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Cloning and expression of organophosphate pesticide, chloropyrifos degrading *opd* gene of *Kocuria* sp.

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ABSTRACT

An organophosphate (OP) pesticide is the most popular type of pesticide family, which effectively eliminates pests owing to its acute neurotoxicity. Organophosphorus hydrolase (*oph*) is a bacterial enzyme that is capable of degrading a wide range of neurotoxic OP pesticides. The present studies aimed at cloning the *opd* gene encoding Organophosphorus hydrolase of *Kocuria* species into the suitable vector *opd* pMAL-c2X and study its expression. The transformed colonies were screened by blue-white screening. The enzyme organophosphorus hydrolase was precipitated at 70% saturation with ammonium sulphate. The SDS-PAGE analysis showed that *oph* was 35 kDa which was expressed in *Escherichia coli* DH5α.

KEY WORDS: Organophosphate pesticides, Organophosphorus hydrolase, *opd* pMAL-c2X

INTRODUCTION

Organophosphate pesticides (OP) are a group of highly toxic agricultural chemicals widely used in plant protection. Their usage has become an indispensable tool in agriculture for the control of weeds, insects and rodent pests. They are poisonous but play an important role in generating plenty of food to the world population (Kurzel and Certrulo, 1981; Akhtar and Ahmed, 2002). Compounds of this family are spontaneously hydrolyzed and cause neurotoxicity in mammals (Sogorb and Vilanova, 2002). Excessive pesticide usage resulted in accumulation of pesticide residues in crops, soils, and biosphere creating an ecological stress (Qiao *et al.*, 2003). Chlorpyrifos is a broad spectrum systemic phosphorothioate ester insecticide patented and introduced by Dow Chemical Company in United States of America in 1965 (Murray *et al.*, 2001). Chlorpyrifos is available in granules, wettable powder, dustable powder, emulsifiable concentrate (Swathi and Singh, 2002) and used for the control of a wide range of pests such as cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, aphids, lice, leptinotarsa and other insects. It is applied to different crops including cotton, nuts, corn, fruits, vegetables, ornamental plants and is highly persistent in foliar application. Chlorpyrifos causes hazardous effects to the environment and also toxic to human beings resulting in headache, nausea, muscle twitching, convulsions, birth defects and even death. It is toxic to a variety of beneficial arthropods including bees, beetles and parasitic wasps. It kills fishes and birds in minute concentrations. Plants are affected by delayed seedling emergence, fruit deformities and abnormal cell division (Thomas and Nicholson, 1989; Richards and Baker, 1993; Giesy *et al.*, 1999; Ragnarsdottir, 2000; Wood and Stark, 2002; Galloway and Handy, 2003). It has antimicrobial property, hence prevents the proliferation of chlorpyrifos degrading microorganisms in soil (Shelton and Doherty, 1997). OP Pesticides in soil and water can be degraded by biotic and abiotic pathways, however biodegradation by microorganisms is the primary mechanism of pesticide breakdown and detoxification in many soils. Thus microbes may have a major effect on the persistence of most pesticides in soil (Surekha *et al.,* 2008). *opd* gene in microbes encodes OPH that hydrolyse OP. The two substrates of this enzyme are aryl dialkyl phosphate and water, whereas its two products are dialkyl phosphate and aryl alcohol (Bosmann, 1972). In light of its importance in agriculture and a need to degrade it in the environment, the present study has been taken up to Clone the *opd* gene of *Kocuria* species into the suitable vector and study its expression levels.

MATERIALS AND METHODS

Isolation of *Kocuria* species for chlorpyrifos degradation

Soil samples were collected from agricultural fields where commercial crops like tobacco and cotton were extensively grown in the West Godavari District of Andhra Pradesh, India lies in the Latitude 20.0 North and Longitude 77.0 East and chlorpyrifos (United Chemicals, Gujarat, India) pesticide was used intensively, by contemplating such soil would contain pesticide contamination and natural micro-flora experiencing pesticide stress. The samples were pooled together and collected into a sterile polythene bag to avoid external contamination. The polythene bag containing soil sample was brought to the laboratory and stored at $4^{o}C$ to maintain the biological activity of the soil microbes. 100 grams of collected soil sample was taken in a conical flask and enriched by adding 1ml of chlorpyrifos. 5 ml of water is added to maintain the moisture and incubated at $37^{o}C$ with pH 7 on a temperature regulated shaking incubator (Abdelnasser and Ahmed, 2007). The enriched soil sample was subjected to serial dilution technique and the samples were inoculated on enriched nutrient agar plates for obtaining pure cultures.

Identification of *Kocuria* species

The pure cultures of bacteria used in the present investigation were sub cultured on enriched nutrient agar medium plates by cross streak method and stored at $4^{o}C$. The purity of the culture was ascertained by microscopic observation, biochemical analysis and molecular identification.

DNA isolation and amplification of *opd* gene of *Kocuria* sp. isolate encoding organophosphorus hydrolase

1. ***Primers used for Polymerase Chain Reaction***

The following two upstream and downstream primers were used for amplification of the *opd* gene. The primers were synthesized by checking the *opd* gene sequence available in the database corresponding to *Kocuria* species in a Primer3 database.

Primers	Sequence
opd-F	5^{I}- GATCGTGGATCCTCGATCGGCACAGGCGATCGG -3^{I}
opd-R	5^{I}- GATCGTAAGCTTTCATGACGCCCGCAAGGTCGG -3^{I}

The template was genomic DNA from *Kocuria* sp. isolate. The *opd* gene of *Kocuria* sp. was amplified in a Master cycler gradient (Eppendorf, Germany). In Polymerase Chain Reaction, the specific primers *Forward* and *Reverse* (Institute of Biological Sciences, Vijayawada) were used to amplify the genomic sequence of the open reading frame (ORF) of the gene.

The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 35 cycles of 92°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. The PCR results were then checked in 1% agarose gel, and an expected band (~1 kb) was excised, extracted and digested with restriction enzymes for subcloning.

2. *Agarose gel electrophoresis*

Required amount of agarose (w/v) was weighed and melted in 1X TAE buffer (0.9M Tris-borate, 0.002 M EDTA, pH 8.2). Then, 1-2 µl ethidium bromide was added from the stock (10 mg/ml). After cooling, the mixture was poured into a casting tray with an appropriate comb. The comb was removed after solidification and the gel was placed in an electrophoresis chamber containing 1X TAE buffer. The products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) at 5:1 ratios and loaded into the well. Electrophoresis was carried out at 60V (Fritsch *et al.*1989).

3. *Eluting DNA from agarose gel fragments*

Ethidium bromide stained agarose gel was visualized under a transilluminator. The fragment of interest was excised with a clean razor blade. After removing the excess liquid, the agarose fragment was placed in the spin column. The tube was centrifuged at 5500 rpm for not more than 45 seconds for the elution of DNA. The eluent was checked by running on an agarose gel and observed on a transilluminator for the presence of ethidium bromide stained DNA. The eluted DNA was used directly in manipulation reactions. This DNA fraction was subjected for sequencing (Institute of Biological Sciences, Vijayawada).

4. *Cloning of opd gene of Kocuria sp Y2 isolate in E.coli DH5α*

Amplified organophosphate degrading gene (*opd gene*) of *Kocuria* sp was cloned into a vector pMAL-c2X (New England Biolabs) to generate the recombinant plasmid *opd* pMAL-c2X. The primer sequence was designed in such a way that a restriction digested, cohesive ended desired gene fragment was obtained.

5. *Restriction digestion of vector pMAL-c2X and opd gene*

Template DNA (*opd* gene) and vector pMAL-c2X (MWG, Bengaluru, India) were digested with two restriction enzymes *Bam*HI and *Eco*R1 restriction endonucleases. Restriction digestion was carried out as per the following protocol. 2 µl of template DNA and 2 µl of vector pMAL-c2X were taken in two separate clean microcentrifuge tubes. For each tube *Eco*R1 buffer (3 µl), *Bam*HI buffer (3 µl), two restriction enzymes *Eco*R1 (1 µl) and *Bam*HI (1 µl) were added and mixed well by gently tapping the tube. The final volume was made up to 25 µl with sterile distilled water. The digested template DNA and digested vector pMAL-c2X were spun separately for 30 seconds and incubated over night at 37^{o}C.

6. *Ligation of vector and opd gene*

The digested *opd* gene (2 µl) and digested vector (6µl) were mixed in 1:3 molar ratios.

Ligase buffer (5µl) was added and mixed well. Ligase enzyme T4 DNA (2µl) was added and diluted with distilled water to make the final volume 25 µl. This ligated sample was refrigerated overnight for further investigations.

7. Transformation

Transformation was performed for the introduction of our recombinant DNA into suitable host system i.e., *E. coli* DH5α by preparing competent cells. 10µl of DNA was added to 200µl of competent cells containing tube and gently swirled or tapped with finger for 10 seconds. The tube was kept on ice for 15 min. The tube was transferred to a rack placed in a preheated 42ºC water bath. The tube was stored for exactly 2 minutes. The tube should not be shaken. The tubes were rapidly transferred to an ice bath.

The cells were allowed to chill for 10 minutes. 200µl of LB broth was added and the cells were incubated for 2 hours at 37ºc to allow the bacteria to recover and to express the antibiotic resistance. 100µl of fresh LB broth was added on top of transformed cells, mixed well and spread thoroughly using a spreader and the plates were incubated at 37ºC overnight. Control plates with competent cells that have not been transformed were also plated to rule out contamination of cells. The screening of the recombinants was done by blue-white screening.

8. Screening of the recombinant colonies

The competent cells were grown in the presence of X-gal. If the ligation was successful, the bacterial colony will be white; if not, the colony will be blue. This technique allows for the quick and easy detection of successful ligation, without the need to individually test each colony.

Expression of *opd* gene of transformed *E.coli* DH5α by SDS-PAGE

Purification of organophosphorus hydrolase

Culture of transformed *E.coli* DH5α grown in LB medium for 48 hours was filtered through wattman filter paper number 5 and the filtrate was subjected to precipitation with 75% ammonium sulphate. The enzyme solution was subjected to dialysis and checked the purity of protein by adding a drop of nessler's reagent. After dialysis, the crude extract was subjected to anion exchange chromatography by using a DEAE cellulose column (IBS, Vijayawada). After the column was washed with 3 volumes of 20 mM Tris- HCl buffer (pH 7.8), protein was eluted with 100 ml linear gradient NaCl (0 to 1 M) in the washing buffer at a flow rate of 1 ml/minute. Fractions with *opd* activity were collected and concentrated against PEG 20,000 for further analysis.Then the samples containing equal amount of protein were loaded into the wells of 12% polyacrylamide gels. The medium ranged molecular weight marker mixed with the sample buffer was also loaded in one of the wells. Electrophoresis was carried out at constant voltage of 75 volts. The gels were stained with 0.2 percent coomassie brilliant blue solution overnight and then destained. Relative mobilities of each protein band were recorded.

RESULTS AND DISCUSSION

Isolation of Kocuria species for chlorpyrifos degradation : Pure cultures of bacteria used in the present investigation were sub cultured on enriched nutrient agar medium plates by cross streak method and stored at 4^{o}C. The bacterial colony was yellow in colour and opaque in appearance that showed maximal growth on the media enriched with Chloropyrifos.

Identification of Kocuria species :The colonies are round in shape and slightly embossed. Simple staining and gram staining of the isolate revealed that the bacteria capable to degrade Chloropyrifos was spherical in shape (cocci) and were gram positive. The bacterium was non-motile and there was no capsule which can be inferred that it is not pathogenic. The sequence analysis demonstrated that all the corresponding bands on agarose gel belonged to *Kocuria* species. The amplified 16S rRNA of bacterial isolate was sequenced and the data obtained correspond to 328 bases. Sequences of the dominant DGGE bands revealed that *Kocuria* species in tested soil was *Kocuria* with the accession no. JF816257 (Nagavardhanam and Vishnuvardhan, 2011).

DNA extraction, Purification and Quantification : The DNA pellet was white, thick thread like mass. This DNA obtained was further quantified by Spectrophotometry and agarose gel electrophoresis. It was observed that *Kocuria* sp DNA fragments were observed to emit orange fluoroscence under UV lamp. The A_{260}/A_{280} ratio for *Kocuria* sp DNA was found to be 1.9 spectrophotometrically. The genomic DNA isolated was checked for the quantity and purity of DNA. The concentration of DNA was adjusted to 0.3 µg/mL with sterile distilled water for carrying out the amplification reactions.

Amplification of *opd gene* gene encoding organophosphorus hydrolase: The amplified fragment of DNA when analyzed by agarose gel electrophoresis indicates that it was of good quality (Fig.1). The highlighted codons are with respect to the start and stop codons encoding *oph.* Then the sample was eluted and sequenced, the sequence of the amplified product was as follows:

GATCGTGGATCCTCGATCGGCACAGGCGATCGGATGCAAACGAGAAGGGTTGTGCT

CAAATCTGCGGCCGCGAGAACTCTGCTCGGCGGCCTGGCTGGGTGCGCGACGTGGCT

GGATCGATCGGCACAGGCGATGCGATCAATACGTGCGCGTCCTATCACAATCTCTGA

AGCGGGTTTCACACTGACTCACGAGGACATCTCGGCAGCTCGGCAGGATTCTTGCGT

GCTTGGCCAGAGTTCTTCGGTAGCGCAAAGCTCTAGCGGAAAAGGCTGTGAGAGGA

TTGCGCGCCAGAGCGGCTGGCGTGCGAACGATTGTCGATGTGTCGACTTTCGATATC

GGTCGCGACGTCAGTTTATTGGCCGAGGTTTCGCGGGCTGCCGACGTTCATATCTGG

CGGCGACCGGCTTGTGGTTCGACCCGCCACTTTCGATGCGATTGAGGTATGTAGAGG

AACTCACGCTAGTTCTTCCTGCGGTGAGATTCAATATGGCATCGAAGTACACCGGAA

TTAGGGCGGGCATTATCAAGGTCGCGACCACAGGCAAGGCGACCCCCTTTCAGGAG

TTAGTGTTAAAGGCGGCCGCCCGGGCCTCCTTGGCCACCGGTGTTCCGGTAACCACT

CACACGGCAGCAAGTCAGCGCGATGGTGAGCGAGGCAGGCCGCCATTTTTGAGTCC

GAAGCTTGAGCCCTCACGGGTTTGTATTGGTCACAGCGATGATACTGATGACTTGAG

CTATCTCACCGCCCTGCTGCGCGGATACCTCATCGGTCTAGACCACATCCCGCACAG

TGCGATTGGTCTAGAAGATAATGCGAGTGCATCACCGCTCCTGGGCATCCGTTCGTG

GCAAACACGGGCTCTCTTGATCAAGGCGCTCATCGACCAAGGCTACATGAAACAAA

TCCTCGTTTCGAATGACTGGCTGTTCGGGTTTTCGAGCTATGTCACCAATATCATGGA

CGTGATGGATCGCGTGAACCCCGACGGGATGGCCTTCATTCACTGAGATCGTAAGCT

TTCATGACGCCCGCAAGGTCGG

The amplified product of *opd* gene contained 1044 bases. The sequence on BLAST search revealed that it is having an open reading frame coding for the enzyme organophosphorus hydrolase (*opd*) encoded by 1014 bases.

Cloning of *opd* gene of *Kocuria* sp.

The isolated *opd* gene of *Kocuria* sp was cloned in a vector pMAL-c2X (Fig.2). The *opd* gene of *Kocuria* sp was ligated into a vector pMAL-c2X placing *opd* gene at the downstream of the constructive *tac* promoter and a recombinant vector '*opd* pMAL-c2X' was constructed**.**

This recombinant vector *opd* pMAL-c2X was then transformed into competent *E.coli* DH5α and was grown in the presence of X-gal. Successful ligations of *opd* gene into vector pMAL-c2X and transformed *E. coli* DH5α with *opd* gene were detected by blue-white screening molecular technique. Presence of white bacterial colonies indicated the successful ligation and such positive recombinant colonies were selected for expression assay (Fig.3). The blue-white screen is a molecular technique, for the detection of successful ligation in vector-based gene cloning. If the ligation was successful, the bacterial colony will be white, if not, the colony will be blue. This technique allows for the quick and easy detection of successful ligation. The transformed cells were observed on LB agar plates and the results were recorded.

Expression of *opd* gene of *Kocuria* sp in *E.coli* DH5α

The enzyme organophosphorus hydrolase was precipitated by saturating with 35% and 70% ammonium sulphate and the organophosphorus hydrolase enzyme was obtained in 70% fraction. This enzyme fraction upon subjecting to dialysis, the pure fraction was obtained. This pure fraction was further purified by anion exchange chromatography. The eluent was collected and checked by SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis). A band appeared corresponding to 35 kDa denoting that it was organophosphorus hydrolase (*oph*) expressed in *E.coli* DH5α (Fig.4).

DISCUSSION

Organophosphorus (OP) pesticides are used worldwide to control major insect pests. The organophosphorus pesticides are the inhibitors of acetylcholinesterase (AchE) an important enzyme present in all vertebrates. The potential damage caused by these pesticides to the non target organisms is very high and hence such pesticides are now being banned in developed countries. Despite of the fact that the OP pesticides pose high risk, still they remain the major group of pesticides used in agricultural pest management of the developing countries.

In general, microorganisms demonstrate considerable capacity for the metabolism of many OP pesticides. Moreover, the bacteria are not adversely affected by OP compounds because they do not contain AChE. At the same time, some of the microorganisms utilize OPs as an energy source (Singh and Walker, 2006). The use of microorganisms in the degradation and detoxification of many toxic xenobiotics especially pesticides is an efficient tool for the decontamination of polluted sites in the environment (Manab and Alok, 2012). OP compounds share similar chemical structures and therefore enhanced degradation of one OP compound in soil might also leads to rapid degradation of other OPs, a phenomenon called cross-enhanced degradation (Singh *et al.,* 2005). Phosphotriesterases (PTEs) are a group of enzymes found in microorganisms that can degrade OP compounds. There are three different types of well characterized bacterial PTEs namely organophosphorus hydrolase (*oph*), methyl parathion hydrolase (*mph*) and organophosphorus acid anhydralase (*opaa*).

The most common enzyme responsible for OP pesticides degradation is organophosphorus hydrolase (*oph*) that was encoded by a gene designated as organophosphorus degrading gene (*opd*). The bacterial enzyme *oph* breaks down the phosphoester bonds of OPs through hydrolysis and in turn reduces the toxicity of OPs (Horne *et al.,* 2002). The aforesaid discussion emphasizes the need to isolate and identify many efficient bacteria that degrade OPs in general and chlorpyrifos in particular. The results of the present study gains prominence in that, the bacterial isolate *Kocuria* sp was screened from agricultural soils contaminated with chlorpyrifos.

CONCLUSION

The above work was to clone the *opd* gene encoding organophosphorus hydrolase of *Kocuria* sp into a *opd* pMal-c2X vector, to assess its expression. SDS–PAGE analysis reviewed that the gene has been properly expressed in the form of protein. The work was carried out in a view to study the expression of a protein in a view that this *E.coli* culture containing foreign gene will enable us in overcoming the deleterious effects of OPs in the environment.

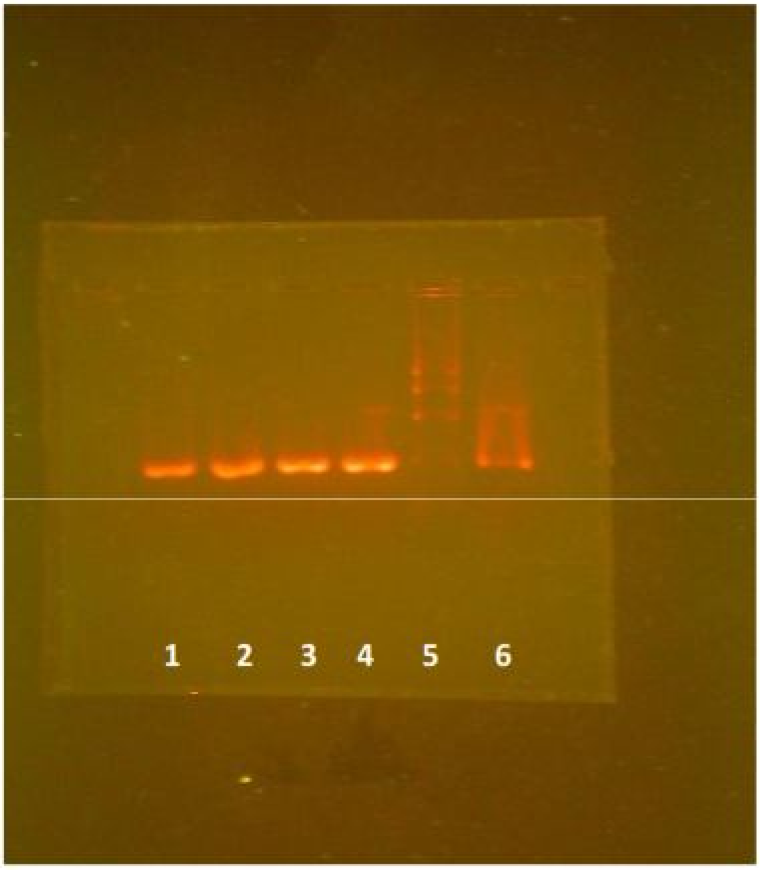

Fig.1: Ampification of *opd* gene of *Kocuria* sp.

1, 2, 3 4 and 6- Amplified DNA samples; 5- 1kb DNA ladder

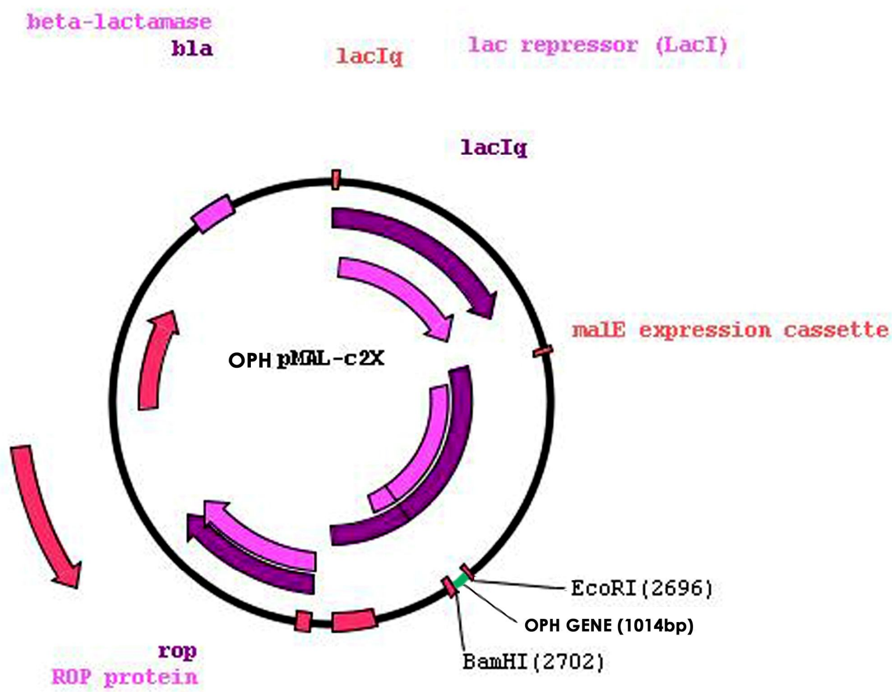

Fig.2: Construction of recombinant vector *opd* pMal-c2X vector

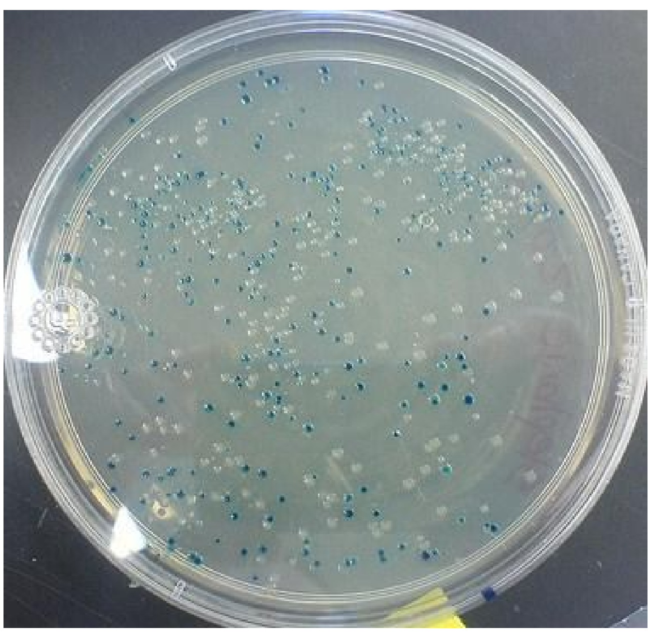

Fig.3: Transformation of *E.coli* DH5α with *opd* pMal-c2X. Blue colonies represent untransformed *E.coli* DH5α; White colonies represent transformed *E.coli* DH5α

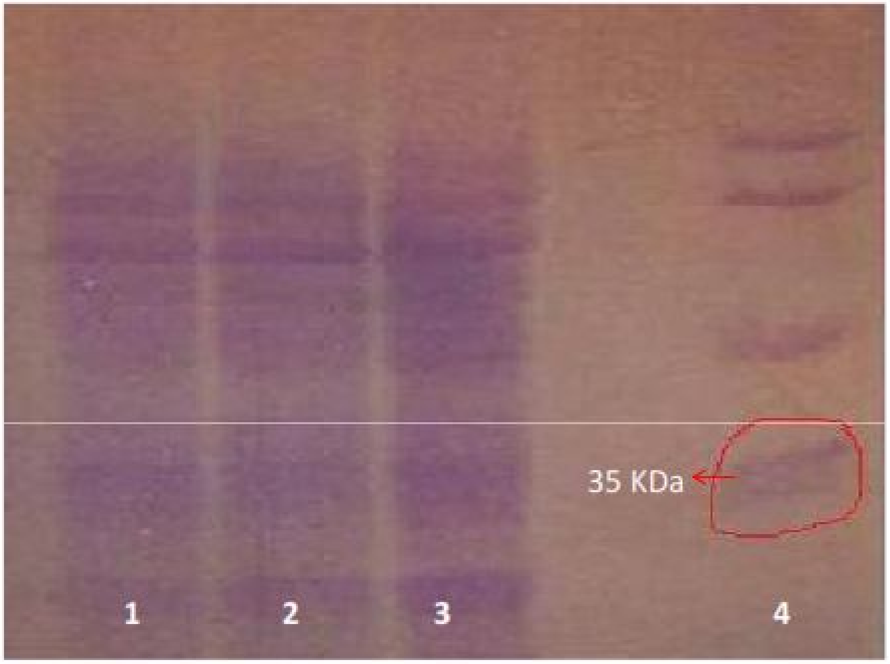

Fig.4: *opd* gene expression of *Kocuria* sp in *E.coli* DH5α.
1,2,3-Marker; 4-Expressed *opd* protein

Abbreviations: OP, Organophosphate pesticides; ***opd***, organophosphate degrading gene; ***opd*-F**, *opd*-Forward; ***opd*-R**, *opd*-Rrverse; **ORF**, open reading frame; **rpm**, revolutions per minute; **µl**, micro litre; **X-gal**, 5-bromo-4-chloro-indolyl-β-D-galactopyranoside; **LB**, luria bertani; **DEAE**, diethylaminoethanol; **NaCl**, sodium chloride; **PEG**, polyethylene glycol; **rRNA**, ribosomal ribo nucleic acid; **UV**,Ultraviolet; **DNA**, deoxyribo nucleic acid; **DGGE**, denatured gradient gel electrophoresis; **SDS-PAGE**, sodium dodecyl sulphate polyacrylamide gel electrophoresis; ***oph***, organophosphorus hydrolase; **PTE**, phosphotriesterases

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