ISSN: 0975-833X

*Available online at http://www.journalcra.com*

*International Journal of Current Research*
*Vol. 7, Issue, 06, pp.17137-17143, June, 2015*

**INTERNATIONAL JOURNAL OF CURRENT RESEARCH**

**RESEARCH ARTICLE**

# MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF FOUR NEW *BACILLUS THURINGIENSIS* STRAINS

**[1]Ankita Das, [1,*]Tanushree Tulsian (Samanta) and [2]Anilava Kaviraj**

[1]Department of Physiology, Raja N. L. Khan Women's College, Midnapore, West Bengal, India
[2]Department of Zoology, University of Kalyani, Nadia, India

**ARTICLE INFO**

***Article History:***
Received 05th March, 2015
Received in revised form
28th April, 2015
Accepted 11th May, 2015
Published online 30th June, 2015

***Key words:***

*Bacillus thuringiensis*,
Gram positive,
Endospore, crystals,
Antibiotic susceptibility,
Biochemical test.

**ABSTRACT**

***Bacillus*** *thuringiensis (Bt),* a Gram positive soil bacterium is a well known biocontrol agent. Its pest specific toxicity arises from crystalline inclusion comprising of one or more polypeptides called insecticidal crystal proteins (ICPs) or delta endotoxins produced in the sporulating cells. Any strain of *B. thuringiensis* may have more than one gene for toxic proteins. Development of *B. thuringiensis* as a microbial insecticide followed from better strains, increased efficiency in production and quality control lead to the development of formulations with high activity and improved characteristics of sprays. In the present study, four new strains i.e. 1953, 6941, 4714 and 4715 of *Bacillus thuringiensis* have been taken and characterized using morphological studies, growth curve analysis, different staining methods and several biochemical tests and antibiotic susceptibility test. The four strains showed similar results in some tests as they are all Gram positive, endospore-forming, crystal forming and sporulating at the same interval of growth phase whereas the results of the different biochemical tests like starch hydrolysis test, catalase test, lecithinase production test, fermentation test, esculin hydrolysis test and Voges-Proskauer tests were quite similar among three strains out of the four strains studied. Antibiotic susceptibility test shows that most of the strains were resistant to Ampicillin.



***Citation*:** Ankita Das, Tanushree Tulsian (Samanta) and Anilava Kaviraj, 2015. "Morphological and biochemical characterization of four new *Bacillus thuringiensis* strains", *International Journal of Current Research*, 7, (6), 17137-17143.

## INTRODUCTION

The competition for crops between human and insects is as old as agriculture itself. The use of chemical substances to control pests was started before 1900s. Early insecticides were some inorganic chemicals and organic arsenic compounds. Organochloride compounds, organophosphates, carbamatespyrethroids and formamides followed them. Many of these chemicals are being used today also. Certain properties made these chemicals useful and effective that time, such as long residual action and toxicity to a wide spectrum of organisms. However, chemical pesticide applications have caused many environmental problems including insect resistance, toxicity to humans and to some beneficial insects (Glazer and Nikaido, 1995). Like all organisms, insects are also susceptible to infection by pathogenic microorganisms. Many of these infectious agents have a narrow host range and, therefore, do not cause uncontrolled destruction of beneficial insects and are not toxic to vertebrates.

***Corresponding author: Tanushree Tulsian (Samanta)***
*Department of Physiology, Raja N. L. Khan Women's College, Midnapore, West Bengal, India.*

*Bacillus thuringiensis* is a major microorganism, which shows an entamopathogenic activity (Glazer and Nikaido, 1995; Schnepf *et al.,* 1998) *B. thuringiensis* can be present in many different habitats such as soil, stored product dust, insect cadavers, grains, agricultural soils, antarctic, olive tree related-habitats, different plants and aquatic environments (Apaydin *et al*., 2004). The organism is a ubiquitous, gram-positive and spore-forming bacterium that forms crystals during the stationary phase of its growth cycle. It is characterized by its ability to produce parasporal crystalline inclusions toxic to larvae of different insect orders and other vertebrates (Crickmore, 2006; Hendriksen and Hansen, 2006).

Its insecticidal activity depends on parasporal crystals encoded by *cry* genes and this insecticidal activity varies according to the type of insect. Natural isolates of *B. thuringiensis* have been used as a biological pesticide since the 1950s for the control of certain insect species among the orders Lepidoptera, Coloeptera and Diptera. The genes of *B. thuringiensis* coding for parasporal crystals are also a key source for transgenic expression which provides pest resistance in plants (Schnepf *et al.,* 1998).

This feature makes *B. thuringiensis* the most important and effective biopesticide on the world market (Bernhard *et al.,* 1997). In 1995, worldwide sales of *B. thuringiensis* based insecticides were estimated about $90 million representing about 2% of the total global insecticide market (Lambert and Pereron, 1992; Schnepf *et al.,* 1998).

The use of chemical pesticides for pest and vector management results in death of some natural enemies and thereby necessitates repeated sprays of the insecticides leading to the development of pest and vector resistance and resurgence. Further, the chemical pesticides also pollute the environment. Biological pesticides are therefore becoming key components of integrated pest management (IPM) strategies (Obeidat *et al*. 2004). The tremendous success in microbial pesticides has come from the uses of *B. thuringiensis* (Obeidat *et al*. 2004). In this context, more such studies are required to characterize *B. thuringiensis* strains in India, so that certain new and undiscovered properties of new strains can be discovered to use them as biopesticide with a broader host range. Hence, the present study was conducted with four new strains of *Bacillus thuringiensis* (1953, 6941, 4714, 4715) which have been bought from IMTECH, Chandigarh. In order to characterize them, various studies like morphological, staining, growth curve analysis and various biochemical tests has been done along with different biochemical tests.

## MATERIALS AND METHODS

**a) Study of Colony morphology**

All the four strains were cultured on Nutrient Agar (NA) plates for 30-48 hrs at 28˚C to obtain single colonies to study the colony morphology.

**b) Growth curve analysis of the strains**

A growth curve was generated for the Bt. strains. The strains were inoculated in sterile 250 ml conical flask containing 150 ml of nutrient broth. The growth curve was generated by taking absorbance readings at an interval of 6hrs (till 110 hrs). Cell density was estimated by the measurement of absorbance at 600nm.

**c) Microscopic analysis**

While taking the reading, the sample was observed under microscope to view sporulation and autolysis stages.

**d) Staining Analysis**

**1. Gram staining**

Gram staining was done to observe the character of the strains. *Bacillus* colonies.

**2. Endospore Staining**

The endospore staining was done using Schaeffer- Fulton method. The strains were observed at different time intervals to see the sporulating phase and the autolysis phase.

**3. Coomassie brilliant blue (CBB) staining**

The *strains* were inoculated into sterile 50 ml conical flask containing nutrient broth. The inoculated nutrient broths were agitated at room temperature on orbital shaker set at 250 rpm for 4days. This is done to induce sporulation by the *Bacillus* colonies. The *strains* were characterized with Coomassie blue staining method at two stage of the *Bacillus* life cycle. The first stage is the sporulated phase while the second is known as the autolysis stage. In the sporulated phase, through CBB method, the parasporal bodies can be viewed clearly and distinguished (Rampersad *et al*., 2002). The study was made to catch the autolysis stage where all the bacterial cells would be completely lysed.

**e) Bichemical Tests**

Different biochemical tests were performed to further characterize the strains.

**1. Starch hydrolysis test**

The strains were inoculated on starch agar. The plates were then incubated for 24 hours at 30˚C. Iodine, which changes color from a yellow-brown to blue-black in the presence of starch, was applied to the agar surface and allowed to stand for 10 minutes.

**2. Mannose, sucrose and salicin fermentation test**

An inoculum from a pure culture of all the four strains was transferred aseptically to a sterile tube of phenol red mannose broth, phenol red sucrose broth and phenol red salicin broth to see the fermentation of mannose, sucrose and salicin respectively. The inoculated tubes were incubated at 25-37˚C for 24 hours. A positive test consists of a color change from red to yellow, indicating a pH change to acidic.

**3. Lecithinase production**

Strains were inoculated on egg yolk agar and incubated for 24 hours at 30˚C. The degradation of lecithin present in the egg yolk results in formation of opaque precipitate around the colonies.

**4. Catalase test**

An inoculum from the pure culture was streaked on a sterile NA plate.

The plate will be incubated at 25-37˚C for 24 hours. Growth from the plate is smeared on a clean slide and a drop of hydrogen peroxide is placed on the smear. Copious bobbles liberated in the hydrogen peroxide indicate presence of catalase.

**5. Voges-Proskauer Test**

An inoculum from a pure culture of all the four strains was transferred aseptically to a sterile tube of MRVP broth. The tubes were then incubated at 35˚C for 24 hours. After incubation, 5 drops of Barritt's A was added and gently mixed. Then 5 drops of Barrit's B was added and kept for 30 minutes. Development of red color indicates a positive test.

**6. Esculin utilization test**

Inoculum were streaked on the bile esculin slant and incubated at 25-37˚ C for 24 hours. A black coffee color indicates the use of esculin sugar.

**7. Antibiotic susceptibility testing**

The antibiotic susceptibility of the Bt strains were tested against Streptomycin, Tetracycline, Ampicilin, Amoxicilin and Oflaxacin, using the disc diffusion method (Bauer et al., 1966).

## RESULTS AND DISCUSSION

The crystal proteins from *B. thuringiensis* have been used for insect control both as biopesticides and in transgenic plants. Discovery of new insecticidal gene is of great importance for delaying the development of resistance in target insects. The diversity of *B. thuringiensis* strains facilitates isolation of new types of cry and vip which is known as vegetative insecticidal protein genes (Kaur, 2006). For better pest control, the cry genes have been transferred to plants. Stacking of more than one insecticidal gene is required for resistance management in transgenic crops. Modification of these proteins through protein engineering for increasing the toxicity and the insecticidal spectrum is also a promising approach, but requires detailed understanding of the structure and function of these proteins (Konecka *et al*., 2006; Ghelardi *et al*., 2007).

More research into this area will provide useful insights for the design of toxins for management of insect resistance (Cannon, 1996). The bacterial strains were maintained as pure cultures on Nutrient agar (NA) plates and were subjected to the above mentioned tests and the following results were drawn:

**a) Colony Morphology**

A number of dilutions were prepared to obtain the single colonies of the strains to study their morphology. The strains showed white to off white color colonies with regular margin, rough surface and slightly raised elevation (Thiery and E. Frachon, 1997). (Fig1).

**b) Growth Patterns**

The growth curve was plotted based on absorbance at 600nm at an interval of 6 hours. All the four strains (i.e. 1953, 6941, 4714, 4715) showed a similar pattern of growth where they reach their lag phase at $12^{th}$ hour and stationary phase on the $18^{th}$ hour. This stationary phase continues till $30^{th}$ hour after which the growth declines (Fig2), but again there is a rise in the curve at 48 hours, which continues till 90 hours, which might be the indication of sporulating phase that can be said by the microscopic analysis. After this the curve again started declining till $110^{th}$ hour which might be the autolysis stage, which can again be said by the microscopic analysis done.

**c) Microscopic Analysis**

Hence, from the generated growth curve and microscopic observations, the strains were identified to sporulate about 90 hours after inoculation and autolysis occurred after110 hours of incubation. It confirmed the presence of crystals in autolysis phase (Rampersad *et al.,* 2002).

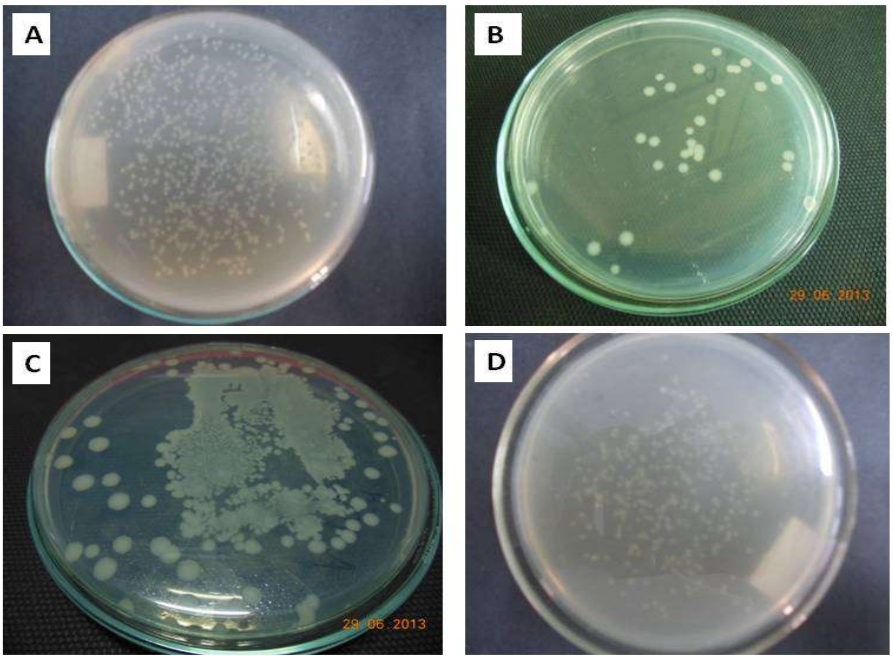



**Fig. 1. Colony morphology of the strains after 30 hours of incubation (A-1953; B-6941; C- 4714; D- 4715)**

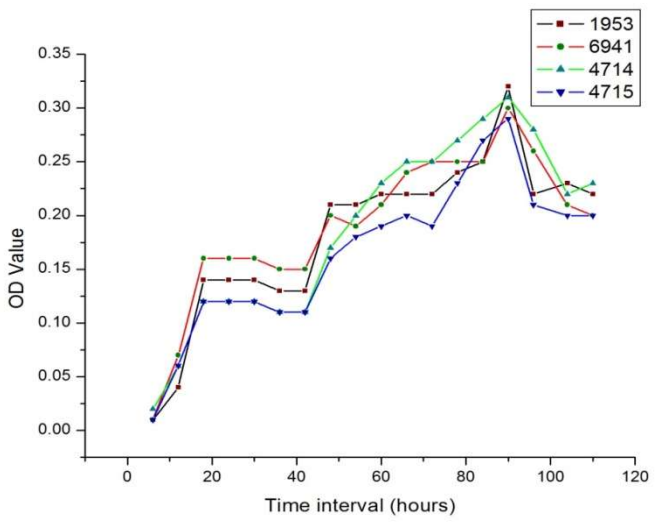



**Fig. 2. Growth curve of the strains based on absorbance at 600nm**

## d) Staining

### 1. Gram staining

Gram staining was carried out to confirm the Gram character of the strains. All strains were rod-shaped and Gram positive, which were violet in color (Fig 3). After 48 hours, the bacilli were observed to be thin and rod shaped indicating its vegetative stage (Obeidat *et al*., 2004).

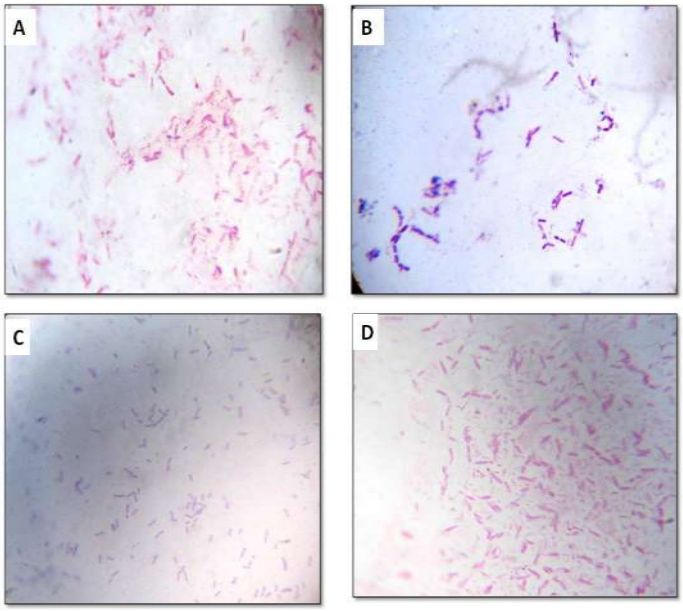



**Fig. 3. Slides showing the Gram character of the strains (1000X) (A-1953; B-6941; C- 4714; D- 4715)**

### 2. Endospore staining

The endospore staining was done using Schaeffer- Fulton method (Fig 4). This test confirmed that the strains are spore-forming (Rampersad *et al.,* 2002).

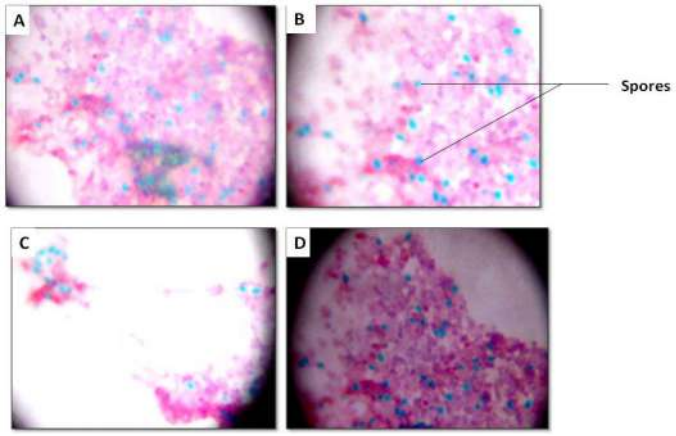



**Fig. 4. Endospore stained slides of the strains (1000X) (A-1953; B-6941; C- 4714; D- 4715)**

### 3. Coomassie Brilliant Blue staining

Spherical and rhomboidal shaped blue-stained crystal inclusions were observed after Coomassie Brilliant Blue staining (Rampersad *et al.,* 2002) (Fig 5).

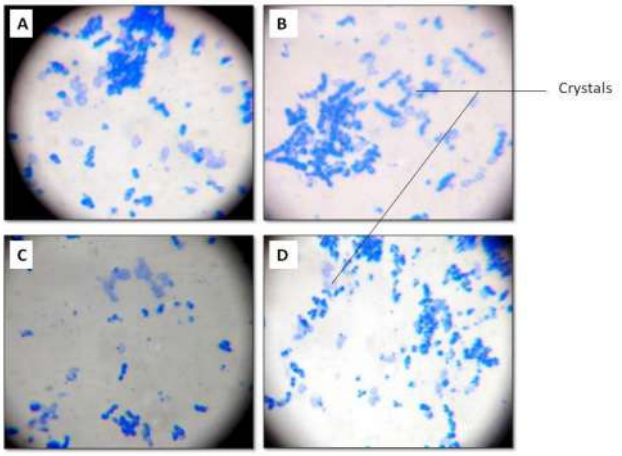



**Fig. 5. Coomassie Brilliant blue stained slides (1000X) (A-1953; B-6941; C- 4714; D- 4715)**

The microscopic analysis results have been summarized in Table 1.

**Table 1. Summary of Microscopic Tests**

| S. No. | Tests for microscopic analysis | Results for the four strains of Bt put to test |
|---|---|---|
| 1. | Gram Staining | Gram Positive |
| 2. | Spore Staining | Presence of spores (48 hours after inoculation) |
| 3. | Crystal Staining | Presence of crystals (110 hours after inoculation i.e. the autolysis phase |

## e) Biochemical Tests

### 1. Starch Hydrolysis test

After 24 hours of incubation period, the plates were flooded with Iodine. All the strains except strain 6941 showed a

positive starch hydrolysis test by giving a clear zone around the streaked colony which indicates hydrolysis of starch in that region.

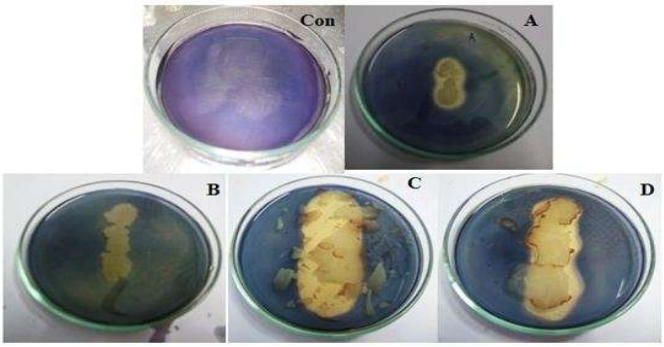
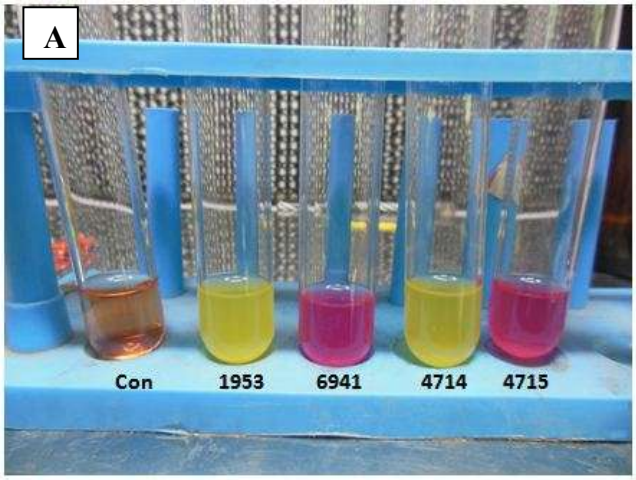



**Fig. 6. Starch hydrolysis test (Con- Control, A-1953, B-6941, C-4714, D-4715)**

## 2. Mannose, Sucrose and Salicin fermentation test

Strain 1953 gave a positive result and strain 6941 gave a negative result for all the three fermentation tests. Strain 4714 showed a positive result for mannose fermentation and negative result for both sucrose and salicin whereas strain 4715 was positive for sucrose and salicin fermentation and negative for mannose fermentation.





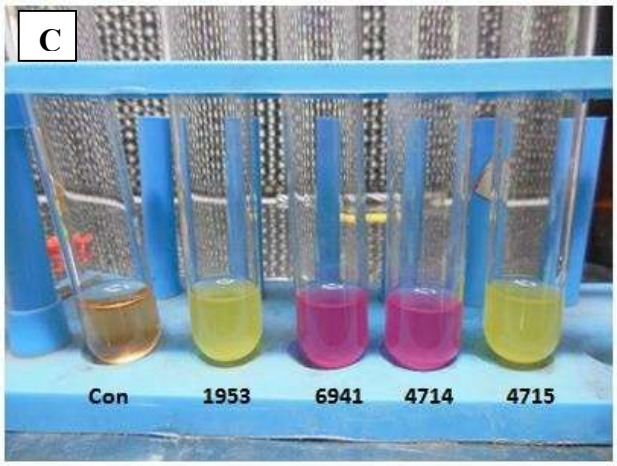



**Fig .7. Fermentation test results (A- Mannose fermentation; B- Sucrose fermentation; C- Salicin fermentation)**

## 3. Lecithinase production test

Opaque white-colored precipitation zones were observed around the colonies that showed a positive lecithinase activity on egg yolk agar. Out of the four strains, only strain 6941 showed a negative lecithinase activity.

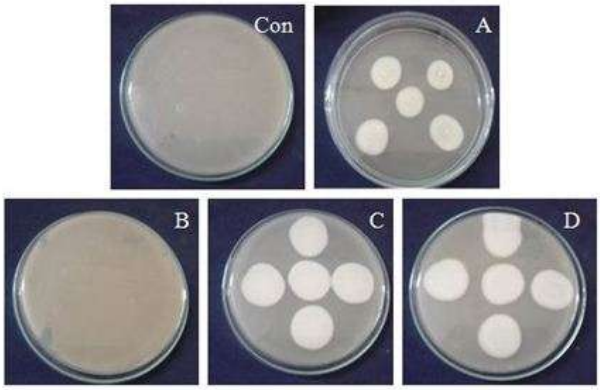



**Fig. 8. Lecithinase production test (Con- Control, A-1953, B-6941, C-4714, D-4715)**

## 4. Catalase test

All the four strains showed a positive catalase activity by bubbling within 5 to 10 seconds after addition of 3% hydrogen peroxide.


**Fig. 9. Catalase test results**

### 5. Voges- Proskauer test

After addition of the reagents (Barrit's A & B), the color of the culture remained the same as the control and that indicated a negative result for all the four strains.

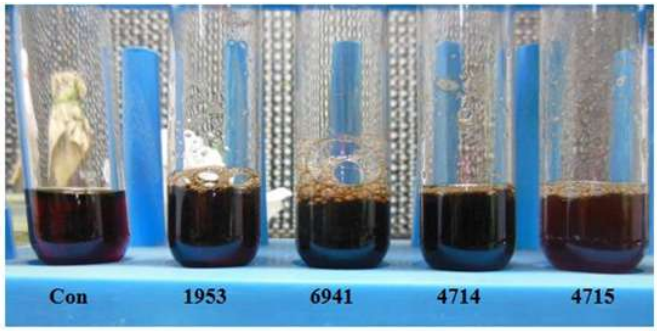



**Fig. 10. Voges-Proskauer test**

### 6. Esculin Hydrolysis test

No color change has been observed in any of the strain. All the strains gave a negative esculin hydrolysis test.

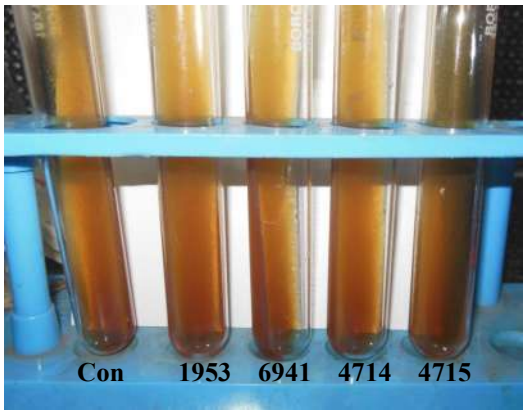



**Fig. 11. Esculin hydrolysis test**

### 7. Antibiotic Susceptibility Test

All the four strains were highly susceptible to Streptomycin, Tetracycline and Oflaxacin and least susceptible to Amoxicilin. Three strains (1953, 4714, 4715) were resistant to Ampicilin but strain 6941 is highly susceptible to it (Table 3).

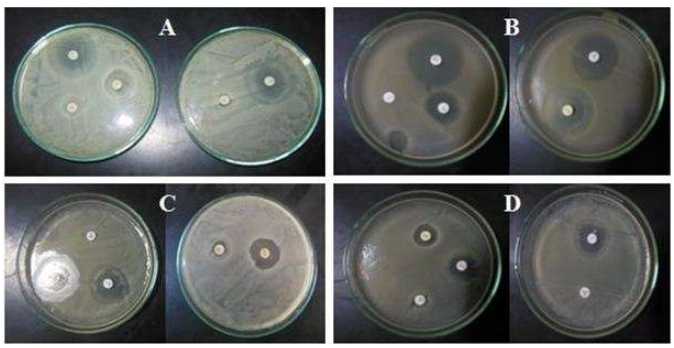



**Fig. 12. Antibiotic Susceptubility Test (A-1953, B-6941, C-4714, D-4715)**

All the biochemical tests have been summarized in Table 2. Based on spore and cell morphology, biochemical and physiological characteristics according to the procedure described in Bergrey's manual of systematic bacteriology, all the biochemical tests performed in the present study are in accordance with the results obtained by B.Eswarapriya et al., 2010.

**Table 2. Summary of Biochemical tests**

| S. No. | Biochemical Tests | Strains | | | |
|---|---|---|---|---|---|
| | | 1953 | 6941 | 4714 | 4715 |
| 1. | Starch hydrolysis test | + | - | + | + |
| 2. | Mannose fermentation test | + | - | + | - |
| 3. | Sucrose fermentation test | + | - | - | + |
| 4. | Salicin fermentation test | + | - | - | + |
| 5. | Lecithinase Production test | + | - | + | + |
| 6. | Catalase test | + | + | + | + |
| 7. | Voges-Proskauer test | - | - | - | - |
| 8. | Esculin Hydrolysis Test | | | | |

+ indicates positive result
-Indicates negative result

**Table 3. Summary of Antibiotic Susceptibility test**

| Strains | Antibiotics | | | | |
|---|---|---|---|---|---|
| | Streptomycin | Tetracycline | Ampicilin | Amoxicillin | Oflaxacin |
| 1953 | +++ | ++ | - | + | ++ |
| 6941 | +++ | +++ | +++ | + | +++ |
| 4714 | +++ | ++ | - | + | ++ |
| 4715 | +++ | ++ | - | + | +++ |

++/+++ indicates degree of susceptibility
+ indicates susceptible
_ indicates resistant

### Conclusion

Employed as a biocontrol agent for many decades against lepidopteron insects, *B. thuringiensis* formulations are known to have a narrow host spectrum, harmless to humans, mammals and non-target insects. The bacterium has a special place in organic agriculture as a bio-pesticide. In the present study, an attempt was made to characterize four new strains of *B. thuringiensis* i.e. 1953, 6941, 4714 and 4715 were characterized morphologically and biochemically in order to reach a new insecticidal protein with a greater efficacy, specificity and wider host range that will also be harmless to non target insects.

### Acknowledgements

This work was supported by grant from Department of Science and Technology (DST), New Delhi, India. Facilities were also provided by UGC-CPE grant, UGC-BSR grant and DST-FIST.

## REFERENCES

Apaydin O, Yenidu FA, Harsa S, Guznes H. 2004. Isolation and characterization of *Bacillus thuringiensis* strains from different grain habitats in Turkey. World. *J. Microbiol. Biotechnol*., 21: 285-292.

Bernhard, K., P. Jarrett , M. Meadows, J. Butt, D. J. Ellis, G. M. Roberts, S.Pauli, P. Rodgers, and H. D. Burges, 1997. .Natural isolates of *Bacillus thuringiensis*: worldwide

distribution, characterization, and activity against insect pests., *Journal of InvertebratePathology*, 70, 59-68.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein-dye binding. *Anal Biochem*., 72:248-254.

Cannon RJC. 1996. *Bacillus thuringiensis* use in agriculture: a molecular perspective. Biol. Rev. Cambridge. *Philos. Soc.,* 71: 561- 636.

Crickmore N. 2006. Beyond the spore - past and future developments of *Bacillus thuringiensis* as a biopesticide. *J. App. Microbiol.,* 101(3):616–619.

Eswarapriya B. *et al*. 2010. Insecticidal Activity of Bacillus thuringiensis IBT- 15Strain against Plutella xylostella. Int.J. PharmTech Res.Vol.2, No.3, pp 2048-2053, July-Sept 2010

Frachon. E, and T. Frachon, Identification, isolation, culture and presevation of entamopathogenic bacteria., in *Biological Techniques, Manual of Techniques in Insect Pathology*, edited by Lawrence H. Lacey, (Academic Press, 1997), 55-72.

Ghelardi E, Celandroni F, Salvetti S, Fiscarelli E, Senesi S 2007. *Bacillus thuringiensis*: Pulmonary infection, critical role for bacterial membrane-damaging toxins and host neutrophils. *Microb. Infect.,* 9(5): 591-598

Glazer, A. N. and H. Nikaido, "Microbial insecticides", in *Microbial Biotechnology Fundamentals of Applied Microbiology,* (W.H. Freeman and Company, New York, 1995), p. 209-229.

Hendriksen NB. and Hansen BM. 2006. Detection of *Bacillus thuringiensis* kurstaki HD1 on cabbage for human consumption. *Fed. Eur. Microbiol. Soc.,* 257:106-111.

Kaur S. 2006. Molecular approaches for identification and construction of novel insecticidal genes for crop protection. *World. J. Microbiol. Biotechnol.,* 22: 233-253.

Kitnamorti T, X Rathinam and S Subramaniam, 2011. Novel isolation and characterization techniques for *Bacillus thuringiensis* strains from the cabbage growing area in Cameron Highlands Malaysia. *African Journal of Microbiology Research,* Vol. 5(20), pp. 3343-3350

Konecka E, Kaznowski A, Ziemnicka J, Ziemnicki K. 2006. Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated during epizootics in *Cydra pomonella* L., *J. Inverteb. Pathol.,* 94: 56- 63.

Lambert, B., M. Peferoen, Insecticidal promise of *Bacillus thuringiensis*. Facts and mysteries about a successful biopesticide., 42, 1992, 112-122.

Obeidat M., Hassawi D. and Ghabeish I. 2004. Char-acterization of *Bacillus thuringiensis* strains from Jordan and their toxicity to the Lepidop-tera, *Ephestia kuehniella zeller*. *African J. Bio-technol.,* 3 (11): 222-226.

Rampersad J., Khan A. and Ammons D. 2002. Usefulness of staining parasporal bodies when screening for *Bacillus thuringiensis*. *J.mInvertebr. Pathol.,* 79:203-204

Schnepf, E., N. Crickmore, J. Van-Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean, .*Bacillus thuringiensis* and its insecticidal proteins.. *Microbiology and Moleculer Biology Reviews,* 62, (1998), 774-806.

*******