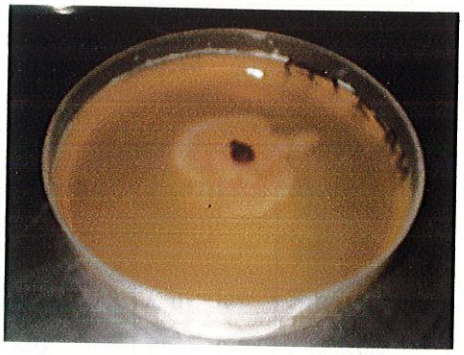


EP3

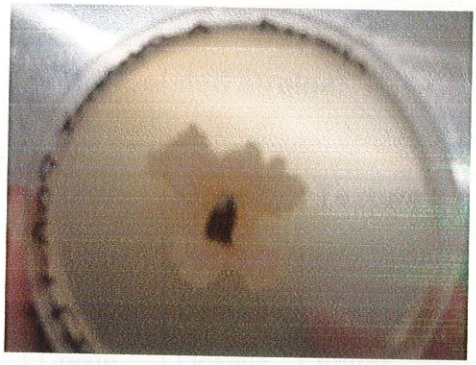


EP4

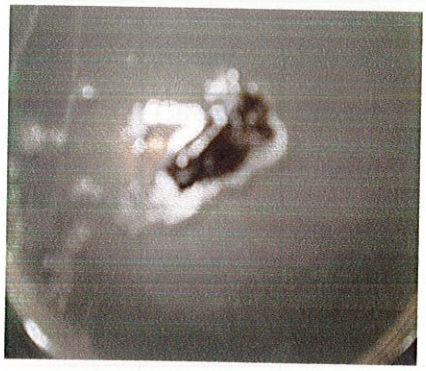
Fig. 4.1.1 Isolated Endophytes EP1, EP2, EP3 and EP4



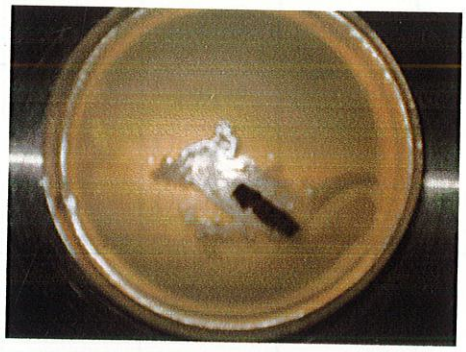
EP5



EP6



EP10



EP11

Fig. 4.1.2 Isolated Endophytes EP5, EP6, EP10 and EP11

From these 8 master cultures a total number of 14 strains were isolated, as endophytes grow in a mixed population. This was done using serial dilution and streaking techniques.

Table 4.1.1:Morphological characteristics of the isolated strains:

Code Name	Form	Margins	Elevation	Color	Opacity	Texture
E1	Circular	Smooth	Flat	cream	Opaque	Mucoid
E2	Circular	Circular	Flat	Cream	Opaque	Rough
E3	Circular	Undulated	Flat	Off white	Opaque	Rough
E4	Circular	Irregular	Elevated	cream	Opaque	Mucoid
E5	Circular	Regular	Slightly raised	Off white	Opaque	Rough
E6	Round	Smooth	Elevated	white	Opaque	Slimy
E7	Circular	Smooth	Flat	cream	Opaque	Mucoid
E8	Circular	Smooth	Flat	cream	Opaque	Mucoid
E9	Circular	Smooth	Flat	cream	Opaque	Mucoid
E10	Circular	Smooth	Flat	cream	Opaque	Mucoid
E11	Round	Smooth	Elevated	Cream	Opaque	
E12	Round	regular	Slightly raised	white	Opaque	Mucoid
E13	Circular	Undulate	Flat	Off white	Opaque	Rough
E14	Round	Smooth	Elevated	Cream	Opaque	Mucoid

Table4.1.2: Biochemical Tests:

Code Name	Gram Staining		Catalase test
	Gram +ve/-ve	Rod/ Coccus	+ve / -ve
E1	+ve	Rod	+ve
E2	-ve	Rod	+ve
E3	+ve	Rod	+ve
E4	-ve	Coccus	+ve
E5	+ve	Coccus	+ve
E6	+ve	Coccus	+ve
E7	+ve	Rod	+ve
E8	+ve	Rod	+ve
E9	+ve	Rod	+ve
E10	+ve	Rod	+ve
E11	+ve	Rod	+ve
E12	-ve	Rod	+ve
E13	+ve	Rod	+ve
E14	+ve	Coccus	+ve

4.2 DNA Isolation from bacterial strains:

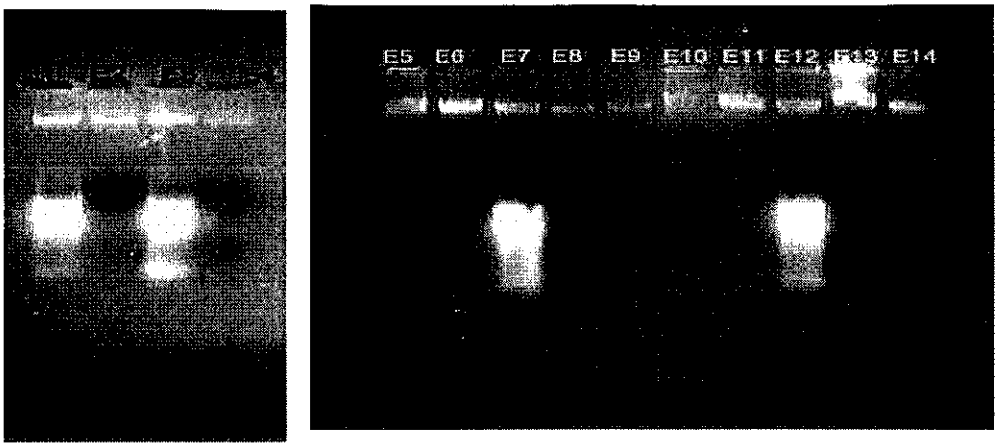


Fig 4.2.1: DNA isolation from isolated 14 endophytic bacterial strains

The isolated DNA 3 µl mixed with 2µl of gel loading dye was loaded on 0.8% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5 µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

4.3 PCR amplifications by using 16S rRNA primers

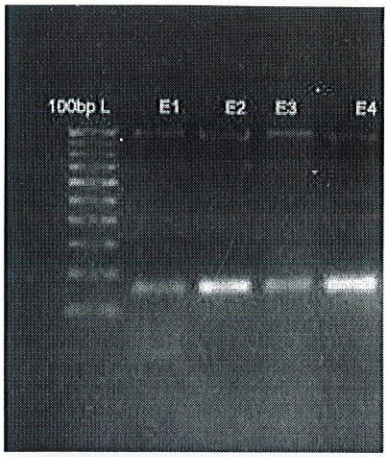


Fig 4.3.1 PCR amplification of four strains E1, E2, E3 and E4.

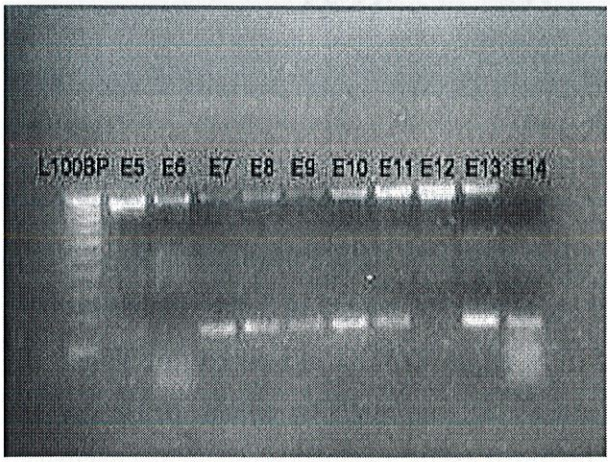


Fig4.3.2 PCR amplification of E5 to E14

The PCR product 8 µl mixed with 2µl of gel loading dye was loaded on 1.2% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

4.4 Plasmid check:

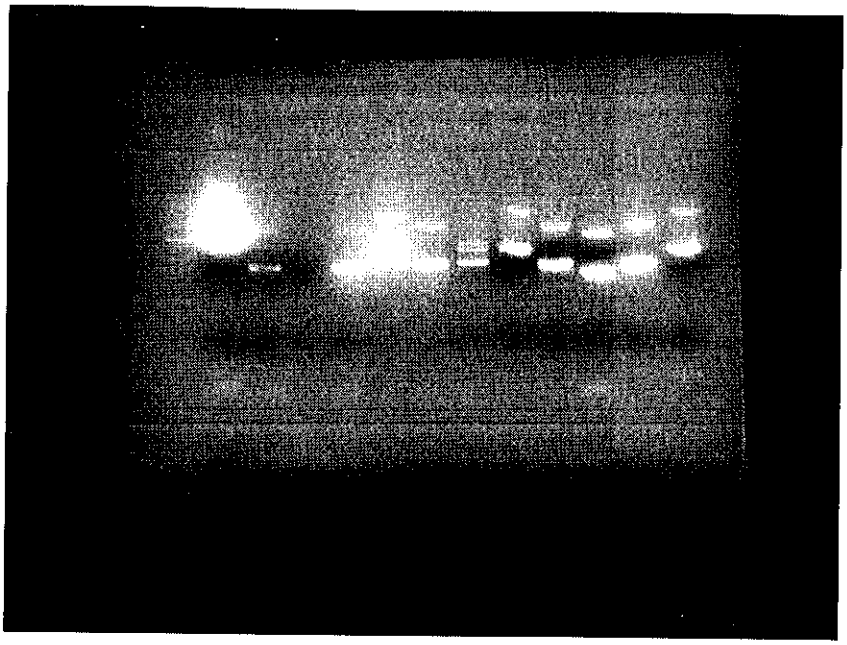


Fig 4.4.1 Checking for isolated plasmid

The isolated plasmid 3 µl mixed with 2µl of gel loading dye was loaded on 0.8% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

4.5 Checking of Insert:

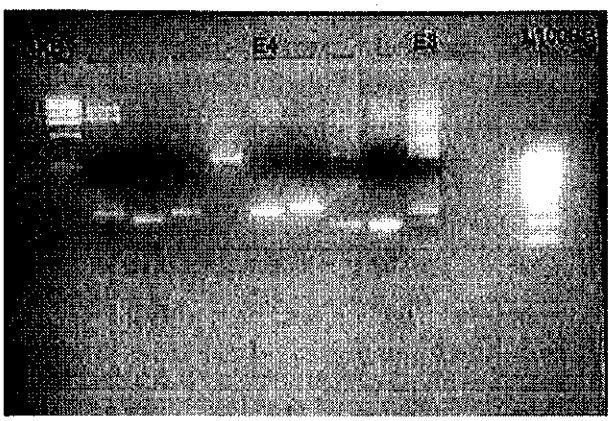


Fig 4.5.1 Insert check with universal T7 & SP6 primers

The amplified product 8 µl mixed with 2µl of gel loading dye was loaded on 1.2% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

Table4.6: Sequencing Results:

Culture Name	Sequence Length	Blast Score	Query Coverage (%)	Max. Identity (%)	Organism	Accession Number
E1	534	932	99	98	Paenibacillus sp.	GU385867.1
E2	512	805	98	98	Filibacter sp.	GQ477174.1
E3	976	1268	100	98	Bacillus subtilis strain	DQ631809.1
E5	832	846	100	99	Acinetobacter sp.	GU977189.1
E6	572	849	100	99	Serratia marcescens strain	GU826157.1
E7	589	1082	99	100	Paenibacillus sp.	FJ487574.1
E8	533	872	90	100	Paenibacillus sp.	EU558287.1
E9	803	1353	92	100	Paenibacillus sp.	GU201854.1
E10	543	973	99	100	Paenibacillus sp.	EU558282.1
E11	881	1510	98	98	Paenibacillus sp.	EU558287.1
E12	983	1782	100	99	Pseudomonas sp.	GU459175.1
E13	714	1281	99	99	Bacillus subtilis strain	HM030757.1

Chapter V

CONCLUSION

Picrorhiza kurroa is a well known herb which has abundant medicinal properties. It is found in Himalayan regions at an elevation of 3,000-5,000 meters. The plant is self-regenerating but unregulated over-harvesting has caused it to be threatened to near extinction.

In the present study we have isolated endophytic bacteria from the healthy rhizomes of *P.kurroa.* The rhizome was chosen because the medicinally active compounds namely Picroside I and Picroside II are mainly present in the roots and rhizomes. The endophytes grow in a mixed population and pure cultures were obtained using streak plate technique and serial dilution method. By this we had 14 bacterial strains as endophytes from *P. kurroa.* The isolated strains were then characterized using 16s rRNA sequence analysis. The sequence thus obtained after sequencing were then used to BLAST with the nucleotide database of NCBI and thus we were able to identify the strains.

The endophytes may protect the host plant against pathogens or produce chemicals which combat the pathogens in the plants or produce chemicals which can help in treating various diseases in humans or animals or can show hepatoprotective, anticholestatic, antioxidant, and immune-modulating activity like as in case of P.kurroa or production of plant hormones which help in growth of P.kurroa during stress condition.

Appendix

0.1% $HgCl_2$

0.1 g of HgCl2 in 100 ml distilled water.

1M Tris

30.28 g of Tris in 250 ml of dH2 O. Adjust the final pH to 8.

0.5M EDTA

46.53 g of EDTA in 250 ml of dH2 O. Adjust the final pH to 8.

TE Buffer

1M Tris – 1 ml

0.5M EDTA – 0.2 ml

Adjust the final volume to 100 ml.

3M Sodium Acetate

40.82 g in 100 ml of dH2 O. pH= 6.8

All the above chemicals are autoclaved before using.

10% SDS

0.1 g of SDS in 1 ml of autoclaved distilled water. SDS is freshly prepared.

50X TAE

242 g Tris Base

57.1 ml glacial acetic acid

100 ml of 0.5M EDTA

Adjust final volume to 1000 ml with dH2 O.

Ethidium bromide (0.5 mg/ml)

0.5 mg of ethidium bromide in 1 ml of autoclaved ddH2O.

6X Gel loading dye

Contents	Quantity
Bromophenol Blue	0.25%(w/v)
Xylene Cyanol	0.25%(w/v)
Glycerol in dH2O	30%(v/v)

0.1 M CaCl2

147.02 g in 100 ml of dH_2O. It is filter sterilized in the laminar air hood.

X-Gal

20 mg in 1 ml N,N dimethyl formamide. It is filter sterilized in the laminar air hood before using and then stored at -20°C.

List of chemicals

DdH_2O (Double Distilled Water)

Ethlenediaminetetraacetic Acid (Merck)

Chloroform (Qualigens)

Hydrochloric Acid (Qualigens)

Isopropyl Alcohol (Merck)

Taq Buffer (SIGMA)

Taq DNA polymerase (SIGMA)

Deoxynucleotide set (SIGMA)

Agarose (Banglore Genel)

Xylene cyanol FF (SIGMA)

Bromophenol Blue (3', 3", 5', 5"Tetrabromophenolsulfonepthalein) (SIGMA)

Ficoll (SIGMA)

List of instruments

Micro litre Centrifuge Z233 MK-2 (Hermle Labor Technik)

Microwave MS-283mc (LG)

Vortex (Banglore GeneI)

Serological Water bath NSW-125

Laminar Flow Bench (Complab)

Autoclave (NSW, India)

Thermal cycler (Applied Biosystem)

Micropipette (Eppendorf and Axygen)

Microtips (Axygen)

Glassware (Borosil)

Gel Doc (Biorad)

Centrifuge (elteck)

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