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Application of Biosurfactant Producing *Kocuria turfanesis* Strain BS-J For Imidacloprid Degradation

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Abstract:
The ability of the biosurfactant producing *Kocuria turfanesis* to mineralize imidacloprid was investigated through batch experimental set up in mineral salts medium using imidacloprid as sole carbon source. Dual capabilities of biosurfactant production and degradation of imidacloprid are the two main attributes of strain BS-J. Present study has shown that in mineral salts medium growth was best at 200 ppm as highest cell number was obtained i.e. $96x10^9$ c.f.u./ml after 72 h of incubation in comparison to lower or higher concentrations of imidacloprid than 200 ppm. 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. After 48 h, 45% of pure Imidacloprid had rapidly disappeared, which slowed down with longer incubation periods. During 72 to 96 h of incubation the surface tension of the cell free broth dropped from 57 dynes/cm to 27 dynes/cm and during this phase of incubation growth associated biosurfactant yield of 0.660g/l was obtained at the expense of 83-96% Imidacloprid utilization. The degradation of Imidacloprid supported cell growth, indicating that isolate BS-J could utilize Imidacloprid as a carbon source and follows first order kinetics. 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.
Keywords: Biosurfactant, biodegradation, imidacloprid, *Kocuria turfanesis* strain BS-J

Introduction:

Imidacloprid is a systemic, chloro-nicotinyl insecticide with soil, seed and foliar uses for the control of sucking insects including rice hoppers, aphids, thrips, whiteflies, termites, turf insects, soil insects and some beetles. It is 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 dustable powder, granular, seed dressing (flowable 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. 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 (Anhant et al. 2007). Numerous metabolic pathways for degradation of imidacloprid in soil have been proposed (Krohn & Hellpointer 2002). Possible metabolites of imidacloprid in soil metabolism includes; imidacloprid–urea, imidacloprid–guanidine and

imidacloprid–guanidine–olefin. Limited reports describing the imidacloprid biodegradation are available. A bacterium from *Leifsonia* sp. has been reported to have the ability to degrade imidacloprid to 6-chloronicotinic acid within three weeks (Anhant et al. 2007), similarly, *Pseudomonas* sp. 1G could transform imidacloprid to desnitro and urea metabolites (Pandey et al. 2009). *Stenotrophomonas maltophilia* CGMCC 1.1788 could hydroxylate imidacloprid (IMI) to 5-hydroxy imidacloprid; which possesses more insecticidal activity compared to parent compound (Dai et al. 2006).

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. 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 remediattiiiion of pesticide contaminated soil ecosystem.

Materials and Methods:

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.

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.

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.*, 2009 was used for the determination of imidacloprid concentration.

Parameters analyzed for assessment of biosurfactant production by the isolates

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 (Dubey & Juwarkar, 2001).

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 (Ramana and Karanth 1987) and was quantified by using analytical balance (Shimadzu AUW220D, Japan).

***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 (Dubey & Juwarkar, 2001).The pH of the cell free culture broth was measured with a digital pH-meter MK VI (Systronics, Naroda, Ahmedabad).

Results and Discussion:

Growth of Kocuria turfanesis 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.

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.

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

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: $t_{1/2} = 0.699/K_{deg.}$ Results presented in Table 3 & 4 shows that degradation constant K_{deg} is found to be 0.6121 aand 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.

Conclusion:

The ability of the biosurfactant producing *Kocuria turfanesis* to mineralize imidacloprid was investigated through batch experimental set up in mineral salts medium using imidacloprid as sole carbon source. Studies have show that the organism has a dual capabilities of biosurfactant production and degradation of imidacloprid. These two main attributes of strain BS-J also 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.

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

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

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

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

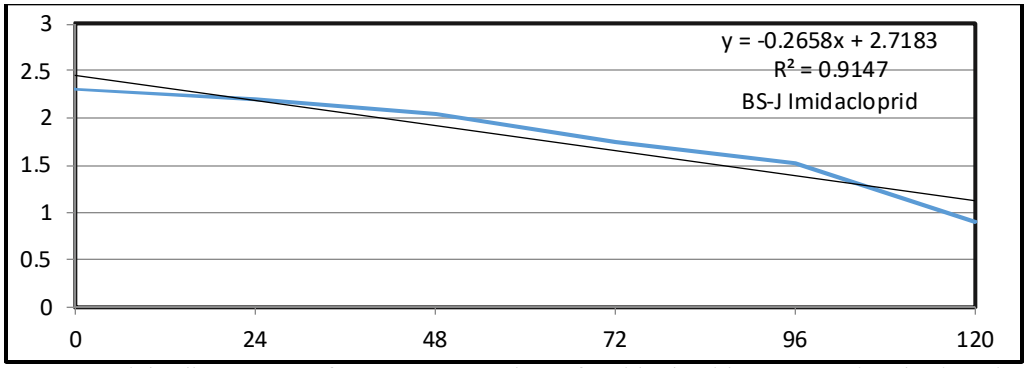

Figure-1: Straight line curve of Log concentration of Imidacloprid verses incubation time for imidacloprid degradation by *Kocuria turfanesis* strain BS-J

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