DEGRADATION OF IMIDACLOPRID IN CONTAMINATED SOIL USING A NOVEL FORMULATION OF BIOSURFACTANT PRODUCED BY KOCURIA TURFANESIS STRAIN BS-J

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A B S T R A C T

Dual capabilities of producing biosurfactant and degrading imidacloprid was identified in a bacterium isolated from soil contaminated with lube oil and distillery spent wash from a distillery unit. Bacterium was identified as a Kocuria turfanesis on the basis of the cellular morphology, physiological and chemotaxonomic characteristics and phylogenetic similarity of 16S rDNA gene sequences and was designated as BS-J. The ability of the biosurfactant producing Kocuria turfanesis to mineralize imidacloprid was investigated through an experimental set up using different treatments of soil with carrier based bioaugmentation package of egg shell coated with biosurfactant and BS-J cells. Results have shown that degradation of imidacloprid in contaminated soil was facilitated after using carrier based bioaugmentation package of egg shell coated with biosurfactant producing microbial isolate BS-J cells and its biosurfactant which resulted 85-91 % degradation of imidacloprid 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. Results indicate that Kocuria turfanesis strain BS-J has great potential in bioremediation of soil
Keywords: Biosurfactant, Imidacloprid, Degradation, Kocuria turfanesis strain BS-J, Bio -formulation, Egg shell

Introduction

Imidacloprid [1-{(6-chloro-3-pyridyl) methyl}-4, 5-dihydroimidazol-2-yl)] nitramide) is a systemic, chloro-nicotinyl insecticide most commonly used on rice, cereal, maize, potatoes, vegetables, sugar beets, fruit, cotton, hops and turf, and is especially systemic when used as a seed or soil treatment. The chemical works by interfering with the transmission of stimuli in the insect nervous system. Specifically, it causes a blockage in a type of neuronal pathway that is more abundant in insects than in warm-blooded animals (making the chemical selectively more toxic to insects than warm-blooded animals). This blockage leads to the accumulation of acetylcholine, an important neurotransmitter, resulting in the insect's paralysis, and eventually death. It is effective on contact and via stomach action.

Imidacloprid based insecticide formulations are available as dust able powder, granular, seed dressing (flow able slurry concentrate), soluble concentrate, suspension concentrate, and wettable powder. Typical application rates range from 0.05 - 0.125 pounds/acre. These application rates are considerably lower than older, traditionally used insecticides. It can be phytotoxic if it is not used according to manufacturer's specifications, and has been shown to be compatible with fungicides when used as a seed treatment to control insect pests.

The half-life of imidacloprid in soil is 48-190 days, depending on the amount of ground cover (it breaks down faster in soils with plant ground cover than in fallow soils). Organic material aging may also affect the breakdown rate of imidacloprid. Plots treated

with cow manure and allowed to age before sowing showed longer persistence of imidacloprid in soils than in plots where the manure was more recently applied, and not allowed to age. Imidacloprid is degraded stepwise to the primary metabolite 6-chloronicotinic acid, which eventually breaks down into carbon dioxide. There is generally not a high risk of groundwater contamination with imidacloprid if used as directed. The chemical is moderately soluble, and has moderate binding affinity to organic materials in soils. However, there is a potential for the compound to move through sensitive soil types including porous, gravelly, or cobbly soils, depending on irrigation practices.

Imidacloprid is relatively stable with aerobic half life period of 997 days whereas anaerobic half life is about 27.1 days. According to the studies of Anhant et al. [1] longer half life period of imidacloprid and its metabolites in the soil is the result of reduced bioavailability of pesticide, for biodegradation, to pesticide degrading microorganisms . Numerous metabolic pathways for degradation of imidacloprid in soil have been proposed by Krohn & Hellpointer [2]. Possible metabolites of imidacloprid in soil metabolism includes; imidacloprid–urea, imidacloprid–guanidine and imidacloprid–guanidine–olefin. Limited reports describing the imidacloprid biodegradation are available. Anhant et al. [1] reported a bacterium from *Leifsonia* sp. to have the ability to degrade imidacloprid to 6-chloronicotinic acid within three weeks. Similarly, Pandey et al. [3] reported *Pseudomonas* sp. 1G that could transform imidacloprid to desnitro and urea metabolites. According to Dai et al. [4] *Stenotrophomonas maltophilia* CGMCC 1.1788 could hydroxylate imidacloprid (IMI) to 5-hydroxy imidacloprid; which possesses more insecticidal activity compared to parent compound.

Surface activity, detergency, emulsification, dispersion, and enhanced solubility properties of biosurfactants make them an ideal choice for remediation of agricultural soil polluted with hydrocarbons, heavy metals, and pesticides. In addition, application of biosurfactant-producing microbes together with biosurfactants could promote the degradation of the pollutants to a safer level, thus improving the soil quality and bringing the soil biology back to a pre-pollution condition. There are many reports of biosurfactant use in hydrocarbon degradation and bioremediation of soils. The present study is aimed to assess the dual capabilities of *Kocuria turfanesis* strain BS-J for biosurfactant production and imidacloprid degradation using novel formulation of biosurfactant coated egg shell calcite material. Till date, there is no report available, describing the imidacloprid degradation by using biosurfactant producing novel culture *Kocuria turfanesis* strain BS-J. Such studies forms an important basis in developing a process for remediation of pesticide contaminated soil ecosystem.

2.0 Materials and Methods:

2.1 Enrichment of *Kocuria turfanesis* strain BS-J for Imidacloprid degradation:

Kocuria turfanesis strain BS-J is a biosurfactant producing microbial culture isolated from lube oil and distillery spent wash contaminated soil which was collected from a distillery unit. The organism was inoculated in 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}) Imidacloprid as sole carbon source. The microbial culture was subjected to selective enrichment with sequential and weekly transfer of strain BS-J to increasing concentrations of imidacloprid from 50 - 500ppm.

2.2 Inoculum preparation for degradation of imidacloprid in mineral salts medium studies:

Strain BS-J was pre-cultured in baffled Erlenmeyer flasks containing LB medium. Flask was incubated overnight at 30 ^oC on a rotary shaker at 150 rpm. The contents of the inoculated flask 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^o C at 150 rpm for 24 h.

2.3 Imidacloprid degradation kinetics of *Kocuria turfanesis* strain BS-J

For the study of kinetics of Imidacloprid degradation, pure culture of *Kocuria turfanesis* strain BS-J was separately suspended in 1 ml

0.9% saline to make a cell suspension of 1×10^6 cells per ml and 100µl of this suspension was inoculated in 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 200 ppm (wv^{-1}) Imidacloprid as sole carbon source and incubated at 28±2 °C for 5 days in an orbital shaking incubator at 150 rpm under aerated culture conditions. Thereafter, Imidacloprid was extracted at time interval of 24 hours uptil 120 hours 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 Imidacloprid was estimated at 560 nm using UV-Vis spectrophotometer. The residual amount of Imidacloprid was calculated by molar absorption coefficient. Studies have shown that degradation kinetics of Imidacloprid follows first order kinetics. Degradation constant K_{deg} was calculated by drawing straight line curve between log of concentration of Imidacloprid at certain incubation duration Vs incubation duration and the slope of the curve was multiplied by 2.303. Thus, the degradation coefficient was calculated by using straight line equation:-

$y= -mx+c$

$K_{deg} = -2.303\times -m$

Half life of Imidacloprid in the mineral salts medium by the strain BS-J was estimated and expressed in hours from the standard Half life formula: $t_{1/2}= 0.699/K_{deg}$

In this work, a rapid, environmentally acceptable and inexpensive first-order derivative spectrophotometric method of Guzsvany *et al*., [5] was used for the determination of imidacloprid concentration.

2.4 Parameters analyzed for assessment of biosurfactant production by the isolates

2.4.1 Surface tension measurement: The surface tension measurement of the cell free supernatant was determined by **du Nouy ring** detachment method. The values reported are the mean of three measurements. All measurements were made on cell-free broth obtained by centrifuging the culture broths at 8000 rpm for 20 minutes according to the method of Dubey & Juwarkar, [6].

2.4.2 Quantitative assessment of biosurfactant yield: Biosurfactant in the form of brown paste and orange paste for culture PP2 and J respectively was recovered by diethyl ether extraction method as per the method of Ramana and Karanth [7] and was quantified by using analytical balance (Shimadzu AUW220D, Japan).

***2.4.3 Biomass and pH measurements*:** Biomass development of biosurfactant producing different isolates in individual and combined wastes was monitored in terms of c.f.u./ml of fermented wastes by serial dilution and pour plate technique using nutrient agar as the growth medium mentioned in Dubey & Juwarkar, [6].The pH of the cell free culture broth was measured with a digital pH-meter MK VI (Systronics, Naroda, Ahmedabad.

2.5 Pot culture studies for Imidacloprid 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 Imidacloprid degradation periodically after 5 days of incubation in green house at a temperature of 30°C over a period of 30 days.

1. Soil amended with Imidacloprid at the rate of 200 ppm + tap water
2. Soil amended with Imidacloprid at the rate of 200 ppm + 10% curd whey
3. Soil amended with Imidacloprid at the rate of 200 ppm + 10% fermented curd whey containing biosurfactant +whole cells of BS-J
4. Soil amended with Imidacloprid at the rate of 200 ppm + 10% cell free fermented curd whey containing biosurfactant.

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

Following treatments were given to the soil to study the role of carrier based bioaugmentation package of egg shell coated with biosurfactant and biosurfactant producing microbial isolates BS-J cells in Imidacloprid degradation in soil, periodically after 5 days of incubation in a green house at a temperature of 30°C over a period of 30 days.

1. Soil + imidacloprid (300 ppm) + 10% tap water
2. Soil + imidacloprid (300 ppm) + 10% egg shell coated with curd whey
3. Soil + imidacloprid (300 ppm) + 10% egg shell coated with fermented curd whey containing biosurfactant producing organism
4. Soil + imidacloprid (300 ppm) + 10% egg shell coated with cell free fermented curd whey containing biosurfactant producing organism

In above experimental setups using pot culture studies 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 Imidacloprid was estimated at 560 nm using UV-Vis spectrophotometer by method of Guzsvany *et al*., [5].

3.0 Results & Discussion:

3.1 Growth of *Kocuria turfanesis* strain BS-J in Mineral Salts Medium containing different concentrations of Imidacloprid as sole C-source:

Results presented in **Table 1** shows that best tolerance of *Kocuria turfanesis* strain BS-J to imidacloprid was 200 ppm as highest growth was obtained i.e. 96x10^{9} c.f.u./ml after 72 h of incubation in comparison that obtained at lower or higher concentrations of imidacloprid than 200 ppm. At 200 ppm, the growth of cells remained almost constant until 96 h and then declined to 76x10^{9} c.f.u./ml. Comparatively less growth at lower concentrations of imidacloprid (50 and 100 ppm) indicated that these concentrations were growth limiting concentrations and moreover, imidacloprid was the only carbon source (a sole carbon source) available for the culture to grow. Concentrations over 300 ppm gradually resulted in decreased growth which later on decreased further over a period of time due to the toxicity of imidacloprid.

Table 1: Growth of *Kocuria turfanesis* in Mineral Salts Medium containing different concentrations of Imidacloprid as sole C-source.

Incubation Time (h)	Growth of *Kocuria turfanesis* strain BS-J at different ppm levels of Imidacloprid (c.f.u/ml)					
	50	**100**	**200**	**300**	**400**	**500**
0	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}
24	32x10^{7}	41x10^{7}	56x10^{7}	35x10^{7}	29x10^{6}	20x10^{5}
48	49x10^{8}	57x10^{8}	99x10^{8}	38x10^{8}	23x10^{6}	15x10^{3}
72	66x10^{8}	78x10^{8}	96x10^{9}	34x10^{9}	20x10^{7}	30x10^{2}
96	74x10^{8}	84x10^{8}	94x10^{9}	30x10^{9}	27x10^{6}	14x10^{2}
120	63x10^{9}	81x10^{9}	76x10^{9}	27x10^{9}	32x10^{5}	218

3.2 Utilization of Imidacloprid and biosurfactant production by isolate *Kocuria turfanesis* BS-J in mineral salts medium:

Imidacloprid degradation by isolate BS-J was monitored by for a period of 120 h (**Table 2**). After 48 h, 45% of pure Imidacloprid had rapidly disappeared, followed by a slower decrease of Imidacloprid with longer incubation times. During 72 to 96 h of incubation the surface tension of the cell free broth dropped from 57 dynes/cm to 27 dynes/cm indicating the maximum production of biosurfactant during this phase of incubation with a yield of 0.660g/l. In this phase, growth associated production of biosurfactant at the expense of 83-96% Imidacloprid utilization was observed. The degradation of Imidacloprid supported cell growth, indicating that isolate BS-J could utilize Imidacloprid as a carbon source. Such an isolate with dual potential of producing biosurfactant along with Imidacloprid degradation can be used as an advanced approach for the bioremediation of wastewater or soil contaminated with Imidacloprid. From the culture enrichment study it was evident that the concentration of Imidacloprid affects the growth of BS-J. The optimal concentration of Imidacloprid for the growth of BS-J was 200

ppm, and a concentration higher than 500 ppm was toxic for the normal growth of BS-J isolate.

Table 2: Process parameters of biosurfactant production and degradation of Imidacloprid by stain BS-J in Mineral Salts Medium containing optimum concentration of Imidacloprid (200 ppm) as C-source.

Incubation Time (h)	Growth of *Kocuria turfanesis* (c.f.u/ml)	Degradation of Imidacloprid (%)	Surface tension (dynes/cm)	Biosurfactant yield (g/l)
0	10^6	0	57	0.0004
24	$56x10^7$	20	42	0.003
48	$99x10^8$	45	34	0.016
72	$96x10^9$	72	27	0.570
96	$94x10^9$	83	27	0.660
120	$76x10^9$	96	27	0.620

3.3 Imidacloprid degradation kinetics of *Kocuria turfanesis* strain BS-J:

Studies have shown that degradation kinetics of Imidacloprid follows first order kinetics. Results of degradation constant K_{deg} calculated from straight line curve between log of concentration of Imidacloprid at certain incubation duration Vs incubation duration and the slope of the curve multiplied by 2.303 are presented in **Figure-1** and **Table-3** . Thus, the degradation coefficient was calculated by using straight line equation:- y= -mx+c and K_{deg} = -2.303× -m

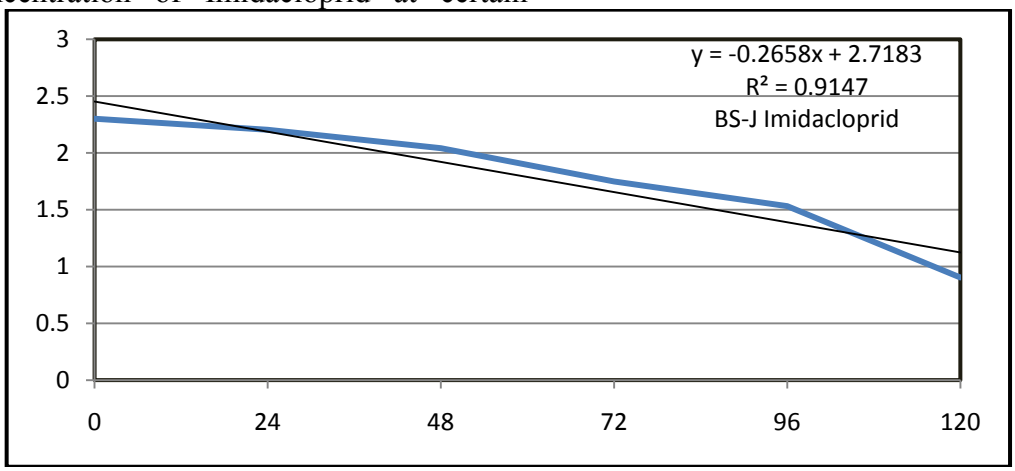

Figure-1: Straight line curve of log concentration of Imidacloprid verses incubation time for imidacloprid degradation by *Kocuria turfanesis* strain BS-J

Table 3: Kinetics of Imidacloprid degradation by *Kocuria turfanesis* strain BS-J

Incubation time (Hours)	Concentration of residual Imidacloprid (ppm)	Log of concentration of residual Imidacloprid
0	200	2.3010
24	160	2.2041
48	110	2.0413
72	56	1.7481
96	34	1.5314
120	08	0.9030

Half life of Imidacloprid in the mineral salts medium by the strain BS-J was estimated and expressed in hours from the standard Half life formula: $t_{1/2}$= 0.699/$K_{deg.}$ Results presented in Table **3 & 4** shows that degradation constant K_{deg} is found to be 0.6121 and the half life of pesticide is estimated to be 1.1419 h. Results indicate that the production of biosurfactant using imidacloprid as sole carbon source has reduced the half life of imidacloprid by several folds and therefore the organism can be used for

remediation of Imidacloprid contaminated ecosystems like soil and waste water.

Table 4: Kinetic degradation constants and half live of imidacloprid degradation by biosurfactant producing microbial isolates

Microbial culture	Pesticide	Slope of the equation (m)	Degradation constant K_{deg} =-2.303 x -m	Half life of pesticide $t_{1/2}$=0.699/ K_{deg} (h)
Kocuria turfanesis **strain BS-J**	Imidacloprid	-0.2658	0.6121	1.1419

3.4 Degradation of Imidacloprid in soil:

3.4.1 Experimental setup for Imidacloprid degradation in soil:

To study the role of biosurfactant produced by strain BS-J for imidacloprid degradation in soil following treatments of imidacloprid simulated soil were used in green house at a temperature of 30^{o} C over a period of 30 days:

1. Soil amended with Imidacloprid at the rate of 300 ppm + tap water
2. Soil amended with Imidacloprid at the rate of 300 ppm + 10% curd whey
3. Soil amended with Imidacloprid at the rate of 300 ppm + 10% fermented curd whey containing biosurfactant +whole cells of BS-J
4. Soil amended with Imidacloprid at the rate of 300 ppm + 10% cell free fermented curd whey containing biosurfactant.

Results presented in **Table 5** have shown that under an experimental set up performed with potential isolate BS-J, application of 10% fermented curd whey containing biosurfactant and whole cells into imidacloprid contaminated soil resulted in maximum imidacloprid degradation ability over a period of 30 days as compared to other treatments of contaminated soil. Results have indicated that during initial phases of degradation i.e. until 10 days high degradation potential was observed in all the treatments. However, later on the degradation capabilities of the isolate became the same.

The treatment in which soil was amended with imidacloprid at the rate of 300 ppm + 10% fermented curd whey containing biosurfactant +whole cells, maximum degradation i.e. 100% was obtained within 15 days of application, however, treatment in which soil was amended with imidacloprid at the rate of 300 ppm + 10% cell free fermented curd whey containing biosurfactant without cells resulted in the same maximum degradation only after 20 days of incubation which otherwise took only 15 days for degradation. Soil amended with imidacloprid at the rate of 300 ppm + tap water and soil amended with imidacloprid at the rate of 300 ppm + 10% curd whey containing biosurfactant +whole cells comparatively promoted and enhanced degradation of imidacloprid in soil.

Table: 5. Time course of Imidacloprid degradation by *Kocuria turfanesis* stain BS-J in various treatments of soil

Treatments of soil	Percent Degradation of Imidacloprid in various treatments of soil at different time intervals (Days)						
	0	**5**	**10**	**15**	**20**	**25**	**30**
1. Soil amended with Imidacloprid at the rate of 300 ppm + tap water	0.0	24	21	35	40	43	49

2. Soil amended with Imidacloprid at the rate of 300 ppm + 10% curd whey	0.0	32	46	69	74	78	82
3. Soil amended with Imidacloprid at the rate of 300 ppm + 10% cell free fermented curd whey containing biosurfactant	0.0	59	66	87	100	100	100
4. Soil amended with Imidacloprid at the rate of 300 ppm + 10% fermented curd whey containing biosurfactant +whole cells	0.0	70	85	100	100	100	100

3.5 Changes in physico-chemical characteristics of Imidacloprid contaminated soil during degradation of Imidacloprid in different treatments of soil with carrier based bioaugmentation package of egg shell

Physico-chemical characteristics of soil contaminated with 300 ppm of imidacloprid used in the present study show that soil had pH of 6.4 with bulk density of 1.21 g/cm^3 and water holding capacity of 56.20% (**Table-6**). Results have also shown that organic carbon content of soil was 0.59% and N, P and K levels of soil were 0.062, 0.073 and 0.124 %, respectively. There was decrease in the pH of the soil from 6.9 to 6.4 after treatment with 300 ppm imidacloprid due to acidic nature of the pesticide. However, after amendment with 10% egg shell coated with curd whey the pH of the imidacloprid treated soil improved to 6.4-7.1 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 imidacloprid 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. N, P and K levels also improved in the soil upon using egg shell carrier based bioaugmentation process.

Table: 6. Changes in physico-chemical characteristics of Imidacloprid contaminated soil during degradation of Imidacloprid 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 imidacloprid (%)
1. Soil + imidacloprid (300 ppm) + 10% tap water	1.21	56.20	6.4	0.59	0.062	0.073	0.124	7.12

2. Soil + imidaclo prid (300 ppm) + 10% egg shell coated with curd whey	1.02	60.14	7.1	0.65	0.075	0.079	0.144	20.45
3. Soil + imidaclo prid (300 ppm) + 10% egg shell coated with fermented curd whey containing biosurfactant producing organism	1.12	60.48	7.3	0.69	0.099	0.072	0.186	90.87
4. Soil + imidaclo prid (300 ppm) + 10% egg shell coated with cell Free fermented Curd whey containing biosurfactant producing organism	1.14	60.05	7.2	0.87	0.094	0.095	0.186	84.76

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 enhances 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 in 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.

4.0 Conclusion:

The ability of the biosurfactant producing *Kocuria turfanesis* to mineralize imidacloprid was investigated in mineral salts medium using imidacloprid as sole carbon source and also in pot culture experiments using imidacloprid contaminated soil. Studies have shown that the organism has a dual capabilities of biosurfactant production and degradation of imidacloprid. These two main attributes of strain BS-J resulted in reducing the half life of imidacloprid by several folds and therefore, the organism has an application in remediation of Imidacloprid contaminated ecosystems like soil and waste water. Degradation of imidacloprid in contaminated soil was facilitated after using carrier based bioaugmentation package of egg shell coated with biosurfactant producing microbial isolate BS-J cells and its biosurfactant resulted in 85-91 % degradation of imidacloprid 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.

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