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बेक्टी बेरियर इंडिया कंपनी
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महोदय

आपको "Microbial Toxicity Testing of Bactibarrier" की रिपोर्ट भेजी जा रही है। कृपया पावती से अवगत करने का कष्ट करें।

धन्यवाद,

संलग्न: उपरोक्त

भवदीय
21/8
(डॉ के सी खुल्बे)
प्रमुख, आरपीबीडी



REPORT

MICROBIAL TOXICITY TESTING OF BACTIBARRIER
Centre for Innovation and Translational Research (CITAR),
CSIR- Indian Institute of Toxicology Research (CSIR-IITR)
November 2017- March 2019

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REPORT

MICROBIAL TOXICITY TESTING OF BACTIBARRIER
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Project summary of Testing Bactibarrier Compound

Bactibarrier Surface Protector is comprised of two ingredients, the chemical that provides the protection i.e. (3- (trihydroxy) silyl propyl dimethyl octadecyl ammonium chloride, CAS no=27668-52-6, % Weight= 0.5%) and water. (3- (Trihydroxy) Silyl Propyldimethyl Octadecyl ammonium chloride is a Quaternary ammonium compound (QACs) which is a major class of cationic surfactants. The mechanism of the QACs antimicrobial property includes the attraction of microbe towards the hydrophobic tail, by the help of positively charged nitrogen atom present in the compound molecular structure. Thereafter, the cell membrane of microorganism is then pierced by the long molecular chain of hydrocarbon which acts like a spear. The long molecular chain not only physically pierces and punctures the microbe but the charge difference created electrocutes it as well, ensuring deactivation of the microbe. Project undertaken to understand the product and mechanism to develop safe and effective product in collaboration.

OBJECTIVES

- To evaluate the microbial inhibitory capabilities for gram-positive and gram-negative bacteria and test the effectiveness of the compound.
- Efficacy of the test compound and standardization.

BACTERIAL STRAINS, CULTURE MEDIA AND CULTURE CONDITIONS:

The microorganisms *Escherichia coli* (MTCC723), *Pseudomonas aeruginosa* (MTCC424), *Bacillus subtilis* (MTCC441) and *Staphylococcus aureus* (MTCC96) were used for assessment of test compound. The subcultures were prepared from stock cultures stored at -20°C , by inoculation in Luria-Bertani, Millerbroth (LB; BD Biosciences, India) and incubated at 37°C for 24 h in aerobic and static conditions

TEST METHOD- The following tests were performed to check the anti-microbial activity of the product.

Minimum Inhibitory Concentration- This assay determines the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism. The details are present below Test 1 and Test 2

Test 1

- 5 plates for each culture – process (spotting and spreading of components to determine the adequate technique)
1. 5 petri plates per sample were prepared
 2. Plate 1 served as a control plate (used for pre-culture for next set of experiments)
 3. Plate 2 and 3 were duplicates for spreading of Bactibarrier and spotting with bacterial culture
 4. Plate 4 and 5 were duplicates for spreading of bacterial culture and spotting with Bactibarrier (5 μl and 10 μl)
 5. The plates were incubated overnight and monitored after 24 hours.

Test 2

- 3 plates- standardizing the parameters like concentration of Bactibarrier used and duration of its effect on microorganisms in said concentration.
 1. 3 petri plates were prepared per sample
 2. Plate 1 served as control (streak of preculture on the plate to testify vitality of the pre culture)
 3. Plate 2 and 3 were duplicates of spreaded culture with spots of Bactibarrier of volume 5 μ l, 10 μ l, 15 μ l and 20 μ l.
 4. Duration of study was one week, to determine the effect of surfactant over the period of time.
- **MICROBICIDAL EFFICACY TEST:** A two level efficacy test was designed and performed.

Efficacy evaluation-

Test 1

This test performed was to understand if application of Bactibarrier on surface allows any microbial growth. Three Petri plates per sample were prepared,

- a) Plate 1 served as a control plate
- b) Plate 2 for spreading of Bactibarrier (200 μ l) and spotting with bacterial culture concentration ranging from 10^{-1} to 10^{-6} (fold serial dilution), duplicate for this test was also performed.
- c) An alteration was made for next test, the protocol was same, but instead of spotting every microbial culture in different plates, all the four microbial cultures of study were spotted in the same Petri plate.

Test 2

This test was performed to understand how well Bactibarrier is able to kill microbes/ to evaluate its disinfectant property. For testing, three plates were prepared per sample,

- a) Plate 1 served as control



b) Plate 2 served as the test plate in which different concentrations of Bactibarrier i.e. 1%, 5%, 10% were mixed in the liquid broth and then plated. After proper drying of plates spotting with bacterial culture concentration ranging from 10^{-1} to 10^{-6} , duplicate for this test was also performed.

c) Also, 10%, 15%, 20% concentrations of Bactibarrier were also studied by the same above mentioned procedure.

The plates were observed after every 24 hours till 28 days. Plates were kept at 37°C in the incubator to determine the growth by calculating colony forming units.

OBSERVATION

No growth was observed on plate with standard type strains even after 96 hours of observation with test compound (200 μl) at 20% concentrations under laboratory conditions.

Minimum Inhibitory Concentration -: Result (Page No. 7-13)

Efficacy evaluation-Test 1: For the study of 28 days, the plates were observed every 24 hours for colony forming units. It was found that the tested concentrations (200 μl) of the compound was not sufficient to access its antimicrobial activity, since from 24 hours only growth can be observed in the cultures. But for *Bacillus subtilis* and *Staphylococcus aureus* cultures growth inhibition was observed from diluted concentration of 10^{-2} and 10^{-3} respectively (on average). The results of which can be seen in Table 1-3 below. (Page No.14-16)

Test 2: From the study for the duration of 14 days, the best effective concentration was 20% of Bactibarrier for its disinfection property was observed. Though this concentration seemed only to be effective against *Bacillus subtilis* and *Staphylococcus aureus* among all the four cultures of study. The results of this test can be seen in Table 4 and 5 below (Page No. 14-18)

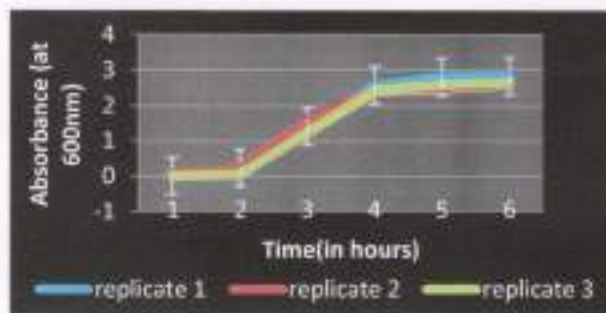
In the process of one month of study, the results remained the same from day 1 to day 28-35 days and no significant variation was observed for the test performed. Effective

concentration of the compound need to be further optimized for the microorganisms especially for *Escherichia coli* and *Pseudomonas aeruginosa*.

- **GROWTH KINETICS OF BACTERIAL STRAINS:**

All the bacterial strains were then studied for their growth under laboratory concentration kinetics. The samples of bacterial cultures were drawn after every two hours and OD was measured at 600nm. The study was done in triplicates and standard growth curves were plotted. This study carried out to understand:

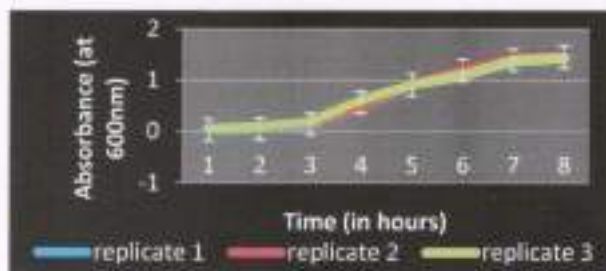
1. Bacteria used for the study were aerobic in nature while among them only *Staphylococcus aureus* is a facultative anaerobe.
2. Normal growth pattern or standard graph is in laboratory condition with and without test compound.



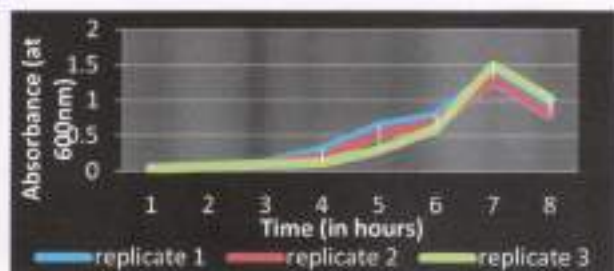
1) GROWTH CURVE of *E.coli*



2) GROWTH CURVE of *B.subtilis*



3) GROWTH CURVE of *Pesudomonas aeruginosa*



4) GROWTH CURVE of *Staphylococcus aureus*

Growth curves of all four microorganisms tested

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Table1- Laboratory conditions of bacteria with their different growth phases

Microorganisms	Relative humidity	Standard deviation	Standard error	Lag phase	Log phase
E.coli	45%	0.11	0.064	2 hours	2 hours
B.subtilis	45%	0.074	0.041	1 hours	3 hours
<i>Pesudomonas aeruginosa</i>	45%	0.029	0.017	2 hours	5 hours
<i>Staphylococcus aureus</i>	45%	0.075	0.043	4 hours	3 hours

TOXICITY ASSESSEMENTS

The various toxicological parameters evaluated for the test compound using *in silico* methodology for designing of a novel bioactive surfactant molecule and a database. The rationale of the development of antimicrobial surface agent database came from the lacuna that there is no specific database already provided on the compiled information about Quaternary ammonium compounds safety/efficiency and other alternate compound.

- 270 Quaternary ammonium chlorides based compounds were collected.
- For these compounds literature analysis has also been conducted for the acute toxicological endpoint that includes LD₅₀ values in mammals. Different Toxicological end points were predicted.
- For the mutagenicity in vitro endpoint with the Sarah Model -2.0 models, the Bactibarrier compound is predicted to be negative with 72% confidence in the prediction.






CONCLUSION







- The optimal growth curve kinetics of standard type strains *E.coli* (MTCC 723), *B.subtilis* (MTCC 121T), *S.aureus* (MTCC 96), and *P.aeruginosa* (MTCC 424) used in the study were 7,14, 28 days respectively under controlled lab conditions in enriched media without the test compound at room temperature for 96 hours.
- The study conducted in triplicates, a reduction in biomass concentration was seen from $\log 10^{-3}$ to 10^{-2} for *B.subtilis* (MTCC 121) and *S.aureus* (MTCC 96) respectively at concentration of 20% formulation of test compound used at 37°C for 96 hours.
- As the test compound was a formulation of concentrate suspended in solvent/carrier, therefore actual individual ingredient and concentrate has to be studied and optimized for dosage evaluation and efficacy.






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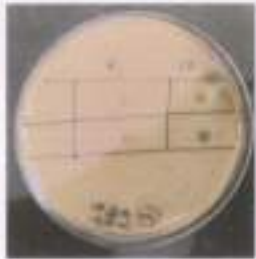




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Results: MIC Test 1

Sample name	Photo	Inference
Control : LB media		Plates show no growth, hence determines that the media was not contaminated.
<i>E.coli</i> strain		These plates had dual purpose : 1. Vitality of the strains was established 2. This pure culture can be used to prepare the pre-culture for further tests.
<i>E.coli</i> with spotting of Bactibarrier at different concentration		Inhibition zone was observed, surfactant does not allow the growth of microorganisms, bacteria in this case.
<i>E.coli</i> with spotting of Bactibarrier at different concentration (duplicate)		Duplicate plate did not show any anomaly, hence supporting the hypothesis.
Layer of Bactibarrier and streaking of <i>E.coli</i> strain		The concentration of Bactibarrier used for <i>E.coli</i> strains ($1.56 \mu\text{l}/\text{cm}^2$) was not enough to stop the growth, this test was repeated with higher concentration of Bactibarrier to achieve positive results.








<p>Layer of Bactibarrier and spotting of <i>E.coli</i> strain</p>		<p>This plate also proved that the vitality of <i>E.coli</i> surpassed the action of the Bactibarrier used.</p>
<p><i>S.aureus</i> strain</p>		<p>Fine growth was seen for SA strain</p>
<p><i>S.aureus</i> with spotting of Bactibarrier at different concentration</p>		<p>5 and 10 μl concentration was enough to stop <i>S.aureus</i> strain from growing.</p>
<p><i>S.aureus</i> with spotting of Bactibarrier at different concentration (duplicate)</p>		<p>Duplicate plates support the hypothesis</p>
<p>Layer of Bactibarrier and streaking of <i>S.aureus</i> strain</p>		<p>No <i>S.aureus</i> growth was observed, hence the Bactibarrier layer worked as an effective control</p>
<p><i>B.subtilis</i> strain</p>		<p>Strain growth observed for future use.</p>




<p><i>B.subtilis</i> with spotting of Bactibarrier at different concentration</p>		<p>Inhibition zone from Bactibarrier was very distinct for both 5 and 10 μl.</p>
<p><i>B.subtilis</i> with spotting of Bactibarrier at different concentration (duplicate)</p>		<p>Duplicate plates show the same result.</p>
<p>Layer of Bactibarrier and spotting of <i>B.subtilis</i> strain</p>		<p>The plate were clear of any growth.</p>
<p>Layer of Bactibarrier and streaking of <i>B.subtilis</i> strain</p>		<p>Marginal growth was observed in the plates.</p>
<p><i>Candida albicans</i> strain</p>		<p>Pure culture of strain obtained for further experimental use.</p>

<p><i>Candida albicans</i> with spotting of Bactibarrier at different concentration</p>		<p>Better efficiency of Bactibarrier was observed at higher concentration in comparison with lower concentration</p>
<p><i>Candida albicans</i> with spotting of Bactibarrier at different concentration (duplicate)</p>		<p>Same result was observed in duplicate plate as well</p>
<p><i>Candida glabrata</i> strain</p>		<p>Proper growth of strain for future experimental usage.</p>
<p><i>Candida glabrata</i> with spotting of Bactibarrier at different concentration</p>		<p>Distinct inhibition zone in both concentrations depict the positive competency of Bactibarrier.</p>
<p><i>Candida glabrata</i> with spotting of Bactibarrier at different concentration (duplicate)</p>		<p>Duplicate plate also portray the similar result showing high efficacy of Bactibarrier.</p>




MIC Test 2

Apart from the culture plates for pure culture, duplicates of following combinations were used to determine the optimum concentration of Bactibarrier which can restrict the growth of microorganisms on nutrient media surface. Volume of surfactant used was 5, 10, 15 and 20 μ l.



Sample	Photo	Inference
<i>E.coli</i> with spotting of Bactibarrier at different concentration		For all the four volumes EC strain showed growth.
<i>E.coli</i> with spotting of Bactibarrier at different concentration (duplicate)		Duplicate plates showed the same result, hence the concentration that should be used for highly viable <i>E. Coli</i> strain should be more.
<i>B.subtilis</i> with spotting of Bactibarrier at different concentration		Higher and lower concentrations were equally efficient, as observed by the inhibition zone.
<i>B.subtilis</i> with spotting of Bactibarrier at different concentration (duplicate)		Duplicate plates showed similar results. (merged due to overflow of spotting)
<i>S.aureus</i> with spotting of Bactibarrier at different concentration		Inhibition zone was observed which became more distinct with increasing concentration.
<i>S.aureus</i> with spotting of Bactibarrier at different concentration (duplicate)		growth of colonies
<i>C.albicans</i> with spotting of Bactibarrier at different concentration		Inhibition zone was observed distinctly for 15 and 20 μ l.

<i>C.albicans</i> with spotting of Bactibarrier at different concentration (duplicate)		Duplicate plates showed similar results.
<i>Candida glabrata</i> with spotting of Bactibarrier at different concentration		Inhibition zone was observed for every volume of Bactibarrier
<i>Candida glabrata</i> with spotting of Bactibarrier at different concentration (duplicate)		Results were consistent for duplicate plate as well

Plates after one week

Sample	Photo	Inference
<i>C.albicans</i> with spotting of Bactibarrier at different concentration.		5 and 10 μ l resisted growth, but the performance of 15 and 20 μ l from 1 week ago was more consistent.
<i>Candida glabrata</i> with spotting of Bactibarrier at different concentration		All the concentrations of the surfactant showed resistance towards fungal growth
<i>S.aureus</i> with spotting of Bactibarrier at different concentration		Distinct inhibition zone observed for different concentrations, a zone of inhibition was also observed.

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<p><i>B.subtilis</i> with spotting of Bactibarrier at different concentration</p>		<p>Very clear zone of inhibition was observed even after 1 week.</p>
<p><i>E.coli</i> with spotting of Bactibarrier at different concentration</p>		<p>EC strain did not show very clear inhibition for microbial growth.</p>

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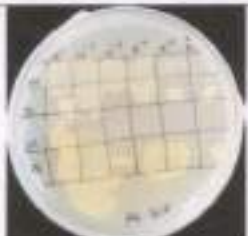

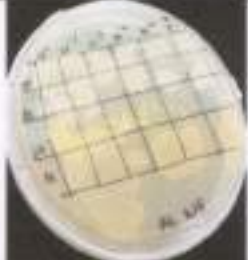

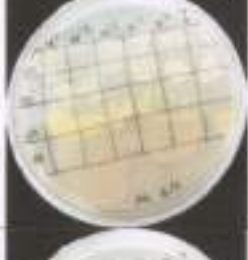
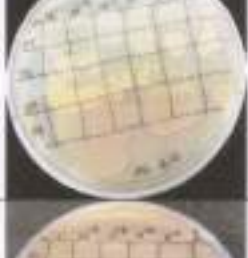

Results: MICROBICIDAL EFFICACY TEST

Table 1: Representation of the experiment test 1 (a):

	<i>E.coli</i>	<i>Bacillus Subtilis</i>	<i>Staphylococcus aureus</i>	<i>Pesudomonas aeruginosa</i>	Control
24 HR					
48 hr					
7 days					
14 days					
21 days					
28 days					

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Table 2: Representation of the experiment test 1 (b):

	All cultures	Control
24 hr		
72 hr		
7 days		
14 DAYS		
21 days		
28 days		

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Table 3: Representation of the experiment test 1 (b-repetition):

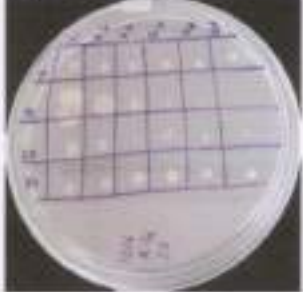

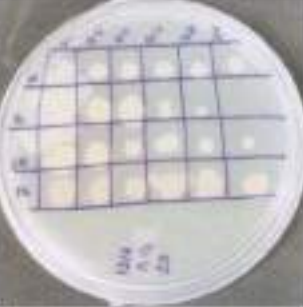
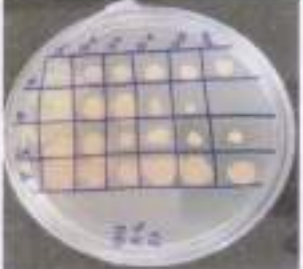

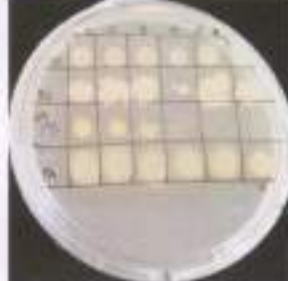
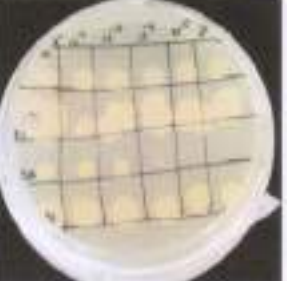
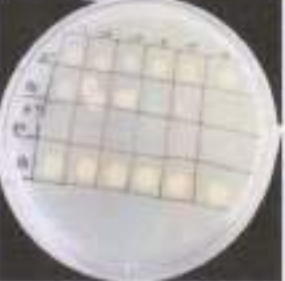
	All cultures	Control
24 hr		
7 DAYS		
14 days		

Table 4: Representation of the experiment test 2 (a):


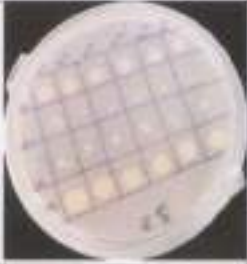
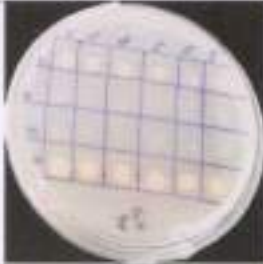
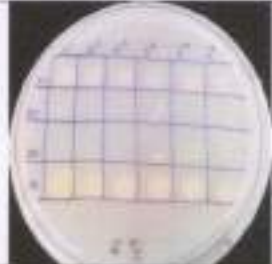


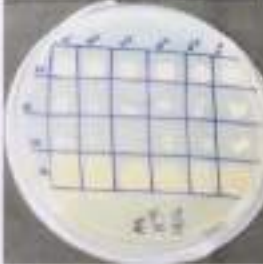



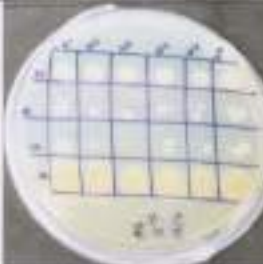
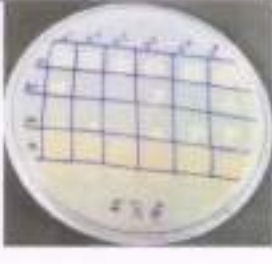

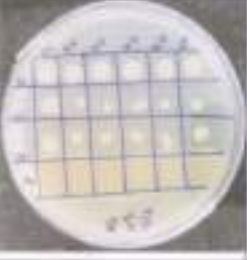


	CONTROL	1 %	5%	10%
24 hr				

7 days				
14 days				
21 DAYS				
28 days				
35 days				

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Table 5: Representation of the experiment test 2 (b):

	CONTROL	10 %	15%	20%
24 hr				
7 DAYS				
14 days				
21 days				

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