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**Research Article**

# Isolation, Screening and Molecular Characterization of Chromium Reducing Cr (VI) Pseudomonas Species

**R.Jayalakshmi*and C.S.V. Rama Chandra Rao**

Department of Biotechnology, DVR &Dr.HS MIC College of Technology,

Kanchikacherla, Andhra Pradesh, India.

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**Abstract***:* Microorganisms are known for their ability to metabolize a variety of chemical compounds, including aliphatic and aromatic hydrocarbons, fatty acids and other environmental pollutants. These capabilities make them useful for application as bioremediation agents. Heavy metals found in wastewaters are harmful to the environment and their effects on biological system are very severe. An efficient and economic treatment for their removal and reuse needs to be developed. Microbial metal bioremediation is an efficient strategy due to its low cost, high efficiency and ecofriendly nature. Recently advances have been made in understanding metal-microbe interaction and their application for metal detoxification. Microorganisms in soil are sensitive to the high concentrations of heavy metals like zinc, manganese, cobalt, copper, chromium, cadmium, mercury and silver. Chromium is a toxic heavy metal, which primarily exists in two inorganic forms, Cr (VI) and Cr (III). Chromate [Cr (VI)] is a serious environmental pollutant. The current study aimed to isolate the bacteria which have the potential capacity to reduce the chromium levels. Chromium contaminated soil was collected and analyzed. From the collected soil samples the bacterial isolates were screened and characterized by using the 16s rRNA based PCR amplification. Biochemical tests were performed for the microorganisms isolated from the effluent. As per the present study the isolated *Pseudomonas sp* was found to reduce 99 % of chromium from the medium in 21 hours.

**Keywords**: Tannery effluents, Hexavalent chromium, Environmental pollution, Diphenycarbazide, 16S rRNA sequence, *Pseudomonas sp*, Bioremediation.

## INTRODUCTION

Chromium is a strong oxidant, Crystalline; steel-gray, lustrous, hard metallic, is an odorless. Found in rocks, animals, plants, and soil. India is the second rank in the world production of chromate generally as an essential micronutrient. Chromium maintaining efficient glucose, lipid, carbohydrate and protein metabolism. Chromium released by a large number of industrial operations such as electroplating, chromate manufacturing, leather tanning industry, dyes and pigment fabrication and wood preservation. Chromium is toxic to the reproductive system and the unborn child. Hexavalent chromium is a widespread industrial waste. Environmentally friendly processes need to be developed to clean-up and protect the environment[2]. Bioremediation can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition. Bioremediation may be employed to attack specific soil contaminants, such as degradation of chlorinated hydrocarbons by bacteria. Several Gram-positive and Gram-negative bacteria species are capable to reduce hexavalent chromium[3]. *Arthrobacter, Escherichia, Bacillus.ES29,B. subtili ,B.pumilus,, P.flourescens, pseudoalcaligenesCECT5344 , P. aeruginosa, Burkhoderia copaiba ,P.aeruginosaPA01*[4]*.P. putida, P. aeruginosaHP104 Streptomyces,, AeromonasEnterobac, E. clocaestrain HO1, Shewanella putrefaciensMR-1, P. fluorescensLB300, Agrobacterium radiobacterEPS-916.*[5]Aquatic plant species, algae, yeast, are also used for chromium reduction these methods have found limited application[6&7]. Traditional methods such as chemical oxidation, filtration, evaporation recovery, membrane technologies, chemical precipitation, freeze separation, reverse osmosis, ion exchange and electrolysis.[8&9]are the used to reduce hexavalent chromium, but huge quantity of reagents, high cost, generation of toxic sludge , incomplete metal removal and creating harmful waste by-products[10].*Pseudomonas putida* has been shown to degrade many heavy metals like chromium, Zinc, copper etc.

## MATERIALS AND METHODS

**Collection of soil sample, Isolation and purification:** Soil Sample was collected from TIE (Tirupur Industrial Estate). It was collected from the surface to a depth of 10 cm, sealed in sterilized polythene bags and stored at 4°C until use. It was subjected to serial dilution[11]and $10^{-5}$ and $10^{-6}$ dilutions were plated on nutrient agar medium (peptone 5g/l, NaCl 5g/l, yeast extract 1.5g/l, beef extract 1.5g/l, agar20g/l, 25ºC, pH at ((25ºC) 7.4±0.2) by spread plate method .cultured and maintained as pure cultures on minimal salt agar medium supplemented with glucose- 5g/l, ammonium phosphate monobasic-1g/l, sodium chloride- 5g/l, magnesium sulphate-0.2g/l, potassium phosphate dibasic- 1g/l for further studies .

**Diphenylcarbazide assay:** Hexavalent chromium in the medium was determined by diphenylcarbazide method[12]. 1000 mg/L $K_2CrO_4$ stock solution was prepared. 0.25gm of diphenylcarbazide reagent dissolved in 9.67ml of acetone along with 330µl of 3M $H_2SO_4$ for the preparation of DPC reagent.100mM of3-Morpholinopropane sulphonic acid (MOPS dissolved in 50ml of distilled water and pH was adjusted to 7. by adding 10 pellets of NAOH such that concentration of MOPS buffer was 100mM which is used as stock and absorbance measured spectromatrically at 540 nm. The absorbance readings of the standard solutions were used to construct a calibration curve. For the test samples the culture supernatant is used and Cr is determined.

## MOLECULAR CHARACTERIZATION

**Isolation of Genomic DNA:** For PCR amplification of 16s rRNA PCR was performed. Genomic DNA was isolated by using[13] method with some modifications 1.5ml of overnight bacterial culture was taken into eppendorf tubes under sterile conditions and centrifuged at 10000rpm for 3 minutes. Supernatant was discarded and 200µl of TE buffer was added to the pellet and was dissolved completely.300ul of ST buffer was added to the tubes and incubated at 65°c for 10 minutes. Tube was inverted for every 2 minutes while

heating for proper mixing. Cooled to room temperature and centrifuged at 10000rpm for 3 minutes .Supernatant was taken and 150 µl of 0.3M sodium acetate was added. These tubes were centrifuged at 10000rpm for 2 minutes. Supernatant was taken and 600 µl of isopropanl was added and incubated in -20 $^{o}$c for 20 minutes and centrifuged at 10000rpm for about 3 minutes. Discard the supernatant and wash the pellet with 300 µl of 70% ethanol. These tubes were centrifuged for 2 minutes at 10000rpm.The supernatant was discarded and pellet was air dried, placing the tube reversely on the tissue paper and 25 µl of TE buffer was added to pellet. Then the sample was subjected to1% agarose gel electrophoresis. The bands were observed on the UV-Transilluminator.

**PCR amplification:** The Genomic DNA was isolated from the overnight grown bacterial culture was amplified with universal bacterial primers. A 25µl of reaction mixture contains, 15µl of master mix (10x assay buffer, dNTP's, Taq polymerase and $MgCl_2$), 1µl of forward primer (5'GGCGAACGGGTGAGTAA3'), 1µl of reverse primer (5'ACTGCTGCCTCCCGTAG3'), 2µl of template DNA and 6µl of distilled water. PCR was carried out by the.Thermal cycling was carried out using a corbett research Australia thermo cycler programmed for the following conditions- An initialization step at 94ºC for 4min followed by 30 cycles of 94ºC for 1min, 52ºC for 1min, 72ºC for 1.15min followed by final extension at 72ºC for 5min and holding temperature at 10ºC for 5 min The PCR products were recovered by 2% agarose gel electrophoresis and then sequenced. The unknown bacterium was identified using GenBank database. The sequences obtained were initially estimated by the BLAST facility of NCBI[14].

**16S rRNA sequencing:** The amplified product of 1330 bp was sequenced by using the ABI 3600 system; Sequencing was carried out at MWG Eurofins India Pvt Ltd, Bangalore .The identification of ribosomal RNA as a premier molecule for evaluating evolutionary relationships and the application of molecular techniques to microbial systematics has revolutionized the concept of phylogenetic relationships among bacteria[15&16]. Genomic DNA was isolated and PCR amplified with universal bacterial primers (MWG Eurofins, Banglore) were used to amplify 16S rRNA gene fragment[17]16S rRNA sequence data and type strains available in the public databases GenBank using the BLAST sequence match routines. The sequences were aligned using the CLUSTAL X program and analyzed with the MEGA software 2001.Evolutionary distances were derived from sequence-pair dissimilarities, calculated as implemented in MEGA, using Kimura's DNA substitution model[18].The phylogenetic reconstruction was done using the neighbor joining (NJ) algorithm, with bootstrap values calculated from 1000 replicate runs, using the routines included in the MEGA software[19].For PCR amplification, Universal bacterial 16S rRNA gene primers forward primers ($5^1$-GGCGAACGGGTGAGTAA-$3^1$_) reverse primers (5-ACTGCTGCCTCCCGTAG- 3_) were used to amplify 16S rRNA gene fragment. Sequence data was aligned and analyzed for finding the closest homology for the microbes

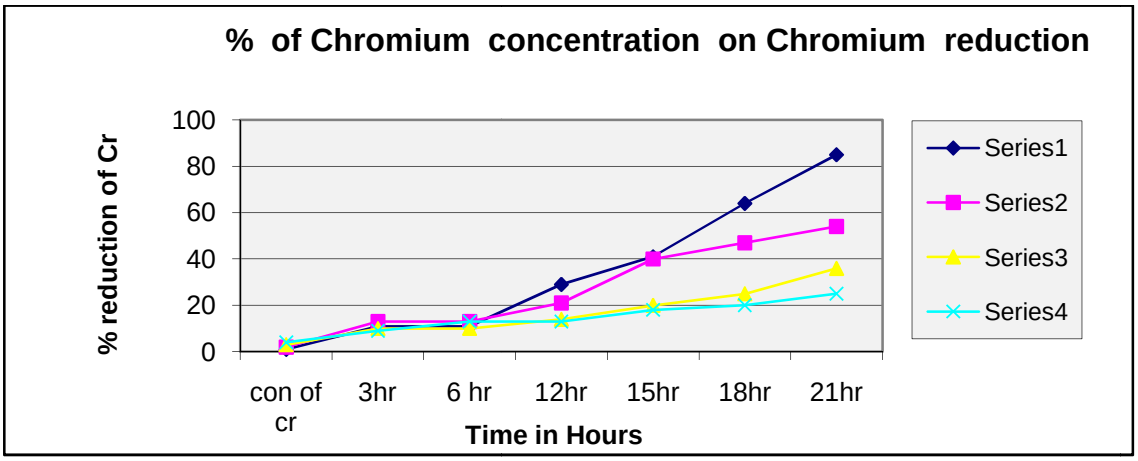
## RESULTS

**Isolation and purification:** Four colonies were obtained on the nutrient agar medium plate which were further purified and single colonies, were isolated and selected based on morphological characters and their biochemical test were performed. The colonies were white and pale yellow in colour which showed irregular shape. Some colonies were also in opaque.

**Chromium reduction:** Hexavalent Chromium in the culture supernatant was measured using diphenylcarbazide (DPC) method as explained above.This Strain **s**howing high Cr (VI) reduction abilities was isolated from Tirupur Industrial Estate Maximum reduction was observed at (194mg/lit) 1mM concentration in 21 hours, 99 % was observed. The optimum condition for hexavalent chromium reduction was reported at temperature $20^0$C, pH 7.0 and substrate fructose as 5% concentrations.

**Table.1- Time and chromium reduction in terms of percentage. Gradual chromium reduction was observed in 1mM concentration**

| Time | 0hr | 3hr | 6 hr | 12 hr | 15 hr | 18 hr | 21hr |
|---|---|---|---|---|---|---|---|
| Blank | | 0% | 0% | 0% | 0% | 0% | 0% |
| 1mM Chromium | | 11% | 11% | 29% | 41% | 64% | 85% |
| 2mM Chromium | | 13% | 13% | 21% | 40% | 47% | 54% |
| 3mM Chromium | | 10% | 10% | 14% | 20% | 25% | 36% |
| 4mM Chromium | | 9% | 13% | 13% | 18% | 20% | 25% |

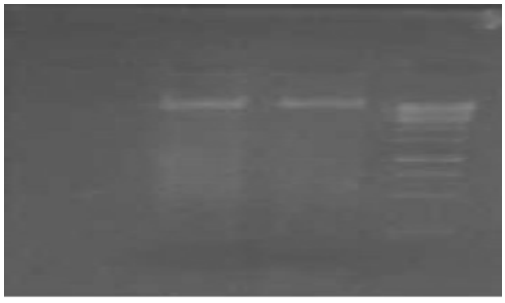



**Graph -1 Percentage of different chromium concentrations profile. X-axis represents time in hours and Y-axis represents chromium degradation in terms of percentage. Series -1 represents the maximum degradation.**

## MOLECULAR CHARACTERIZATION

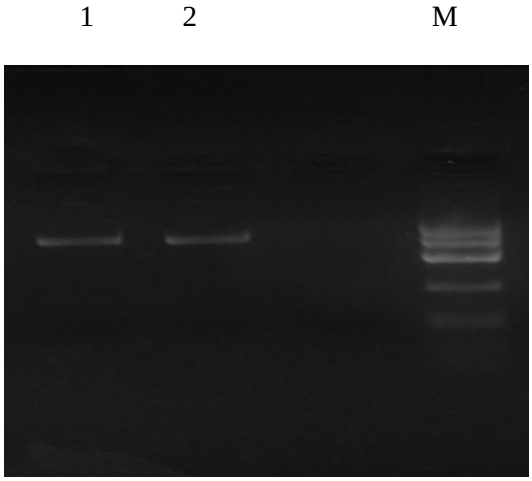
**Genomic DNA isolation:** The highest yield of DNA to be obtained was preserved with TE buffer, yields of 25 µl total DNA from the 1.5ml broth. The extracted DNA was electrophoresed on 1% Agarose gel with ethidium bromide. The bands were observed under UV- transilluminator.

1 2 M



**(Picture-1).Agarose gel electrophoresis of Genomic DNA**
Lane 1 and 2= Bacterial genomic DNA, M =.Molecular Marker.

**PCR amplification:** The DNA extraction provided a good yield of DNA, which could be used in 16S gene amplification with bacterial universal primers (Picture-2) satisfactory amplification products were obtained after adding large quantities of Taq DNA polymerase. The amplified sample and 100bp DNA Ladder was electrophoresed on 2% agarose gel and compared. The bands were observed under UV-transilluminator.




**(Picture-2)–. Agarose gel electrophoresis of PCR products.**

Lanes**:** M = Molecular Marker, 1 & 2 = PCR amplified isolated genomic DNA with universal primer product of desired gene.

**16S rRNA based identification :**> TIE2 16srRNA (1330bp)

The amplified product of 1330 bp was sequenced by using the ABI 3600 system; Sequencing was carried out at MWG Eurofins India Pvt Ltd, Bangalore.

GGTGGGAGCTACCTGCAGTCGACGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGA
GTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTC
GCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT
GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAA
ACACGGTCCAAACTCCTACGGGAGGCAGCAGTGGGGAAATTTGCCCATGGGGCGCAAGCCGGATGCAGCC
ATGCGGGGGGGATACATAGGGCCTACGGTTGTAAACCTTCTTTACCGGGAAAGAACTCGGTTAGAGACAT
AAAATTCTTCCGAATGACCTTAACCCGCAAAAAATATACCGCGTTAATTCCATTCCTACCTTCCTCGAAT
ATATTGATGGTGCAAGCTTTAATCACGACTTACAGCGTCGTACACCTCACGACAGGCGGTTTGATCACTC
AGATGTAAAATTCCCCTCGCATCAATCCTGGTAACTTGCATTCGAAAACTGGCCTAGGCTAGCACTCTTG
GTAGAAGGCGGTAAGAAATTCTTCAGCAGAAGCACTGTAAATGCCTAACACATCTTGGACCGAATACCGC
TATGGCGTAATGGCGGCCCTCCTGGACAAACCACTGATCGACTCAAGTAGTCGATAACCCTGGCGCAGCA
CAAAAGGATTTAGAAAACCCTCGGTTAGTCCACCCCTGTAAAACGATAGTCCAAATAGAGGTATGACTGC
CCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA
CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC
CTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATC
CTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGAT
GACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGC
GACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCA

**PHYLOGENETIC TREE**

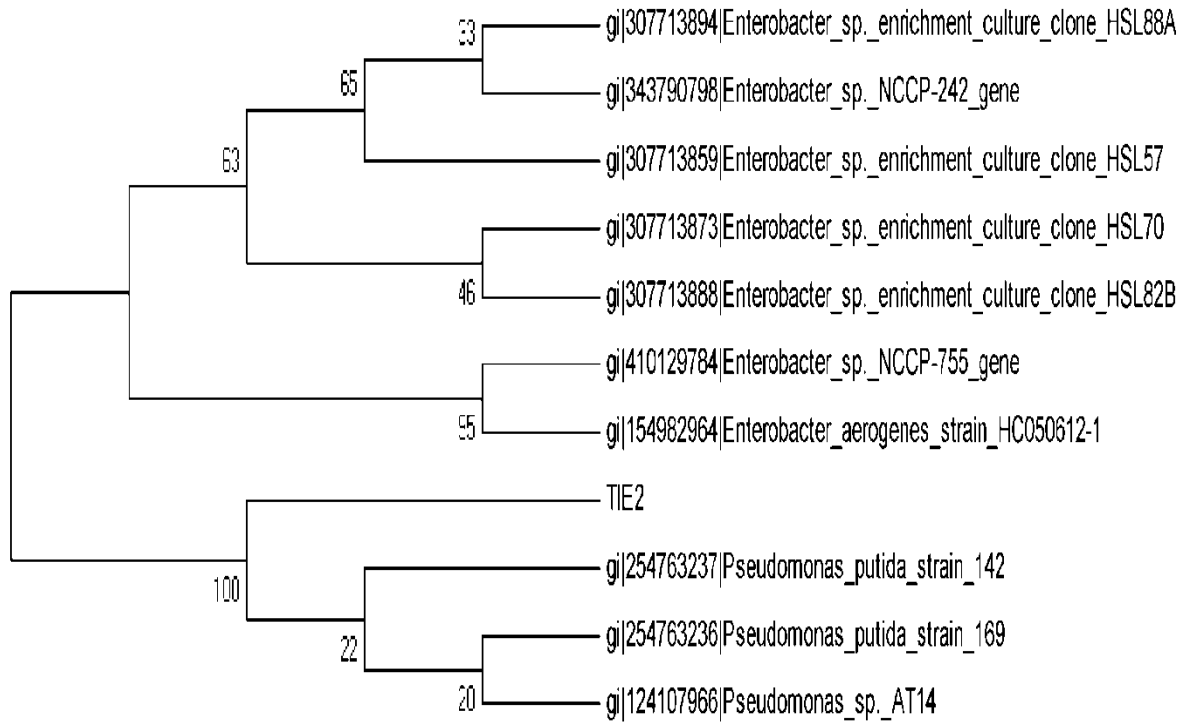



**Fig. 1: Phylogenetic tree of *Pseudomonas sp* strain from TIE2. The tree was constructed using the neighbor-joining method.**

The 16S rRNA results suggested that the culture belongs to genus *Pseudomonas* of *Pseudomonadaceae* family having the nearest neighbor as *Pseudomonas sp*according to phylogenetic tree (Figure1) *pseudomonas putida* strain(Acc.No.142) with 98.2% similarity, *Pseudomonas putida* strain(Acc.No.169) with 98.3% similarity. And *Pseudomonas sp* strain (Acc.No.AT14) with 98.4% similarity. The phylogenetic tree (Figure 1) was done by neighbor joining tool, which shows the relation between the isolates and their respective neighbor type strains along with their respective distances.16S rRNA nucleotide sequences from NCBI genbank were compared with known sequences *Pseudomonas sp* strain with 98.4% similarity. (Acc.No. TIE 2KC17812).

## DISCUSSION

A total of 4 Chromium resistant bacteria were isolated from Tirupur Industrial Estates (TIE Park) effluent in the present study. Four selected isolates according to their morphological shape were plated in media amended with 194 mg/l Cr (VI) (1Mm). Out of 4 isolates (TIE 1, TIE 2, TIE 3 and TIE 4), the maximum degradation ability was observed in TIE2 strain and was identified as *Pseudomonas sp.* Showed excellent bioremediation ability the degradation efficiency of the four respective isolates follows the sequence: TIE2>TIE1>TIE3>TIE4. The TIE2 isolate was selected for further experiments that isolate TIE2 showed different abilities to degrade Cr (VI) in the medium, which was directly related to varying Cr (VI) concentrations. Genomic DNA was isolated from the pure culture. Using universal primers totally~700 bp of the 16S rDNA of TIE2 strain was amplified using high-fidelity PCR Polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and primer. Sequence data was aligned and analyzed for finding the closest homolog for the microbe. Based on nucleotides homology and phylogenetic analysis the strain-TIE2 was detected to be *Pseudomonas sp*. nearest homolog species was found to be *Pseudomonas sp* strains. The 16S rDNA were partially sequenced following PCR amplification and

compared with sequences deposited in databases. The phylogenetic tree (Fig. 1) showed that isolate TIE2 is closely related to the genus *Pseudomonas* exhibiting similarity values greater than 90%. Cr (VI) reduction kinetics and bio-mass growth at varying Chromium concentrations. The optimum time required for maximum Cr (VI) removal was estimated within 18-21 hours at an initial Cr (VI) concentration of 194mg/l (1mM). The maximum Cr (VI) removal and biomass growth by *Pseudomonas sp* was recorded during 21 hours of incubation (table 1.) The percentage of Cr (VI) reduction initially increased and extended up to 21 hours and after that there was only marginal increase. The rate of Cr (VI) reduction was observed in two phases, an initial phase of faster degradation followed by the phase of slower degradation. The initial faster uptake might be due to the availability of abundant Cr (VI) species and empty metal binding sites of the microbes. The slower phase may be attributed to saturation of metal binding sites *Arthrobacter sp.* and Bacillus sp. reduced 30µg/ml of Cr (VI) during 46 h incubation[20]**.** While *Microbacterium sp.* MP30 completely reduced 20 µgml−1 Cr (VI) within 72 h[21].Strain B. casei thus seems to be more efficient than all of these reported strains. However, rates of Cr (VI) reduction decreased over time with all Cr (VI) concentrations. It was probably due to Cr (VI) toxicity towards biological activity.

## CONCLUSION

The present investigation has examined the ability of indigenous organisms from the Cr (VI) contaminated soil. It identifies an organism that has high degrading ability Cr (VI) and has significant potential to degrade the toxic Hexavalent chromium. The organism was molecular characterised and the organism was revealed as *Pseudomonas sp*. The optimum condition for maximum degradation was yet to study. The optimum condition moreover, the effect of various operating conditions such as initial pH of the medium, Temperature, initial concentration of Cr (VI) on Cr (VI) degradation helps to estimate the hydraulic resilience time of the species in the reactor to completely detoxify the environment.

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**Corresponding authors: R.Jayalakshmi; and C.S.V. Rama Chandra Rao**

Department of Biotechnology, DVR & Dr.HS MIC College of Technology,

Kanchikacherla, Andhra Pradesh, India.