RESEARCH ARTICLE

Impact of Biosurfactant from *Kocuria rosea* and *Pseudomonas aeruginosa* on Germinating Seedlings of *Glycine max*, *Pisum sativum* and *Spinacia oleracea*

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ABSTRACT- Biosurfactant is a structurally diverse group of surface-active molecule, synthesized by microorganisms. *Kocuria rosea* and *Pseudomonas aeruginosa* strains isolated from pesticide contaminated soil, which produces biosurfactant were studied. Curd whey was used as a cheap source of growth medium for biosurfactant production. There was formation of stable emulsions of biosurfactant containing broth with vegetable oil and kerosene. These strains produced a clear zone in oil spreading test, which is an indicative of the good biosurfactant activity. Both the strains produced extra cellular biosurfactant in the culture media and showed good foam stability in the culture medium. Biosurfactant was efficiently extracted from the culture broth by acetone-HCl precipitation. The biosurfactants from the two species, namely *Kocuria rosea* and *Pseudomonas aeruginosa* were found to have no effects on germinating seedlings of *Glycine max, Pisum sativum* and *Spinacia oleracea,* when treated with 25%, 50%, 75% and 100% with the combination of curd whey in the making of 100ml volume. Curd whey as a control was taken with no surfactant. Our study suggested an efficient use in surfactant aided bioremediation in agricultural land.

Key-words- Biosurfactant, Kerosene, Emulsification, Oil spreading, *Kocuria rosea*, *Pseudomonas aeruginosa, Glycine max, Pisum sativum*, *Spinacia oleracea*

INTRODUCTION

Surfactants and emulsifiers are indispensable components of daily life. Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi [1]. Biosurfactant is extensively used worldwide as it is preferred to chemical surfactants for it being non toxic and easily biodegradable [2]. Biosurfactants have gained importance in the fields of enhanced oil recovery, food processing, environment, bioremediation, and pharmaceuticals [3]. The cost of biosurfactant is much cheaper as compared to chemical surfactants. They are found to have lower c*ritical micelle concentration* value as compared to chemical surfactant [4]. These compounds find applications in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization [2,5]. Biosurfactant producing microorganisms are naturally present in the oil contaminated soil. Oil contaminated environment contain large amount of hydrocarbons. Hydrocarbons are composed of complex chemical structure i.e., aliphatic and aromatic hydrocarbons. Microorganisms exhibit emulsifying activity by producing biosurfactants and utilize the hydrocarbons as substrate often mineralizing them or converting them into harmless products [6].

One of the major biosurfactant applications in environmental protection is bioremediation. The most cost

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effective methods include *in-situ* bioremediation. Surface active agents are needed for the hydrophilization of heavy soil for obtaining good wettability and also to achieve equal distribution of fertilizers and pesticides in the soils [5]. Most of the pesticides are water soluble and are marketed in powder or in liquid concentrate form. Before spraying in field, these are diluted with water and mixed with surface active compound for spontaneous distribution of the water insoluble pesticides in the aqueous phase as well as an equal distribution on wetting of the treated areas [4].

Glycine max (Soyabean) and *Pisum sativum* (Pea) belong to the family fabaceae while *Spinacia oleracea* (Spinach) belongs to the family amaranthaceae. The United States, Brazil and Argentina are the world's largest soybean producers and represent more than 80% of global soybean production followed by India, and China. China, India, United States of America, France and Egypt are green pea producing countries. Spinach is grown mainly in China, United States of America, Japan, Turkey and Indonesia. The seeds are rich in protein, carbohydrates, fats and vitamins making them a highly nutritious food [7-8].

The present study focused on the biosurfactant production by *Kocuria rosea* BS-4 and *Pseudomonas aeruginosa* BS-14 isolated from pesticide contaminated soil. The biosurfactant production was screened by oil spreading technique and emulsification stability test. Curd whey was used as a cheap and cost effective growth medium. In this work we report the effect of biosurfactant from *Kocuria rosea* and *Pseudomonas aeruginosa* on germinating seedlings of *Glycine max, Pisum sativum* and *Spinacia oleracea.*

MATERIALS AND METHODS

This study was conducted in the Department of Microbiology and Biotechnology, Shri Shivaji Science College, Amravati (Maharashtra), India in the duration of 2015-2016. All the chemicals were of analytical grade and were obtained from Himedia Laboratories Private Limited, India. Acetone was obtained from Merck, India. The seeds of *G. max*, *P. sativum* and *S. oleracea* were obtained from an authorized dealer.

Enrichment culture and isolation of microbes

Three pesticide contaminated soil samples were collected at four different locations from Yawalkar Pesticide Industry, Nagpur, Maharashtra, India. One gram each of the soil samples was added to 100 ml of mineral salt medium with 2% (v/v) liquid paraffin and incubated at 37^0C for 7 days at 180 rpm. The composition of the mineral salt medium was modified in our laboratory as follows: $NaNO_3$ -3 g; KH_2PO_4 -1.5 g; Na_2PO_4 -1.5 g; $MgSO_4.7H_2O$ -2 g; $CaCl_2.2H_2O$ -0.005 g; $FeSO_4$ -0.001 g; $ZnSO_47H_2O$ -70 µg; $CuSO_4.5H_2O$ –50 µg; H_3BO_3 -10 µg; MoO_3 -10 µg per liter; pH 7±0.2. After 7 days 1 ml inoculums were transferred to the same medium and incubated at 37^0C for another 7 days. The process was repeated for three times and 1 ml inoculum from the third enrichment culture was appropriately serially diluted and 100 µl aliquots from the last two dilutions were streaked on nutrient agar plate. Isolated pure colonies were picked up and maintained at 37^0C on nutrient agar slant containing hydrocarbon.

Screening for biosurfactant production

The isolated pure cultures were transferred separately to culture tubes containing 30 ml mineral salt medium with 2% (v/v) liquid paraffin and incubated in orbital shaker for 5 days at 180 rpm at 37°C. After the incubation period the tubes were vortexed for 2 min to record the foaming and turbidity of the growth medium. Medium without inoculum served as control.

Culture supernatant

The mineral salt medium containing Biosurfactant was centrifuged at 10,000 rpm for 10 min and supernatant was used for emulsification index and oil spreading test.

Emulsification index E24

Emulsification index of culture samples were determined by adding 2 ml of oil (kerosene, vegetable oil, petrol and diesel) to the same amount of culture supernatant, mixing with a vortex for 2 min, and leaving to stand for 24 hours. The emulsification index was calculated as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm) [9].

Oil Spreading Technique

50 ml of distilled water was taken in glass Petri dish with15 cm diameter and then followed by the addition of 20µl of oil to the surface of water and finally 10µl of culture supernatant was added to observe the clearance zone [10].

Identification of the selected culture

Best two isolates (BS-4 and BS-14) screened for biosurfactant production were identified according to the standard microscopical, cultural and biochemical tests [11] and comparing the results with Bergey's Manual of Systematic Bacteriology [12]. BS-4 and BS-14 were further identified to species level using sequencing as *Kocuria rosea* and *Pseudomonas aeruginosa* from Yaazh Xenomics, Mumbai, India.

Curd whey as a low cost growth medium

The processing of curd whey was done by adjusting the pH 4.1 to 7 by using 5 N NaOH and heating for 2-3 times. After adjusting the pH, whey was cooled and centrifuged at 8000 rpm for 12 min to remove casein. Supernatant was adjusted to pH-7.0 again and 30 ml of processed curd whey was distributed in a big culture tube and sterilized at 121^0C for 20 minutes. Sterile whey was separately inoculated with isolated colonies and incubated in orbital shaking incubator for 96 hours (optimum biosurfactant production time). Fermented whey containing biosurfactant was centrifuged at 10,000 rpm for 10 min. Supernatants of biosurfactant producing organisms were used for testing the effect of biosurfactant on total length proliferation of germinating

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seedlings [13].

Extraction of biosurfactant

Extraction was done by using acetone and then precipitation by HCI, overnight at 4°C. The product recovered was dried under vacuum and preserved. The culture broth was centrifuged (10000 g, 15 min) to remove the cells and thereafter sterilized with millipore membrane filter. The clear sterile supernatant served as the source of the crude biosurfactant. The biosurfactant was recovered from the cell free culture supernatant by cold acetone precipitation [9].

Effect of biosurfactant on germinating seedlings

Biosurfactant produced by *Kocuria rosea* BS-4 and *Pseudomonas aeruginosa* BS-14 was evaluated for its effect on germinating seedlings of *Glycine max* (Soyabean), *Pisum sativum* (Pea) and *Spinacia oleracea* (Spinach). Four different concentrations of biosurfactant 25%, 50%, 75% and 100% v/v produced by *Kocuria rosea* and *Pseudomonas aeruginosa* were prepared in curd whey. Curd whey at a concentration of 25%, 50%, 75% and 100% v/v in distilled water was used as a control. Ten healthy seeds of the above plant species were presoaked in the respective concentration for 24 hours. The soaked seeds were spread on filter paper in petridishes and the total length (cm) proliferation of the germinating seedlings was recorded after 4 days.

RESULTS AND DISCUSSION

Isolation of biosurfactant producing microbes

The production of surfactants by microbial cells often results in foaming and emulsifies hydrophobic substrates [5]. These properties were used in screening for surfactant producing strains. A total of 14 isolates were isolated in pure form from three pesticide contaminated soil samples. All the isolates showed good growth in mineral salt medium containing 2% (v/v) liquid light paraffin as the sole carbon source. On the basis of the foaming, turbidity and emulsification index two best isolates BS-4 and BS-14 were considered for further investigation. Combination of three tests, foaming, emulsification index and oil spreading tests are commonly used to identify microbes as potential biosurfactant producers [14].

Aqueous solutions of both BS-4 and BS-14 biosurfactants showed good foaming stability. Total disappearance of the foam was detected after 2 hours. Stabilization of an oil and water emulsion is commonly used as a surface activity indicator.

The results of emulsification activity and clearance zone produced by oil spreading technique are shown in Table 1. All the hydrocarbons tested served as substrates for emulsification by the biosurfactant. Diesel and kerosene were the best substrates for both the strains while vegetable cooking oil was less good substrates for emulsification. Overall BS-4 exhibited higher emulsification activity than BS-14 with all the hydrocarbons used. Furthermore cell free broth culture of BS-4 had highest emulsification activity of 72% with kerosene while BS-14 had highest emulsification activity of 68.85% with diesel.

Table 1: Emulsification activity (E24) and clearance zone of biosurfactant for different hydrocarbons

E24 value (%)/Clearance zone (mm)				
Strains	**Vegetable oil**	**Kerosene**	**Petrol**	**Diesel**
BS-4	67/10	72/46	69/48	70/51
BS-14	64/06	67/40	68/42	68.85/48

In oil spreading technique, isolate BS-4 and BS-14 produced the maximum clearance zone of 51mm and 48mm respectively in diesel.

Characterization and identification of the selected cultures

Strain *Kocuria rosea* (BS-4) and *Pseudomonas aeruginosa* (BS-14) found to be Gram-positive coccus and Gram-negative rods respectively. Strain *Kocuria rosea* was found to be non-motile whereas *Pseudomonas aeruginosa* proved to be motile. Both the strains showed negative tests for indole production and MR-VP. *Kocuria rosea* was negative for citrate reduction where as *Pseudomonas aeruginosa* was positive for citrate reduction. Both were catalase positive and showed positive test for urea hydrolysis. *Kocuria rosea* showed growth at temperature of 4^0C, 10^0C, 20^0C, 30^0C, 37^0C and 45^0C. Growth of *Pseudomonas aeruginosa* was observed at 20^0C, 30^0C, 37^0C, and 45^0C. However *K. rosea* and *P. aeruginosa* showed maximum growth at 37^0C and did not grow at 55^0C. The pH range of 6 to 11.5 supported the growth of both strains with optimum growth at pH 7.

Curd whey as a growth medium

Processed curd whey had been used as a good and cheap substitute for the production of biosurfactant. Both the strains showed good biosurfactant productivity when inoculated in processed curd whey. After centrifugation of the curd whey, supernatants of biosurfactant producing organisms were used for testing the effect of biosurfactant on total length proliferation of germinating seedlings.

Extraction of biosurfactant

The biosurfactant was separated without loss of its activity. The yields of biosurfactants of *Kocuria rosea* and *Pseudomonas aeruginosa* were 3.68 g/l and 4.25 g/l, respectively. A maximum yield of around 5.5 g/l was reported by Pruthi and Cameotra [15] by three *Pseudomonas* species grown on n-dodecane.

Effect of biosurfactant in germinating *Glycine max*, *Pisum sativum* and *Spinacia oleracea*

The total proliferation seedling length was measured on 5th day of treatment (Table 2-4). Each value represents the mean of three replicates.

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Table 2- Effect of curd whey as a control on germinating seedlings of *Glycine max, Pisum sativum and Spinacia oleracea*

Curd Whey	Seed type	No. of seeds 1 / Length of proliferation in centimeters	2	3	4	5	6	7	8	9	10
25%	*Glycine max*	2.1	2.9	2.6	3.1	3.2	1.9	2.5	3.4	2.8	2.0
	Pisum sativum	2.4	1.6	3.7	2.9	3.1	2.9.	3.8	3.3	2.5	3.8
	Spinacia oleracea	1.0	0.8	1.1	1.3	0.9	1.2	0.8	0.7	1.4	1.3
50%	*Glycine max*	1.9	2.7	3.1	2.8	1.7	3.0	2.2	2.7	2.9	2.1
	Pisum sativum	0.9	2.2	2.4	1.8	3.0	2.3	2.4	3.1	0.9	1.9
	Spinacia oleracea	2.0	1.5	0.2	1.5	0.1	1.2	0.6	0.1	0.0	1.1
75%	*Glycine max*	1.2	1.8	0.8	1.6	2.1	2.5	0.4	0.8	1.8	2.3
	Pisum sativum	2.3	1.9	2.1	1.9	0.2	0.7	0.0	2.5	3.1	2.7
	Spinacia oleracea	1.4	1.6	1.1	0.2	0.0	1.4	2.1	1.7	1.0	0.8
100%	*Glycine max*	1.2	1.1	0.9	0.8	1.6	1.3	1.2	0.6	1.0	0.9
	Pisum sativum	3.1	0.1	2.7	2.9	3.1	2.2	1.9	1.8	2.1	2.0
	Spinacia oleracea	0.7	1.1	0.2	0.0	1.3	0.9	1.9	2.0	0.0	0.1

Table 3- Effect of Biosurfactants produced by *strain Kocuria rosea* on germinating seedlings of *Glycine max* (Soyabean), *Pisum sativum and Spinacia oleracea*

Kocuria rosea	Seed type	No. of seeds 1 / Length of proliferation in centimeters	2	3	4	5	6	7	8	9	10
25%	*Glycine max*	1.9	2.0	2.2	1.9	2.7	1.5	3.0	3.1	2.1	1.8
	Pisum sativum	1.8	3.2	2.1	2.8	2.0	3.2	3.4	3.9	2.8	3.4
	Spinacia oleracea	0.5	1.4	1.6	0.8	1.4	1.5	1.3	0.9	0.4	1.7
50%	*Glycine max*	2.9	1.7	3.0	2.2	3.2	2.6	1.9	2.8	2.4	1.7
	Pisum sativum	3.1	2.4	3.3	2.5	2.7	1.8	2.2	3.0	2.7	3.1
	Spinacia oleracea	0.0	1.2	0.9	1.4	0.7	0.4	1.6	0.4	1.3	1.5
75%	*Glycine max*	2.0	1.5	1.4	2.3	2.7	0.7	2.2	1.5	2.1	2.6
	Pisum sativum	3.3	0.4	2.5	2.1	0.8	1.6	0.6	3.2	2.4	3.4
	Spinacia oleracea	1.6	0.1	1.3	0.0	0.9	1.7	2.3	2.4	1.2	0.7
100%	*Glycine max*	2.1	1.9	1.2	1.3	2.2	1.9	2.1	0.2	2.3	2.7
	Pisum sativum	2.4	2.1	3.3	2.9	3.0	1.9	2.3	3.1	2.9	2.4
	Spinacia oleracea	0.1	1.3	1.7	0.3	1.9	2.2	2.1	2.4	0.8	0.0

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Table 4- Effect of Biosurfactants produced by *strain Pseudomonas aeruginosa* on germinating seedlings of *Glycine max* (Soyabean), *Pisum sativum and Spinacia oleracea*

Kocuria rosea	**Seed type**	**No. of seeds 1 / Length of proliferation in centimeters**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
25%	*Glycine max*	3.2	2.8	3.6	3.5	1.9	2.2	2.9	3.1	2.8	2.9
	Pisum sativum	3.4	2.8	2.9	2.6	3.0	3.1	2.8	3.4	3.5	3.7
	Spinacia oleracea	1.3	2.0	0.9	1.3	0.9	1.3	1.0	1.9	1.6	1.5
50%	*Glycine max*	3.1	2.9	1.9	3.4	2.5	3.1	2.9	3.2	2.9	2.3
	Pisum sativum	2.9	3.2	2.6	3.2	2.0	1.8	2.6	3.2	1.6	0.7
	Spinacia oleracea	1.4	2.1	0.7	1.8	2.0	0.5	0.6	0.3	0.0	1.8
75%	*Glycine max*	2.0	3.2	2.6	1.8	2.8	2.7	3.1	0.8	2.6	2.8
	Pisum sativum	3.3	2.7	2.4	0.9	3.0	0.9	2.6	2.9	2.8	2.1
	Spinacia oleracea	2.0	1.8	0.4	0.7	0.9	0.0	2.1	1.8	0.7	0.9
100%	*Glycine max*	2.3	0.8	1.7	0.7	1.4	2.3	2.1	0.8	1.3	0.9
	Pisum sativum	2.9	1.8	3.2	2.7	1.1	3.1	1.4	1.8	2.3	3.0
	Spinacia oleracea	2.2	1.7	0.6	0.0	1.1	0.9	1.8	2.3	0.0	0.0

The biosurfactant produced by *Kocuria rosea* and *Pseudomonas aeruginosa* had no adverse effect on the total length proliferation on the germinating seedlings of soyabean, peas and spinach.

Biosurfactant producing microorganisms are naturally present in the pesticide contaminated soils. We successfully isolated bacteria with the ability to produce biosurfactants from the soil samples collected from pesticide industry. Further study on the utilization of curd whey as a growth medium for the large-scale production of biosurfactants was done. The concentration of biosurfactant used in this study ranged from 25%- 100% which is suggestible for practical application of biosurfactant in surfactant aided bioremediation of cultivated land. Also, the biosurfactant could possibly be used in hydrophilization of heavy soil and even distribution of pesticides and fertilizers in agriculture.

CONCLUSIONS

This study represented surfactant activity of the bacterial strains isolated from pesticide contaminated soils from the pesticide industry. The present study is an attempt to find economically cheaper sources for the large scale production of microbial biosurfactant. Results obtained in biosurfactant production with curd whey waste suggested the possibility of industrial production of biosurfactant using economically cheaper source. We concluded in our result that the biosurfactant from strains *Kocuria rosea* and *Pseudomonas aeruginosa* have no effect on the germination seedlings of *Glycine max*, *Pisum sativum* and *Spinacia oleracea.*

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