ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation, Characterisation& Strain Improvement of 2, 4-D Degrading Bacteria from Sewage Sample In Bangalore

Gaurav Bhide, Archana L, Shruthi SD*, Chetana SH

PG Dept. of Biotechnology,The Oxford College of Science, Bangalore 560102, Karnataka, India.

ABSTRACT

2,4-Dichlorophenoxyacetic acid (2,4-D) degrading bacteria were isolated from sewage samples collected from selected sites in Bangalore, which have no history of 2,4-D exposure. A herbicide 2,4-D was used in a minimal salt medium as a sole source of carbon to isolate and enumerate the 2,4-D degraders. Isolated bacteria found to be Bacilli, having ability to degrade 1gm of 2,4-D in 32.88 hours after successful strain improvement.

Key words: 2,4-Dichlorophenoxyaceticacid, Bacilli, bioremediation, minimal salt medium, Xenobiotics

Corresponding author
Email:Email: sdshruthi@gmail.com

ISSN: 0975-8585

INTRODUCTION

Xenobiotics have been used extensively during the past 5 decades both in industry and agriculture [1]. This use has led to the accumulation of these compounds in the environment polluting natural resources. The widespread use of pesticides in agriculture to increase crop production has generated a series of toxicological and environment problems [2]. The use of herbicides has increased in last few years to replace mechanical weeding. Chlorinated phenoxy acid derivatives like esters and amines are the most common and the most widely used herbicides. 2,4-D is the one that is used to defoliate jungle in many areas worldwide [3]. This herbicide is extensively used in many crops such as wheat, rice, corn, sorghum and sugar cane [4]. Because it is highly selective and systemic, this herbicide is transported through the plant, being accumulated in the growing roots, inhibiting the growth of weeds [5]. It effectively controls unwanted and invasive weeds across agricultural fields, lawns, public parks, lakes and more [6].

2,4-D is classified by WHO as a hormonal herbicide of level II toxicity [7][8]. It is considered as a carcinogen agent, affecting liver, heart and central nervous system, leading to convulsions [9]. This herbicide is usually commercialized as salt, amine and ester formulations; and has post-emergence action. After its application in field, the excess of the herbicide is easily transferred to the groundwater, due to its high solubility in water (600mg/l at 25ºc) [10]. Even after a long period of disuse, considerable amounts of either 2,4-D or its main product of degradation, 2,4-dichlorophenol (2,4-DCF), might be found in surface waters and groundwater [11][12][13]. Therefore, the development of an efficient degradation process for this herbicide is extremely relevant and necessary. The efficiency of the degradation methodology can be monitored and easily determined by using high-performance liquid chromatography. Since this technique allows the determination of the amount of herbicide and its metabolites present in the samples [1]. Many investigations have suggested bioremediation as an alternative process for the removal of xenobiotic compounds from the soil, due to the smaller environmental impact and greater efficiency [14][15].

2,4-D has frequently been used as a chemical model to investigate the evolution and diversity of catabolic genes involved in the degradation of anthropogenic contaminants in the environment [16]. Although the chemical structure of 2,4-D is relatively complex, it is readily degraded and used as a carbon source by various environmental microorganisms [17]. On the other hand, pollution has induced rapid evolution of specific biodegradative pathways in terrestrial and aquatic bacteria. Several bacterial genera which can degrade and metabolize 2,4-D are *Alcaligenes, Pseudomonas, Acenitobacter, Arthrobacter, Corynebacterium*and *Bacillus* [14][18]. However, abiotic factors such as photooxidation and adsorption of 2,4-D to particulates could also contribute to the degradation of some toxic substances partially but never to mineralization [17]. It was reported that plasmid carried genes in the strains of *Pseudomonas* and *Bacillus* showed the degradation of 2,4-D. Furthermore, plasmids often carry genes responsible for degradation of other structurally related chlorinated organic compounds [14][15].

ISSN: 0975-8585

The major aim of this work was to explore the metabolic versatility of microorganisms, and identify pure microbial cultures highly specialized in the degradation of the largely employed herbicide 2,4-D.Large amounts of man-made chlorinated organic chemicals have been used in agriculture as herbicides and pesticides. Among these 2,4-D has received widespread use as a herbicide for more than 45 years. Unlike many of the synthetic compounds released into environment, 2,4-D is rapidly mineralized by sewage bacteria by enrichment media technique. Organisms that has reported to be capable of degrading 2,4-D belong to bacilli genera [19].

MATERIALS AND METHODS

Media preparation

Minimal salt medium (MSM) with some modifications was used throughout the study. Composition is as shown in table 1. The media was boiled for 10mins and autoclaved at 15 bars and 121^0c without significant loss in 2,4-D. (pH 6.5-7). Nutrient agar medium containing 300ppm 2,4-D was prepared. The media used in characterization of isolated strains of 2,4-D degraders were prepared as described in Collins and Lyne's microbiological methods [20].

Table 1: Media components used for isolation of 2,4-D degrading bacteria

Component	Quantity (gm)
K_2HPO_4	0.5
$(NH_4)_2SO_4$	0.5
$MgCl_2$	0.4
$FeCL_3$	0.1
$CaCL_2$	0.01
$MnSO_4$	0.01
$ZnSO_4$	0.001
2,4-D	0.3
Agar	20

Processing of sewage samples

Sewage samples from the upper 10cm sewage water were taken from the sewage canal in HSR Layout, near The Oxford College of Science, Bangalore and kept in sterile test tubes, at ambient temperature until processing. The tube was kept closed to avoid further contamination. 1ml of sample was taken and aseptically transferred to 10ml of sterile distilled water, which served as 10^{-1} dilution. This was further diluted serially to get 10^{-2}, 10^{-3} and used for further processes.

Isolation of 2,4-D degraders

The medium used for isolation of 2,4-D degrading bacteria was MSM supplemented with 0.008gm/100ml Eosin B and 0.0013gm/100ml of Methylene blue. The colour of media was

blue, 2,4-D was the sole carbon source in the media hence the degradation of 2,4-D produced certain enzymes which turned the colour of 2,4-D degrading bacterial colony to black by reacting with Eosin B and Methylene blue [21][22]. 100µl of the 10^{-3} dilution of sewage sample was spread plated on MSM medium supplemented with Eosin B and Methylene blue; plates were incubated at 30^{o}c for 48h [23]. After incubation black coloured colonies were observed on the MSM plate. The colony was pure cultured and kept in MSM slant at 0.3% of 2,4-D for further use [24].

Characterization of bacterial isolate

The isolated colonies on MSM supplemented with Eosin B and Methylene Blue were further transferred to above mentioned MSM media without any stains [6]. The agar concentration kept as 4% because it was observed that at higher concentration of 2,4-D, 2% agar does not get solidify. The organism isolated was found to be mesophilic as more growth was observed at room temperature which was around 30^{0}c [22]. The isolated strains were characterised using standard procedures described in Collins and Lyne's Microbiological Methods and Bergey's manual of systematic Bacteriology.

Strain Improvement

Strain improvement of isolatedbacillus strain was achieved by 2 methods:

1)Enrichment Technique method: This was showed to be slow method of strain improvement. Initially strain was isolated on 0.03% of 2,4-D concentration. The isolated colonies were inoculated in 50ml MSM broth containing 0.03% of 2,4-D, and incubated for 24h at 30^{0}c on rotary shaker at 90rpm. After incubation 1ml of the media was aseptically transferred to 50ml of MSM broth containing 0.06% of 2,4-D and incubated for 24h at 30^{0}c on shaker. Same procedure was followed to culture 2,4-D degrading bacteria on higher concentrations of 2,4-D as 0.06%, 0.09%, obtaining final concentration of 2,4-D to be 0.1%.

2) Ultraviolet Mutation Method: It was observed that this method is faster and efficient for strain improvement. 0.1ml of the media obtained from above procedure was spread plated on MSM agar medium containing 2,4-D concentration 0.1%, and were incubated for 24h at 30^{0}c. This plate was kept as a master plate. Colonies from this plate were replica plated on 5 plates of MSM agar medium containing 0.15% of 2,4-D concentration. These plates were exposed to UV for different time intervals as 1, 5, 10, 15 and 20mins. After exposure plates were immediately transferred to dark conditions to avoid photo-reactivation, incubated for 48h at 30^{0}c. After incubation period growth was observed in decreasing manner from 1 to 15mins. No growth was observed in plate exposed for 20mins, as it is lethal time of UV exposure for isolated bacteria. The colonies from the plate exposed for 15mins was streaked on MSM agar plate containing 0.2% and incubated for 36h at 30^{0}c. Very few colonies were observed after incubation. This colonies were again replica plated on MSM agar medium containing 0.3% of 2,4-D and exposed to UV for 5mins and incubated in dark conditions. After 48h of incubation very few and small colonies appeared.

ISSN: 0975-8585

Estimation of Degradation of 2,4-D

Estimation of the amount of 2,4-D degraded by bacteria is done by Volhard's method of chlorine estimation [8]. Amount of chlorine in 100ml of MSM+0.1% of 2,4-D was estimated. 0.1ml of 24h old culture containing isolated bacillus was inoculated in above media, culture flask was incubated for 24h at 30^0c, 90rpm shaking condition. After incubation amount of chlorine present in medium was determined again, this found to be increased after incubation. Increased amount of chlorine corresponds to the degradation of 2,4-D, as 1gm of 2,4-D liberates 0.321gm of chlorine upon degradation [25][27]. 10ml of sample was taken from inoculated 2,4-D-MSM medium, 10 drops of concentrated HNO_3 and 2ml of 10% ferric alum was added, 10ml of 0.17N $AgNO_3$ was added drop by drop with constant shaking. White precipitate formed of AgCl sample was kept for 10mins at room temperature, made up to 50ml by adding distilled water and filtered. 20ml of filtrate was taken for further titration with 0.17N Ammonium thiocynate [25]. This procedure was repeated 2 times after each 24h of incubation and the amount of chlorine was determined.

RESULTS AND DISCUSSION

Morphological and physiological characteristics of isolated strain

Bacterial isolate was the outcome of purification of mixed cultures from the sewage sample. Isolate was identified and characterised according to Microbiological methods and Bergey's manual of Systematic Bacteriology [26]. The 2,4-D degrader was identified as bacillus. Characteristics of isolated colonies are as shown in table 2. Results of biochemical tests are denoted in table 3. Biochemical tests which are intended for the diagnosis of gram positive rods, was conducted for identification of the 2,4-D degrader. According to the results obtained from microscopic observations, colony characteristics and biochemical tests it was confirmed that isolated strain of bacteria is from Bacillus Species [6][24][23].

Table 2: Colony characteristics of 2,4-D degrader colony

Character	Appearance
Size	Small (1-2mm)
Margin	Entire
Opacity	Opaque
Elevation	Convex
Colour	Pale White, Creamish
Consistency	Mucoid
Shape	Circular

ISSN: 0975-8585

Table 3: Biochemical properties of 2,4-D degrader organism

Test*	Inference
Gram's staining	Positive rods
Indol	Negative
MR	Positive
VP	Positive
Citrate utilization test	Positive
Starch utilization test	Positive
Gelatin Liquefaction test	Negative
Motility	Motile
Endospore	Endospore forming
Catalase	Positive
Oxidase	Positive

*Reactions obtained after 24h incubation at 30^0c

Strain Improvement

Strain was improved in its resistance capacity towards 2,4-D. The first isolated colonies were sensitive to concentration of 2,4-D more than 0.03%, after strain improvement strain was resistant to 0.3% of 2,4-D. Colonies were streaked on plates containing 0.1% of 2,4-D and agar prepared in double distilled water without any additions, which proved that strain was able to grow on only 2,4-D.

Biodegradation of 2,4-D

On the day 1, chlorine concentration of the medium determined by Volhard's method was 0.045gm/100 ml, after 24h of incubation 0.407gm/100 ml, at 48h it was found to be 0.512 gm/100ml. Hence increase in chlorine concentration after first incubation period was 0.362gm and after second incubation it got increased by 0.105gm. So the average increase in chlorine concentration was found to be 0.2335gm in 24h of incubation. This increased concentration was due to the biodegradation of 2,4-D. Hence the amount of chlorine corresponds to 0.72786gm of 2,4-D degradation in 24h. Further it is estimated that, it requires 32.88h to degrade 1gm of 2,4-D. The characterised 2,4-D degrader showed that it has ability to metabolize and mineralize 1000ppm of 2,4-D in 32.88h when incubated at 30^0c. Toxicity of 2,4-D at higher concentration (3000ppm) was observed, whereas at 3500ppm the colonies of 2,4-D degraders were not detected [28].

Growth of 2,4-D degrader

Sewage was chosen as a source of organism for many reasons such as sewage is a pool of wide variety of microorganism and sewage contains large number of organic as well as inorganic compounds. Both of these conditions support adaptation of organism to environmental conditions. Hence, isolated organism might be exposed to 2,4-D or any other phenoxy acid chemicals. Earlier it was observed that the growth of organism is very slow at

lower concentrations of 2,4-D in MSM media. But interestingly after UV treatment the resistance capacity of organism against 2,4-D as well as mineralizing capacity of organism was increased remarkably. Shaking conditions at 90rpm, pH 6.9 and 30^0c incubation are optimum conditions for maximum degradation with isolated bacillus strain.

Identification of bacterial isolates and degradation

Characterisation studies of the isolate in this work indicated that bacillus species are consistently made up of significant proportion of phenoxy acid herbicide-degrading communities. Bacilli are a vast heterogeneous group of bacteria that occur in substantial numbers in the sewage and soil where they are active agents of mineralization of organic matter. Most species grow well in simple minimal medium with a single organic compound as a carbon and energy source [14]. Bacilluspossess a variety of diverse catabolic pathways that enable them to metabolize an equally diverse number of low molecular weight organic substrates, including chlorinated aliphatic hydrocarbons such as the phenoxyalkanoic acid herbicides [3]. Some species ofbacillusare capable of using 100 different organic compounds for growth [20]. The presence of substantial numbers of bacillusin sewage likely accounts for the reason why these microorganisms are so readily isolated from sewage [29]. Furthermore, experimental conditions of standard enrichment culture techniques and time of sample collection employed here are no doubt optimal for the selection of bacillus and related species. Bacillus showed its ability to entirely degrade 2,4-D at concentration below 2000ppm, but higher concentration (3500ppm) become toxic and inhibit the growth of the bacteria [8][28].

Our research indicates several important points. First, 2,4-D degrading microorganisms were widespread in sewage environment but were difficult to culture. Second, most of the 2,4-D degraders were slow-growing microorganisms and were sensitive to high concentrations of organic nutrients. Third, high concentration of 2,4-D (3500ppm) were very toxic to bacillus after strain improvement. Fourth, the isolation of 2,4-D degrader from sewage might be suggested that selection for chloroagromatic degradation has occurred [19][11]. Further work will focus on the metabolic pathway of 2,4-D degradation and genetic analysis of 2,4-D degrading genes in isolated bacillus strain [30].

CONCLUSION

In this study we succeeded in isolating 2,4-D metabolizing organism Bacillus from Bangalore sewage. The strategy of using progressively less organic supplements and higher concentration of 2,4-D in each of the enrichment steps seems to have worked, although UV mutation method for strain improvement was more successful in increasing the ability of organism to withstand and degrade higher concentration of 2,4-D. Some of the most interesting features of this organism are that it is slow-growing microorganism and sensitive to high concentration of nutrients. Further work related to genetic makeup of organism is in progress.

.

REFERENCES

[1] Harker AR, Oslen RH, Seidler RJ. J Bacteriol 1989; 171: 314-320.

[2] Lewis DL, Hodson RE, Freeman FL. Appl Environ Microbiol 1984; 13: 679-690.
[3] Smith Grenier LL, Adkins LL. Can J Microbiology 1996; 42: 221-226.
[4] Anderson WP. Weed Science Principles and applications. 3rd edition, West Publishing company, New York, 1996, pp. 193-197.
[5] Rodrigues BN, Almeida FS. Guia de herbicidas. 5th edition, Londrina, Grafmarke, 2005, pp. 591.
[6] Miwa N, Kuwatsuka. Soil Sci Plant Nutr 1991; 37: 575-581.
[7] Torang L, Nyholm N, Albrechtsen HJ. Environ SciTechnol 2003; 37: 3095-3103.
[8] Volhard JJ. PraktChem 1874; 9: 217.
[9] Garcia GB, Konjuh C, Duffard RO, Evangelista de Duffard AM. Drug ChemToxicol 2006; 29(4): 435-42.
[10] Maloney EK, Waxman DJ. ToxicolApplPharmacol 1999; 161(2): 209-218.
[11] Evans WC, Smith BSW, Fernely HN, Davis J. Biochem J 1992; 122: 543-566.
[12] AmaranteJunio OP, Brito NM, Santos TCR, Nunes GS, Ribeiro ML. Talanta 2003a; 60: 115-121.
[13] AmaranteJunio OP, Santos TCR, Nunes GS. Quím Nova 2003b; 26: 223-229.
[14] Don RH, Pemberton JM. J Bacteriol1989; 145:681-689.
[15] Greer LE, Shelton DR. Appl Environ Microbiol 1992; 58: 1459-1465.
[16] Pandey G, Jain RK. Appl Environ Microbiol 2002; 68: 5789-5795.
[17] Grotzschel S, Koster J, De Beer D. MicrobEcol 2004; 48: 254-262.
[18] Haugland RA, Shlemm DJ, Lyonsl RP, Sferra RR, Chakarabarty AM. Appl Environ Microbiol 1990; 56: 1357-1362.
[19] Kelly MP, Hallberg KB, Tuovinen O. Environmental microbiology 1989; 55: 2717-2719.
[20] Collins CH, Lyne PM, Grange JM. Collins and Lyne's Microbiological Methods. 6th Edition, Butterworth and Co. Ltd. London, 1989.
[21] Bell GR. Can J Microbiol 1957; 3: 257-262.
[22] Fournier JC. Chemosphere 1980; 9: 169-174.
[23] Shaw LJ, Burns RG. Environ Microbiol 2005; 7: 191-202.
[24] Getenga ZM, Madadi V, Wandiga SO. Bull Environ ContamToxicol 2004; 72: 504-513.
[25] USEPA. The registration eligibility decision (RED) for prevention, pesticides and toxic substances. Washington DC, 2000.
[26] Palleroni NJ.Genus *Pseudomonas.* InBergey's Manual of Systematic Bacteriology, Vol.1. Krieg, N.R., andHolt, J.G.(eds). Baltimore, MD, USA: Williams & Wilkins, 1984, pp.141–199.
[27] Thill D. Growth regulator herbicides. In: Herbicide action course. West Lafayette: Purdue University, 2003, pp. 267-291.
[28] WHO. The WHO recommended classification of pesticides by hazard and guidelines to classification 2004, WHO/PCS/90.1, World Health Organization, Geneva, Switzerland. 2004.
[29] Lee TH, Kurata S, Nakatsu CH, Kamagata Y. MicrobEcol 2005; 49: 151-162.
[30] Kukor JJ, Oslen RH, Siak JS. J Bacteriol 1989; 171: 3385-3390.