Screening of glyphosate herbicide degrading bacteria from soil

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Abstract:
Glyphosate (N-phosphonomethylglycine) is a broad spectrum most widely used systemic herbicide worldwide.The present study was performed for isolation and characterization of bacteria having the efficiency to grow in presence of glyphosate. An enrichment culture technique was used to obtain bacterial strains from soil by using mineral salt medium containing 0.25 G/L to 2.0 G/L concentrations of the glyphosate as sole carbon or phosphorus source. Isolates having ability to grow at maximum concentration were selected and subcultured in mineral salt medium containing 1.25g/L glyphosate as sole carbon and phosphorous source. Total 75 isolates were obtained after enrichment and plating on nutrient agar plate. 45 isolates were found to gram negative rod shaped bacteria.25 isolates were gram positive spore former bacteria and five bacterial isolates gram positive nonspore forming bacteria.Five isolates GL1,GL2,GL3,GL4,GL5 were selected on the basis of growth study. Bacterial strains were identified by using standard microbiological characteristics as *Pseudomonas aeuroginosa* (GL1),*Bacillus spp. , Serratia marscences , Pseudomonas aeuroginosa*(GL4) *Pseudomonas aeuroginosa* (GL5). Microbial degradation of glyphosate determined by spectrophotometric analysis of inorganic phosphate and thin layer chromatography. These bacterial strains possess potential to degrade glyphosate and could be used for bioremediation of glyphosate-contaminated sites**.**

Key words: Herbicides, glyphosate -Excel MERA 71, biodegradation, bioremediation, consortium

Introduction:

Pestcides are chemicals applied to control pests on agricultural crops. Herbicides are pesticides widely used in agriculture for weed control. They specially kill weeds, grasses that compete with commercial crops and its application has become indispensible for agriculture Glyophosate is a systemic nonselective herbicide used worldwide (Franz et al., 1997, Woodburn, 2000). Only a small fraction of the chemicals available to the microorganisms leading to potential impact of residues of herbicides in soil and water on human animal and crop health (Pimentel,1995).The surfactant polyoxyethylene amine (POEA) used in the formulation of glyophosate is more toxic as compared to glyphosate alone (Atikinson,1985). A number of herbicides have been introduced as pre- or post-emergence weed killer (Ayansina and Oso, 2006). These herbicides release unwanted residues in soil, which are ecologically harmful (Haney *et al.*, 2000; Derksen *et al.*, 2002). Herbicides not only affect the target organisms, but also affect microbial communities important in soil activities (Perucci and Scarponi, 1994) and poses a risk to the entire ecological system (Kalia and Gupta, 2004).Microbes have key roles in organic matter transformations, nutrient cycling and degradation of organic pollutants, including pesticides (Beck et al., 2005). Bioremediation is attractive, less expensive and eco friendly method for degradation of chemicals from contaminated site (Alexander, 1999).

Glyophosate is organophosphate herbicide and account for half of the pesticide used world wide (Franz et.al. 1997, Woodburn; 2000). Excel MERA 71, is a widely used glyphosate based formulation. It belongs organophosphorus group of herbicides. It is broad spectrum systemic nonselective and post emergent herbicide used in INDIA for controlling weeds in agriculture, forestry, urban areas,and even in aquatic bodies.

Glyphosate inhibit an enzyme 5-enol pyruyl shikimate-3-phosphate synthase, which is involved in the synthesis of aromatic amino acids-tyrosine, tryptophan and phenylalanine biosysnthesis in plants. It also acts as competitive inhibiter of phosphonel pyruvate which is one of the processor for biosynthesis of aromatic amino acids. Hence it is effective in growing plant. Glyphosate possess tendency of absorption of soil particles and organic matters Thus limits bio avalibility to microorganisms. In soil half life of Glyphosate vary from 3 days to 141 days(Andrea et.al.2003,Annsworth et.al.1993). Biodegradation is considered to be the most important of the transformation processes controlling its persistence in soil (Araujo et al., 2003).

Therefore present study was performed for the isolation and identification, characterization of glyophosate utilizing bacteria in soil. Capability of these of these isolates to degrade glyophosate herbicide confirmed by determination of inorganic acid phosphate and chromatographic analysis.

Materials and methods:

Chemicals and media used:

The isopropylamine salt of glyphosate known as Excel MERA 171 purchased from local supplier of Latur district, (M.S.) INDIA.

For the enrichment of glyphosate degrading bacteria mineral salt medium (MSM) containing glyphosate as sole carbon and phosphorous source was used. The composition of MSM (in g/L) was: (1.5), NaCl (0.5), NH_4SO_4 (2), $MgSO_4$ $7H_2O$ (0.2), $CaCl_2$ (0.01) and $FeSO_4$ $7H_2O$ (0.001). pH (7.0 to 7.2). Trace mineral solution 2 mL was added to MSM. Composition Trace mineral solution (in mg/L), NaCl (0.5), KCl (0.5) NH_4SO_4 (2), $MgSO_4$ $7H_2O$ (0.2) $CaCl_2$ (0.01) and $FeSO_4$ $7H_2O$ (0.001) $Na_2.MoO_4.2H_2O$,(0.020). Flasks of MSM (100mL) after sterilization were incoporaterd with 0.25G, 0.50G, 0.75G, 1.0 G, 1.25G, 1.50G, 2.0 G filter-sterilized (0.2 µm filter) glyphosate .

Collection of soil sample:

Soil samples were collected from two agriculture fields having previous history glyphosate application from Latur district. Soil samples were collected from depth of 0-15 cm in sterile petriplates. On arrival to laboratory stored in refrigerator.

Enrichment of glyphosate utilizing bacteria

Effect of different concentrations of glyphosate on total viable count of bacteria

About 1 g of soil sample added to 100 m L sterile physiological saline, after uniform mixing centrifuged at 3000 rpm for 5 min. 1 m L soil suspension inoculated to each flask containing 0.25G/L 0.50G/L, 0.75G/L 1.0 G/L, 1.25G/L, 1.50G/L, 2.0/L glyphosate as sole source of carbon and phosphorus. Flasks were incubated for 72 hr in shaking incubator at 150 rpm and 30°C.

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After incubation 0.1 m L culture inoculated on nutrient agar plates. Plates were incubated at 30°C for 24 hr. After incubation growth was measured in terms of colony forming units per m L (cfu/ml).

Isolation of glyphosate degrading bacteria

For the isolation of bacteria that were survived highest concentration of MERA 171, from the flask containing 1.25G/L concentration of glyphosate 1.0 ml of culture was transferred to fresh MSM incorporated with 1.25G/L glyphosate. Flasks were incubated for 72 hr in shaking incubator at 150 rpm and 30°C. After incubation 0.1 m L culture was inoculated on the mineral salt agar plate containing 1.25 G/L Excel MERA 171 as a sole source of carbon and phosphorous. Plates were incubated for 48 hr. for growth of glyphosate utilizing bacteria. Macroscopic and microscopic characteristics were studied.

Colonies of glyphosate degrading bacteria were purified by sub culturing on nutrient agar plates .After incubation sub cultured on nutrient agar slant. After growth slants were stored at 4° C for further study.

Identification of glyphosate degrading bacteria

Isolates were subjected to morphological, biochemical studies including Gram staining , motility, endospore staining(Schaffer and Fulton's),capsule staining(Maneval's).Standard biochemical test including carbohydrate fermentation (glucose, lactose, sucrose, mannose), enzyme activities-oxidase , catalase , amylase , gelatinase, Indole formation, methyl red test, VP test, citrate utilization. The isolates were identified according to Bergey's Manual of Systematic Bacteriology.

Growth study of glyphosate utilizing bacteria

Inoculums of glyphosate utilizing bacterial cultures GL1,GL2,GL-3,GL-4,GL-5were prepared by inoculation of pure culture of bacteria in to flask containing nutrient broth. Flasks were incubated at 30°C for 24 hr. After incubation culture were centrifuged at 5000 g for 5 min. Cell pellet was washed twice with sterile distilled water and resuspended in 2m L sterile distilled water cell density adjusted to 0.3 O.D. at 660 nm. 1.0 ml of culture was transferred to fresh MSM incorporated with 1.25G/L glyphosate. Flasks were incubated for 15 days in shaking incubator at 150 rpm and 30°C. Growth was monitored in terms of cfu /m L at the interval of 24 hr. by inoculating 0.1 m L culture on nutrient agar plate and after incubation for 24hr at 30°C.

Determination of glyphosate biodegradation

Spectrophotometric analysis of inorganic phosphate

Culture (3mL) from the above flask were centrifuged at 5000 g for 5 min and supernatants were used for analysis of inorganic phosphate(Fiske andSubbaraw,1925).To the 1.0 m L of the supernatant,2mLTrichloroacetic acid, 1.0 m L ammonium molybdate solution and1,2,4-aminonaptholsulphonic acid was added. The mixture was thoroughly mixed and allowed to react for 5 minutes. The absorbance was read 660 nm using spectrophotometer

(Shimadzu) against blank. Analysis was carried out in triplicate for supernatant obtained from each flask at the interval of 24 hr. Control flasks (un inoculated) were also analyzed.

Thin layer chromatographic analysis

Cultures from the above flasks after incubation for 15 days were centrifuged at 5000 g for 5 min. Supernatant was extracted with acetonitrile and thin layer chromatography was carried out on TLC sheets pre coated with silica gel (Merck).Chromatography was performed using solvent system butanol-acetic acid-water (24:6:10). Spots were developed with ninhydrin reagent. Standard compounds glyphosate, glycine and sarcosine were used as the reference for identification of compounds.

Results and discussion

Enrichment and isolation of glyphosate utilizing bacteria

In order to obtain population of bacteria having ability to grow at higher concentration of glyphosate enrichment was carried out in different flasks containing mineral salt medium incorporated with 0.25G/L to 2.0G/L concentrations Growth was monitored at the interval of 24 hr. in terms of cfu/ mL. Total count was decreased as concentration of glyphosate was increased. Marked decrease in cfu/m L was found at concentration 1.25G/L.

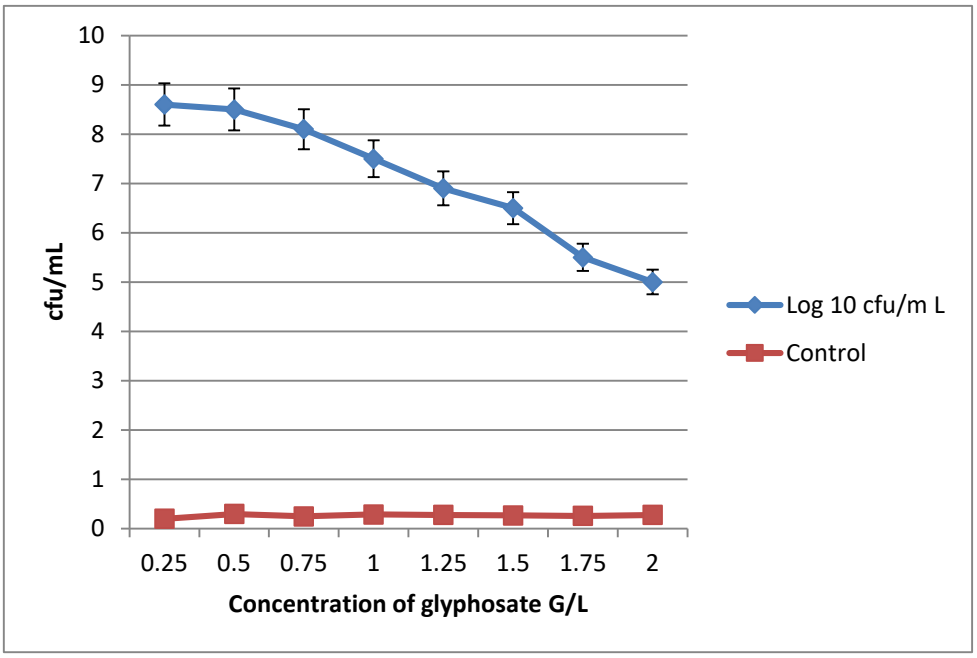

Fig.1: Total viable count of glyphosate utilizing bacteria at different concentrations of glyphosate

Initially total count of glyphosate utilizing bacteria was constant at the 0.25G/L and 0.50 G/L. Total count was decreased with increasing concentration up to 2G/L. Total 75 isolates were obtained after enrichment and plating of culture(1.25G/L) nutrient agar plate. 45 isolates were ound to gram negative rod shaped bacteria.25 isolates were gram positive spore former bacteria and five bacterial isolates gram positive nonspore forming bacteria. Only five isolates were predominated at 2mg/L glyphosate. Isolates were designated as GL-1, GL-2, GL-3,GL-4, GL-5.Glyphosate is an organophosphonate that can be used as a source of P,C or N by either gram positive or gram negative bacteria reported by van Eerd et al.,2003.Glyphosate was found to be lethal to bacteria above 50 mg/ L concentration Glyphosate reduced bacterial viability on solid media and complete inhibition of fungal growth reported by Busse et al.2000.

Identification of glyphosate degrading bacteria

Cultural,morphological and biochemical characters of five glyphosate degrading isolates were studied and isolates GL-1, GL-2, GL-3,GL4,GL-5 were identified on the basis of Bergey's Manual of systematic Bacteriology. Table-1 and table-2

Table-1. Cultural characteristics of glyphosate degrading bacterial isolates

Isolate	Colony size	Shape	Chromogenesis	Surface	Elevation	Edge	Opacity	Consistancy
GL-1	2mm	circular	Yellowish	smooth	convex	entire	Translucent	Butyrous
GL-2	1mm	circular	white	smooth	flat	irragular	opaque	Butyrous
GL-3	2mm	circular	pink	smooth	umbonate	entire	Translucent	Butyrous
GL-4	1mm	circular	yellowish	smooth	raised	entire	Translucent	Butyrous
GL-5	3mm	circular	orange	smooth	convex	entire	Translucent	Butyrous

Table-1. Morphological and biochemical characteristics of glyphosate degrading bacterial isolates

Isolates → / Characteristics	GL1	GL2	GL3	GL4	GL5
Morphology	rod	rod	rod	rod	Rod
Gram staining	-	-	-	-	-
Endospore staining	-	+	-	-	-
Motility	+	+	+	+	+
Oxidase	+	+	-	+	+
Catalase activity	+	+	+	+	+
Indole production	-	-	-	+	-
MR	-	-	-	-	-
VP	-	+	+	-	-
Citrate utilization	+	+	+	+	+
Starch hydrolysis	-	+	-	-	-
Gelatinase	+	+	+	+	+
Sugar fermentation					
1.Glucose	-	+	-	-	-
2.Fructose	-	+	+	+	+
3.Sucrose	+	+	+	+	+
4.Lactose	-	+	+	+	-
5Mannose	+		+	+	+
6.Mannitol	+		+	+	-
Arabinose	+	+	-	ND	ND
H_2S production	-		-	-	-
Lysine decarboxylation	+	+	+	+	-
Tentative identification	*Pseudomonas aeuroginosa*(stra	*Bacillus pumilus*	*Serratia marcesce*	*Pseudomonas aeuroginosa*(stra	*Pseudomonas aeuroginosa*(stra

	in-1)		*ns*	in-2)	in-3)

ND: Means not determined ,+ means positive test,- means negative test

Five bacterial isolates GL1, GL2, GL3, GL4, GL5 were tentatively identified as *Pseudomonas aeuroginosa*(strain-1),*Bacillus pumilus* , *Serratia marcescens, Pseudomonas aeuroginosa*(strain -2), *Pseudomonas aeuroginosa*(strain-3)

Growth study of glyphosate degrading bacteria

Growth study of glyphosate degrading bacteria carried out by using mineral salt medium containing 2G/L glyphosate. Strain GL1, GL2, GL3 showed maximum growth 4.2 Log 10 cfu/ml,3.2 Log 10 cfu/ml,3.7 Log 10 cfu/ml after twelve days of incubation. Strain GL4, GL5 showed 3.9 Log 10 cfu/ml , 4.2 Log 10 cfu/ml after incubation for 10 days. Growth kinetics of glyphosate degrading bacteria was as shown in figure 2a and figure 2b.

Spectrophotometric analysis of inorganic phosphate-

Glyphosate degradation by isolated bacteria was determined by monitoring sectrophotometric analysis of inorganic phosphate using Fiske Subbarow method Concentration of inorganic phosphate determined at the interval of 24 hr in the culture broth containing 2G/L of glyphosate. Results were as per table-3

Chromatographic analysis of glyphosate biodegradation:

Chromatographic analysis of extracted biodegradation products from both *Pseudomonas aeuroginosa* GL4 showed sarcosine Rf 0.54Chromatographic analysis of extracted biodegradation products from culture of *Bacillus pumilus.* showed presence of sarcosine (Rf=0.54) and glycine Rf. 0.25 .Complete degradation of glyphosate observed after 15 days by GL4-*Pseudomonas aeuroginosa* , *Serratia marcescences*- showed presence of glycine Rf-0.25

Discussion :

Glyphosate is an organophosphonate that can be used as a source of P,C or N by either gram positive or gram negative bacteria reported by van Eerd et al.,2003.Application of herbicide to the soil may adversely affect sensitive microorganisms and simultaneously those microorganisms that have ability to degrade herbicide get stimulated.(Wikinson and Lucas,1969;Ayansina and Oso,2006) Glyphosate was found to be lethal to bacteria above 50 mg/ L concentration and glyphosate reduced bacterial viability on solid media and complete inhibition of fungal growth reported by Busse et al.2000.In the present study also marked inhibition observed at higher concentration above 200mg/L concentration. However ,Amoros et al.2007,studied effect of different concentration of glyphosate on Aeromonas count and reported increase in count as compared to the control. M. Baboo et al. (2013), reported that herbicide treatment resulted an initial increase in biomass upto 14th day followed by significant drop in biomass and indicated transient impact herbicide.

Capacity to metabolize of glyphosate have been reported in Several species of Pseudomonas (Moore et al., 1983; Jacob et al., 1988; Quinn et al., 1989). Metabolism of glyphosate by *Bacillus megaterium* strain 2BLW (Quinn et al., 1989) also reported , *B. cerus* (Fang et al.2012).

Three *Pseudomonas aeuroginosa* strains, *Bacillus pumilus* , *Serratia marcescens* were found to be predominated while other population was found to be inhibited at 2G/L. Growth study of all the *Pseudomonas aeuroginosa* strains showed higher growth as compared to *B. pumilus* and *Serratia marcescens.* These observations are consistent with studies of Gimsing et al.(2004) who reported a high correlation between glyphosate mineralization rate and Pseudomonas sp counts in five different Dannish soils.

Microbial degradation of glyphosate produces the major metabolite aminomethylphosphonic acid and ultimately leads to the production of CO_2, phosphate and water (Forlani et al.,1999;Araujo et al.,2003) In the present study concentration of inorganic phosphate determined at the interval of 24 hr in the culture broth containing 2G/L of glyphosate. Concentration of inorganic phosphate in the culture of *Pseudomonas aeuroginosa* found to be increased during 144 hr of incubation it indicate increased transformation of glyphosate. In case of *Bacillus pumilus*, *Serratia marcescens* no significant concentration was detected, it might be utilized for growth.

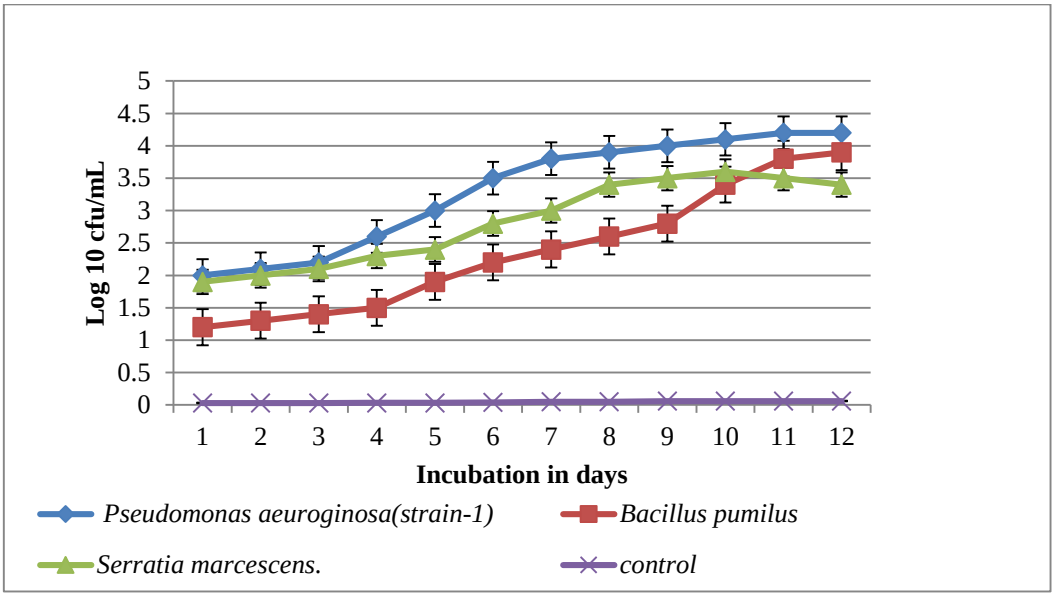

Figure2a. Growth study of glyphosate degrading bacteria in medium containing glyphosate as sole source of carbon and phosphorous

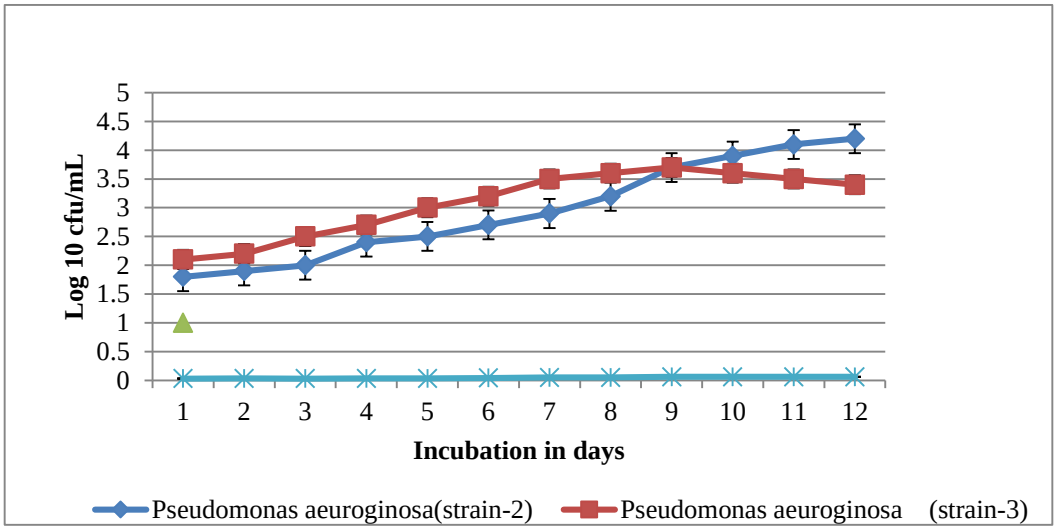

Figure2b. Growth study *Pseudomonas aeuroginosa* (GL1) and *Pseudomonas aeuroginosa*

(GL5) in medium containing glyphosate as sole source of carbon and phosphorous

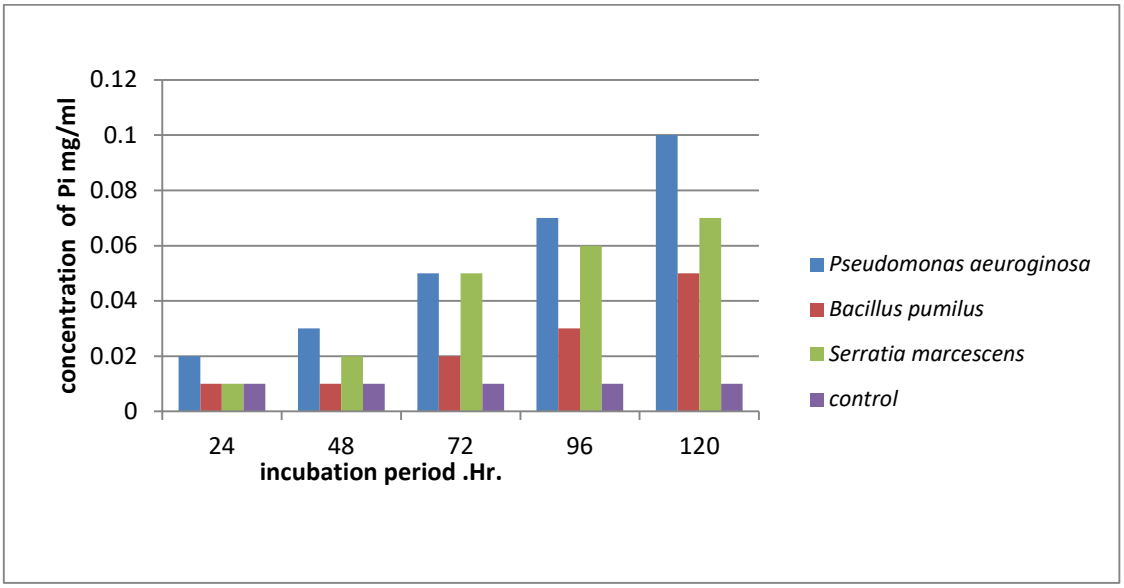

Figure 3: Analysis of inorganic phosphate in broth cultures containing glyphosate

Chromatographic analysis of glyphosate biodegradation:

Chromatographic analysis of extracted biodegradation products from both *Pseudomonas aeuroginosa* showed presence of AMPA. Chromatographic analysis of extracted biodegradation products from culture of *Bacillus pumilus* . showed presence of degradation product sarcosine and glycine , *Serratia marcescens* showed presence of glycine.

Pseudomonas sp. PG2982 degrade glyphosate by initial cleavage of the C-P bond resulting in the production of sarcosine(*N*-methyl-glycine) by C-P lyase activity ((Kishore & Jacob, 1987).

Conclusion

Present study confirmed ability of *Pseudomonas aeuroginosa* strains, *Bacillus pumilus*, *Serratia marcescens* to degrade glyphosate. Chromatographic analysis and analysis of inorganic phosphate confirmed transformation of glyphosate in vitro by these isolates. Consortium of these microorganisms may be employed for detoxification of contaminated sites.

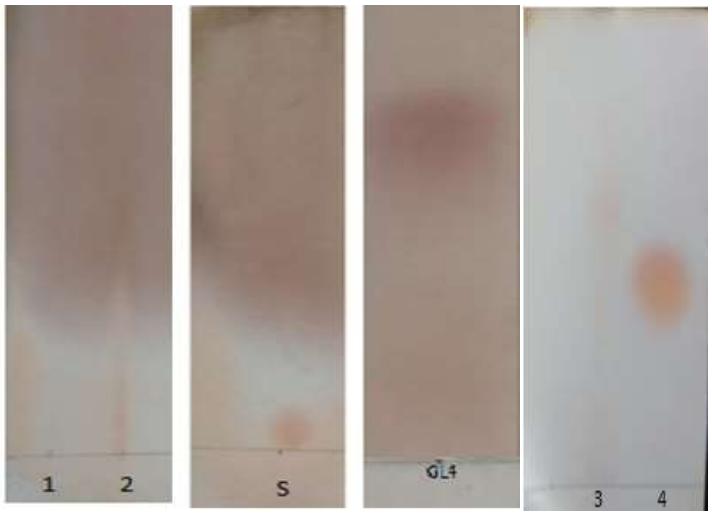

Fig. Thin layer chromatography

1.Complete degradation of glyphosate after 15 days by GL4-*Pseudomonas aeuroginosa* ,
2.*Serratia marcescences*-Presence of glycine, Rf-0.25
3.*Bacillus pumulus*--Presence of glycine ,Rf. 0.25 and sarcosine Rf 0.54

4.glycine Rf. 0.25

S-Glyphosate Rf 0.12

GL4-*Pseudomonas aeuroginosa* -Sarcosine Rf 0.54

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