KOCURIA TURFANESIS STRAIN BS-J A NOVEL MICROBIAL ISOLATE WITH COMBINED POTENTIAL OF BIOSURFACTANT PRODUCTIONAND DEGRADATION OF MONOCROTOPHOS

K. V. Dubey[1*] , G. L. Boosreddy[1], P. N. Charde[1], V. S. Dubey[2], A. A. Juwarkar

[1] Sevadal Mahila Mahavidyalaya, Sakkardara Square, Umrer Road, Nagpur-09.

[2] Hislop College, Civil Lines, Nagpur-440001.

[3] National Environmental Engineering Research Institute, Nagpur

*Author for correspondence: Email- kirtivijay_dubey@yahoo.com

Abstract

A bacterium, having dual capabilities of producing biosurfactant and capable of degrading monocrotophos, was isolated from lube oil and distillery spent wash contaminated soil collected from a distillery unit and was named as BS-J. On the basis of the cellular morphology, physiological and chemotaxonomic characteristics and phylogenetic similarity of 16S rDNA gene sequences, the strain was identified as a *Kocuria turfanesis.* The ability of the biosurfactant producing *Kocuria turfanesis* to mineralize monocrotophos was investigated under different culture conditions viz. in mineral salts medium containing monocrotophos (150 mg/l) as sole carbon source and in soil simulated with monocrotophos and BS-J whole cells in fermented curd whey containing biosurfactant and soil simulated with monocrotophos and biosurfactant containing cell free fermented curd whey. The addition of BS-J whole cells broth with biosurfactant to soil containing monocrotophos (500 mg kg^{-1}) resulted in a higher degradation rate than that obtained in soil containing fermented curd whey with biosurfactant alone without cells. Results indicate that *Kocuria turfanesis* strain BS-J has great potential utility for the bioremediation of wastewater or soil contaminated with monocrotophos.

Key words: biosurfactant, biodegradation, curd whey, monocrotophos, soil.

Introduction

Monocrotophos [dimethyl-(E)-1,2-methylcarbamoylvinylphosphate] is an organophosphorus insecticide. It has been widely used to control a variety of insects on crops, such as cotton, sugar cane, peanuts and tobacco, because of its low cost and effectiveness (Sha, 1999).

Monocrotophos was amongst the top 15 pesticides used in the 20th century. Monocrotophos works systemically and, on contact, it is highly toxic to organisms, including humans (Isoda et al., 2005). Monocrotophos is weakly sorbed by soil particles because of its hydrophilic nature. Leaching of monocrotophos may pollute the surface and/or groundwater, ultimately resulting in adverse effects on biological systems (Subhas & Singh, 2003). In general, pesticide degradation in soil can be influenced by both biotic and abiotic factors, which act in tandem and complement one another in the microenvironment (Singh et al., 2003). Microbial activity has been deemed to be the most influential and significant cause of organophosphorus pesticide removal. Therefore, biodegradation is a reliable and cost-effective technique for pesticide abatement, and a major factor determining the fate of organophosphorus pesticides in the environment (Kertesz et al., 1994; Munnecke & Hsieh, 1974). Monocrotophos is characterized by a P–O–C linkage and amide bond, and has been reported to be degraded as a sole carbon or phosphorus source in liquid media by *Pseudomonas aeruginosa* sp., *Clavibacter michiganense* ssp. (Subhas & Singh, 2003), *Arthrobacter atrocyaneus* sp., *Bacillus megaterium* sp. and *Pseudomonas mendocina* (Bhadbhade et al., 2002a, b, c).

The majority of studies concerning the fate of monocrotophos in soils have focused on tropical soil systems (Racke et al., 1996; Vijay et al., 2006). There have been no reports of the degradation of monocrotophos in tropical soils in presence of microbial surfactants. Hence, a study was undertaken to determine the ability of *Kocuria turfanesis* strain BS-J to produce biosurfactant in curd whey and to utilize the whole fermented curd whey that contains the cells and biosurfactant for degradation of monocrotophos in contaminated soils. In the present work, studies were also carried out on the degradation of monocrotophos under different culture conditions viz. in mineral salts medium containing monocrotophos as sole carbon source and in

monocrotophos amended soil treated with fermented curd whey containing cells of BS-J and biosurfactant produced by the same culture and in that treated with BS-J cells alone and cell free fermented curd whey containing biosurfactant under separated sets of experiments. This study will open new avenues for not only cost- effective remediation of pesticide contaminated soil.

Materials and methods

Screening of biosurfactant producing microorganisms

Biosurfactant producing microorganisms were isolated from soil collected from an area just below the spent wash pumping device of a distillery unit which was contaminated with lube oil and distillery spent wash by using selective enrichment procedure and plating serially diluted enriched culture on sterile nutrient agar followed by incubation at 37°C for isolated colonies (Dubey and Juwarkar, 2001). Isolates so obtained were individually screened for biosurfactant production from processed curd whey waste on the basis of stability of foam, emulsification index, surface tension reduction, and biosurfactant yield as per the methods described by Dubey and Juwarkar, 2001. Among five isolates tested, BS-J was an efficient isolate with potential of having high emulsification index indicating powerful biosurfactant which can be used to solubilize / mobilize pesticide in contaminated ecosystem and produce high yield of biosurfactant in curd whey.

Identification of the efficient biosurfactant producing isolate

Biosurfactant producing microbial isolate designated as BS-J was identified on the basis of morphological, cultural and biochemical methods described by Cappuccino and Sherman, 1999 and the results

were compared with those in Bergey's Manual of Systemic Bacteriology (Holtz et al., 1994).

16S rRNA gene amplification and sequencing of biosurfactant producing isolate BS-J

The 16S rRNA gene amplification and sequencing of biosurfactant producing microbial isolate BS-J was performed by the following procedure:

DNA was extracted by using ZR fungal/bacterial genomic DNA extraction kit (Zymo Research Corporation 17062 Murphy Ave. Irvine, CA 92614, U.S.A.) according to the manufactures instructions and used for PCR amplification of 16S rRNA gene. The 16S rRNA gene was amplified by PCR using universal bacterial primers 6-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 r (5'-TACGGYTACCTTGTTACGACTT-3'). The amplified product was electrophoresed on 1% agarose gel and a band corresponding to 1.5 Kb was cut and was eluted using QIA quick PCR purification Kit (Qiagen, Hilden, Germany). This purified amplicon was then sequenced using Big Dye Terminator cycle sequencing kit and an ABI PRISM model 3130xl analyzer automatic DNA sequencer (Applied Biosystems, USA) with 3 different internal sequencing primers (27f, 535r and 1492r). The sequences obtained were aligned, edited manually and the aligned sequence was used for BLAST search.

Alignment of 16S rRNA sequence and construction of phylogenetic tree of biosurfactant producing isolates

The sequence of 16S rRNA gene was aligned with closely related sequences using CLUSTAL_X Windows interface (Thompson et al., 1997) and edited manually. Neighbour-joining analysis was done with Kimura 2-parameter model (Kimura, 1980), using TREECON; version 1.3b (Van de Peer and De Watcher, 1997). The stability among the groupings of

phylogenetic tree was assessed by taking 1000 replicates. For J-strain *Kytococcus sedentarius* (X87755) was used as an out-group. Strain J formed a clade with *Kocuria turfanesis* Ho-9042T. Based on nucleotide homology and phylogenetic analysis, the biosurfactant producing BS-J was identified as *Kocuria turfanesis* at IMTECH, Chandigarh, India, and MTCC allotted number was 10635.

Enrichment of biosurfactant producing *Kocuria turfanesis* strain BS-J for monocrotophos degradation:

Kocuria turfanesis strain BS-J is a biosurfactant producing microbial culture isolated from lube oil and distillery spent wash contaminated soil collected from a distillery unit and was added to 500 ml Erlenmeyer flasks containing 100 ml of synthetic medium which consisted of (g/l) $NaNO_3$, 2.0; K_2HPO_4, 1.0; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5; KCl, 0.1 and $FeSO_4.7H_2O$, 0.01 amended with 50 ppm (wv^{-1}) Monocrotophos as sole carbon source. The microbial cultures were subjected to selective enrichment with sequential and weekly transfer of strain BS-J to an increasing concentrations of Monocrotophos from 50 - 500ppm.

Inoculum preparation for degradation of monocrotophos in mineral salts medium studies:

Strain BS-J was pre-cultured in baffled Erlenmeyer flasks containing LB medium. Flasks were incubated overnight at 30 ^oC on a rotary shaker at 150 rpm. The contents of the inoculated flasks were centrifuged at 8000 rpm for 10 min and the cell pellet was washed three times with fresh medium and quantified by the dilution plate count technique. For all experiments, 10^6 CFU ml $^{-1}$ was used and samples were incubated at 30° C at 150 rpm unless otherwise stated.

Determination of monocrotophos in cell free fermented mineral salts medium

After periodic intervals of 24 hours the fermented broth was centrifuged at 8000 rpm for 10 min and the supernatant samples were collected and filtered through a 0.45 micron membrane filter paper. Amount of monocrotophos in the filtered samples was estimated by method of Janghel et al. 2006.

Degradation of monocrotophos in soil and its estimation

Experimental setup for monocrotophos degradation in soil:

Garden soil with sand, mixed in 2:1 proportion were filled in experimental polypropylene pots at the rate of 1 Kg in each pot and following treatments were given to the soil to study the role of biosurfactant produced by Strain BS-J in monocrotophos degradation periodically after 5 days of incubation in green house at a temperature of 30 ^{o}C over a period of 30 days.

1. Soil amended with monocrotophos + 10% tap water
2. Soil amended with monocrotophos + 10% curd whey
3. Soil amended with monocrotophos at the rate of 500 ppm + 10% fermented curd whey containing biosurfactant +whole cells of BS-J
4. Soil amended with monocrotophos at the rate of 500ppm + 10% cell free fermented curd whey containing biosurfactant

During incubation distilled water was added to adjust the moisture content to 40% of the maximum water-holding capacity. The samples were incubated at 30 ^{o}C in the dark. Subsamples were extracted periodically extracted at time interval of 5 days over a period of 30 days, twice with equal amount of ethyl acetate (1:1). The solvent was evaporated and the residue was re-dissolved in 3 ml of ethyl acetate. Amount of monocrotophos was estimated at 560 nm using UV-Vis spectrophometer by method of Janghel et al. 2006..

Results and Discussion

Collection of curd whey wastes for biosurfactant production and their physico-chemical characteristics

Industrial waste viz. curd whey is a viable alternative source for biosurfactant production and it has been already reported (Dubey and Juwarkar, 2001). However, in the present study was carried out with an aim to replace mineral salts medium with curd whey so that no-cost medium for biosurfactant production by strain BS-J can be assessed for its use in remediation of pesticide contaminated soil. Results presented in **Table 1** show that curd whey i.e. lactic acid whey had high COD of 56000 mg/l , sugar and nitrogen levels of 6.8 g/l and 987.0 mg/l respectively indicating that it had sufficient organic load required for the growth of biosurfactant producing microbial isolate. Curd whey is generated during the preparation of Chakka which is an intermediate product obtained by draining of curd (a type of fermented milk from lactic acid bacteria) for preparation of “Shrikhand”, a well known popular Indian dessert. During preparation of 1 Kg Chakka, 9 l o lactic acid whey is generated (Bandyopadhyay and Mathur, 1987). Suitability of curd whey as a fermentation medium for biosurfactant production was assessed by studying the growth profile of biosurfactant producing isolate and its biosurfactant production potential. Use of curd whey in the present study has been taken up instead of using sweet whey as it is feasible to transport the curd whey to the biosurfactant production site without using any special cryo-preservation techniques because continued fermentation of curd whey by indigenous lactic acid bacteria of curd will lead to more production of lactic acid which is a preferred substrate for biosurfactant production since the biosurfactant producer used in this study is an efficient utilizer of lactic acid for growth (Holtz et al., 1994).

Isolation and screening of efficient biosurfactant producing microorganism

The use of biosurfactants is an attractive option because of its versatility, biodegradability, ecological safety and environmental acceptance. However, their high production cost limits their use in bioremediation processes. In this context, it is necessary to evaluate the culturing conditions that optimize their production, assess the economic use of new substrates, such as those arising from industrial waste, and to evaluate techniques of isolation and purification to make production more economically feasible.

In order to form a basis for using no-cost wastes as potential alternative fermentative medium for biosurfactant production, present study focused on the use of industrial waste viz. curd whey as nutrient medium for biosurfactant production by new microbial isolate. For isolation and screening of biosurfactant producing microorganisms, the phenomenon of reduction of surface tension of the culture medium and emulsification index was selected as described earlier (Dubey and Juwarkar, 2001). The success of biosurfactant production depends on the development of cheaper processes and the use of low-cost raw materials, which account for 10-30% of the overall cost (Rodrigues et al., 2006; Makkar et al., 2002). A great variety of agro-industrial wastes have been studied as potential substrates for biosurfactant production such as by-product of the sugar cane industry, fruit processing industry, whey wastes represents an alternative medium for the biosurfactant production process as these have no-cost as compared to other known substrate sources, and they possesses valuable nutrients required for the fermentation process (Dubey and Juwarkar, 2001 and 2004, Dubey et al., 2005). Results presented in **Table 2** show that BS-J isolate had biosurfactant production capacity which was evident by production of stable foam formation in

the fermented curd whey waste that lasted up to two hours of standing. The isolate reduced the surface tension of the fermented curd whey waste from an initial range of 56 mN/m to 27 mN/m indicating the production of effective biosurfactant and also showed good emulsification property as the emulsification index E24 of 94% was obtained. Biosurfactant yield produced by isolate was 0.98 g/l. Results have shown that the isolate produced high yields of biomass and biosurfactant in curd whey owing to the presence of very rich source of organic carbon, nitrogen and minerals like calcium, phosphorus, potassium, sodium, copper and iron. It is also a good source of vitamins of B-complex group viz. riboflavin and pantothenic acid that are readily available for the growth of biosurfactant producing isolates (Nickerson, 1974 Reductions in COD and total nitrogen, sugars, and phosphate contents were observed in the whey waste which indicates decrease in pollutional load of the waste during biosurfactant production.

Identification of efficient biosurfactant producing strain:

Based on morphological, biochemical, physiological characteristics, the efficient biosurfactant producing isolate BS-J was identified as *Kocuria turfanesis.* This biosurfactant producing isolate is a new strain as revealed by 16S rRNA sequence pattern and phylogenetic tree analysis performed at IMTECH, Chandigarh, India, and MTCC allotted number to this isolates as 10635 (Dubey *et al.* 2011).

Utilization of monocrotophos and biosurfactant production by isolate BS-J in mineral salts medium

Monocrotophos degradation by isolate BS-J was monitored by for a period of 120 h. After 24 h, 50% of pure monocrotophos had rapidly disappeared, followed by a slower decrease of monocrotophos with longer incubation times. During 72 to 96 h of incubation the surface tension of

the cell free broth dropped from 62 mN/cm to 28 mN/cm indicating the production of biosurfactant which was maximum at 96 h of incubation with an yield of 0.58g/l. The degradation of monocrotophos supported cell growth, indicating that isolate BS-J could utilize monocrotophos as a carbon source. Such an isolate with dual potential of producing biosurfactant along with monocrotophos degradation can be used an advanced approach for the bioremediation of wastewater or soil contaminated with monocrotophos. From the culture enrichment study it was evident that the concentration of monocrotophos affects the growth of BS-J. The optimal concentration of monocrotophos for the growth of BS-J was 200 mg l^{-1}, and a concentration higher than 600 mg l^{-1} was toxic for the normal growth of BS-J isolate.

Degradation of monocrotophos in different treatments of soil with carrier based bioaugmentation package of egg shell coated with biosurfactant and biosurfactant producing microbial isolates BS-J cells

The experimental set up used to study the degradation of monocrotophos in different treatments of soil with carrier based bioaugmentation package of egg shell coated with biosurfactant and biosurfactant producing microbial isolates BS-J cells is presented in **Figure 1.** Physico-chemical characteristics of soil used in the present study is presented in **Table 3** . Results show that soil had pH of 6.9 with bulk density of 1.16 g/cm^3 and water holding capacity of 61.04%. Organic carbon content of soil was 0.56%. The N, P and K levels of soil were 0.52, 0.073 and 0.144, respectively. There was decrease in the pH of the soil from 6.9 to 6.4 after treatment with 500 ppm monocrotophos due to acidic nature of the pesticide. However, after amendment with 10% egg shell coated with curd whey the pH of the monocrotophos treated soil improved to 7.8-7.9 which is due to calcium carbonated nature of the egg shell that was used as a carrier material for coating biosurfactant for facilitated degradation of monocrotophos in

contaminated soil. This improvement in pH of soil is very important to maintain the physiological state of the native microbial population present in the contaminated soil that maintains the biogeochemical cycle in soil. This is owing to the fact that microorganisms are a major component of the ecosystem and play a considerable role in the degradation of insecticides. This study indicates that use of calcite nature of the egg shell can act as soil conditioning agent. It has been proven that addition of biosurfactants, bioemulsifiers, and/or biosurfactant-producing microorganisms can be used in soil biodegradation techniques, soil washing, and water and waste treatment (in *situ* and *ex situ)* (Urumand Pekdemir, 2004; Zhou and Zhu, 2008). Biosurfactants have also been found to be useful for oil spill remediation and for dispersing oil slicks into fine droplets and converting mousse oil into an oil-in-water emulsion (Toledo *et al.,* 2008). In the present study, degradation of monocrotophos in contaminated soil was found to be facilitated after using carrier based bioaugmentation package of egg shell coated with biosurfactant and biosurfactant producing microbial isolate BS-J cells and its biosurfactant resulted 86-89 % degradation of monocrotophos after 7-days of treatment. Without biosurfactant producing microbial cells the degradation of pesticide was comparatively lower indicating the need of live biosurfactant producing microbial cells and their surfactants for efficient degradation of pesticide. In general, most pesticides used in agriculture are moderately hydrophobic compounds, with complex molecular structures that differ from hydrocarbons in their lower hydrophobicity and in the presence of a polar functional group. These compounds are also strongly adsorbed by soil organic matter and desorption is limited (Rodríguez-Cruz *et al.,* 2004). Wattanaphon *et al.* (2008) evaluated the ability of a BS biosurfactant produced by *Burkholderia cenocepacia* BSP3 to enhance pesticide solubilization for further application in environmental remediation. The results showed that the application of the BS

biosurfactant to facilitate pesticide solubilization demonstrated that this biosurfactant at concentrations below and above its CMC could enhance the apparent water solubility of methyl parathion, ethyl parathion and trifluralin. In the present study the chemical composition of soil in terms of total organic carbon, nitrogen, phosphorus and potassium also improved with the addition of the bioaugmentation package which facilitates the biodegradation of pesticide. The efficiency of degradation in the active soil samples (without inoculation) was generally better than that in the sterile soil samples (without inoculation), suggesting that microorganisms in the soil may play a role in the degradation of monocrotophos. The best degradation of monocrotophos was in the soil samples with the addition of the BS-J, indicating that BS-J isolate from lube oil and distillery spent wash contaminated soil collected from a distillery unit can compete and survive with the local microflora in the soils. The result of monocrotophos degradation in soils proved that BS-J could be used successfully for the removal of monocrotophos from contaminated soils. This is the first report on a *Kocuria turfanesis* strain BS-J which, showed the combined capabilities of producing biosurfactant from monocrotophos as the carbon source and also degradation of monocrotophos in soil. Earlier our studies have shown fairly stable surface active properties of biosurfactant produced by *Kocuria turfanesis* strain BS-J in regard to emulsification of pesticides viz. monocrotophos and imidacloprid used routinely for agricultural purposes at extremes of different environmental conditions (Dubey et al 2012).

Conclusion:

The carrier based technology used in the present study for the cleanup of pesticide contaminants in soil, included bioaugmentation package of egg shell coated with biosurfactant, and microbe. This unique formulation stimulates and enhance the bioremediation processes designed to disperse and augment remediation of pesticide in

soil. Carrier based biosurfactant technology for pesticide decontamination is a simple, highly effective, simple to use, cost-effective and completely non-toxic, environmentally friendly, as all its ingredients are organic in origin and completely biodegradable. This bioaugmentation package of egg shell coated with biosurfactant and biosurfactant producing microbial isolate BS-J resulted 86-89 % degradation of monocrotophos after 7-days of treatment. Application of egg shell waste as a carrier of biosurfactants that improves the physico-chemical and microbiological status of disturbed soil thereby improving the overall fertility and rejuvenation of disturbed soil ecosystem.

Table 1: Characteristics of curd whey collected for biosurfactant production

S. No.	Type of waste	Sources of collection of wastes	Parameters					
			pH	COD (mg/l)	BOD (mg/l)	Total sugars (g/l)	Total Nitrogen (mg/l)	Total Phosphorus (mg/l)
1.	Whey waste (WW)	Amruta Dairy, Umred Road, Nagpur, India.	4.3	56,000	28,000	6.8	987.0	352.0

Findings are the mean values of the three replicate readings

Table 2: Variation in biosurfactant production potential and other related parameters of the new microbial isolate BS-J using curd whey as fermentation medium (After 120 hours of incubation)

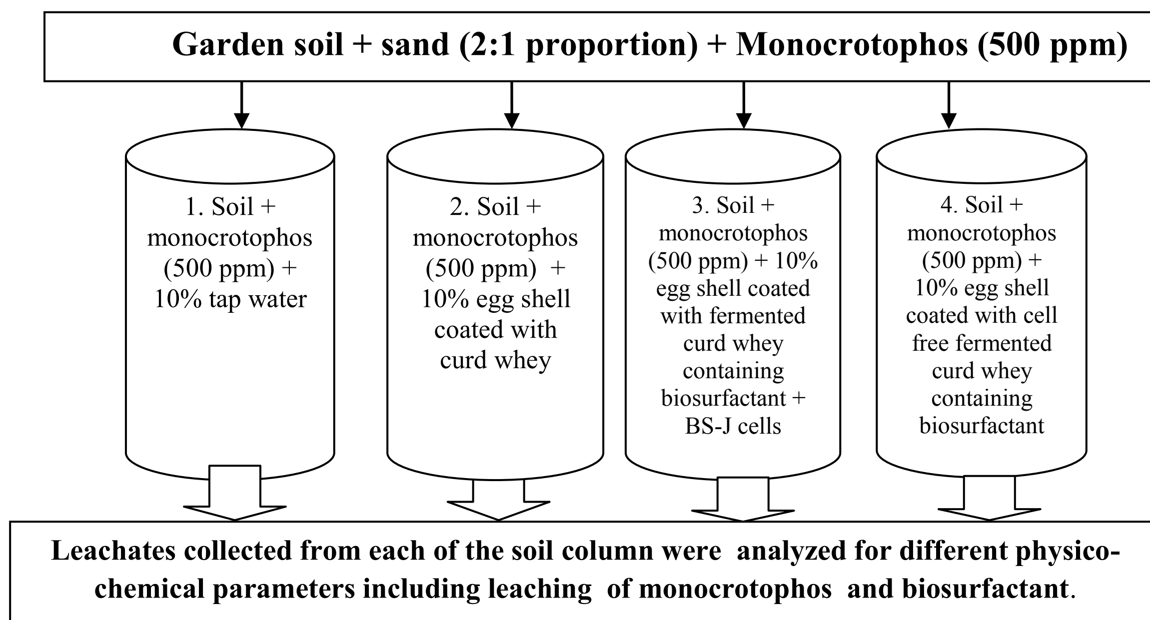
Parameters	Control	Microbial isolate BS-J
Foaming	-ve	+ve
Emulsification index (%)	-	94
Surface tension (mN/m)	56	27
pH	7.0	8.57
Biomass yield (c.f.u./ml)	$12x10^2$	$83x10^8$
COD (mg/L)	37000	19340
Biosurfactant yield (g/l)	0.0011	0.9897

Results are the mean values of the three replicate readings

Table 3. Physico-chemical characteristics of Monocrotophos contaminated soil and degradation of monocrotophos in different treatments of soil with carrier based bioaugmentation package of egg shell coated with biosurfactant and biosurfactant producing microbial isolates BS-J cells

Different treatments of the soil sample	Bulk density (g/cm^3)	Maximum water holding capacity (%)	pH	Organic carbon (%)	Nitrogen (%)	Phosphorus (%)	Potassium (%)	% degradation of monocrotophos
Soil + monocrotophos (500 ppm) + 10% tap water	1.16	60.20	6.6	0.63	0.059	0.083	0.156	57
Soil + monocrotophos (500 ppm) + 10% egg shell coated with curd whey	1.13	60.34	6.5	0.75	0.073	0.089	0.167	61.45
Soil + monocrotophos (500 ppm) + 10% egg shell coated with fermented curd whey containing biosurfactant + BS-J cells	1.12	60.48	7.8	0.79	0.08	0.092	0.180	88.72
Soil + monocrotophos (500 ppm) + 10% egg shell coated with cell free fermented curd whey containing biosurfactant produced by BS-J	1.14	60.45	7.9	0.77	0.07	0.090	0.178	85.71

Figure 1: Flow sheet of an experimental setup for monocrotophos degradation in soil in presence of biosurfactants adsorbed on egg shell as carrier material



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