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EVALUATION OF COST EFFECTIVE METHODOLOGY FOR THE ISOLATION OF *BACILLUS THURINGIENSIS* AND ITS TOXIN PRODUCTION

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ABSTRACT: A modified approach has been employed for the isolation of *Bacillus thuringiensis* from soil. Thirteen bacterial isolates were presumptively identified as *Bacillus* species on the basis of their morphological and biochemical characteristics. Most of the bacterial isolates were found as positive for MR, VP, Catalase, Starch and Gelatin hydrolysis test and negative for Indole and Citrate utilization test. Large number of bacterial isolates showed glucose, sucrose, and fructose fermentation whereas, many isolates were found as negative for lactose and xylose fermentation. Agricultural wastes based mediums along with Luria broth (L.B.) were evaluated for the cost effective production of Bt toxin. Significant differences were observed in toxin content among different mediums and days of incubation. Maximum mean protein content (47.85 mg/L) was observed in L.B. medium followed by mean protein content (36.84 mg/L) found in corn flour based medium. Maximum mean protein content (31.32 mg/L) was observed on third day of incubation which suggests that third day of fermentation may be appropriate period of toxin harvesting. However, a positive correlation was observed in protein content and CFU/ml count in different mediums of toxin production at successive days of incubation. Present study suggests, this modified methodology can be used for the isolation of *B. thuringiensis* from soil. Moreover, corn flour and oat flour based mediums can be used for the production of Bt toxin at industrial level; yet there is need of more optimization for production of Bt toxin on commercial level.

KEYWORDS: *Bacillus thuringiensis*, Biopesticides, Bt toxin and Culture media

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1. INTRODUCTION

Environmental and health concerns about the use of chemical pesticides to reduce large-scale insect pest infestation undermine their applicability [1]. There is a growing urgency to develop alternative pesticides. Biological control has become a key component of crop protection worldwide and relies on the introduction of naturally occurring biological control agents (BCAs) which suppress pests and persist in the environment [2]. Among all the BCAs, *Bacillus thuringiensis* is most exploited biological control agent to produce biopesticides on the commercial level [3].Many studies have shown the potential of *B. thuringiensis* to control several agriculturally important insects of the orders Lepidoptera, Coleoptera, Diptera, etc [4-6].There are several subspecies of *B. thuringiensis* which are effective on different insects groups, such as Lepidoptera (*Bt kurstaki* and *Bt aizawai*), Coleoptera (Chrysomelidae) (*Bt tenebronis*), and Diptera (Nematocera) (*Bt israeliensis*). A large number of *B. thuringiensis* species exists in nature and there is a need for the isolation and characterization of bacterial species to produce more effective biocontrol agents. So, present study focus on the search of new isolates which can produce new combinations of insecticidal toxins.Moreover, the effectiveness of *B. thuringiensis* is due to its ability to produce protein crystals during sporulation that contain insecticidal, toxins known as endotoxins [7]. However, the low-cost production of these endotoxins (*Bt* toxins) at the commercial level is a major concern and it can be achieved by optimizing the media components and fermentation conditions. Media formulation and optimization are the key considerations in the development of bioprocesses that can produce affordable biological agents; yet limited progress has been, made in this area to satisfy market opportunities for affordable commercial biological insecticide products based on *B. thuringiensis*[8]. Hence, the present study is to determine thecost-effectiveness of potential substrates in the production of Bttoxin.

2. MATERIALS AND METHODS

1. Isolation of native *Bacillus thuringiensis* isolates

Twelve soil samples were collected from different field crops of Punjab, India. The samples were collected from depths of 10-12 cm using a sterilized spatula and placed into sterile 50 mL Falcon tubes. Samples were brought to the laboratory and stored at 4^0 C till the further use. Isolation of *B. thuringiensis* was done according to the methodology of Travers et al[9] with modifications. One gram of soil sample was suspended in ten ml of sterilized distilled water. One ml of above suspension was added to ten ml of Luria-Bertani (L.B.) broth (HiMedia, Mumbai) which was buffered with 0.25 sodium acetate pH 6.8 [10]. The broth was incubated in a shaker at 200 rpm for 4 hours at 30 ± 1^0 C. One ml aliquot was taken out from the above suspension and heated to 80^0 C for 8 minutes to eliminate vegetative cells. Resulting suspension was diluted up to 10^{-6} and the serial dilutions of 10^{-5} and 10^{-6} were used. 0.1 ml aliquot from each dilution was plated on Nutrient Agar (N.A.) (HiMedia, Mumbai) plates. Plates were incubated at 30 ± 1^0 C for 2-3 days. After incubation bacterial

colonies were selected on the morphological characteristics typical of Bt, such as lack of pigmentation, wavy edges and a circular form [11]. Colonies with typical Bt characteristics were purified and maintained on N.A. slants for further characterization.

2. Morphological and Cultural Characterization

All the bacterial isolates were streaked on N.A. plates and characterized for the morphological characteristics like colony color, colony shape, colony margin and colony surface. Bacterial isolates were cultured in L.B. broth and suspension of culture was used for gram staining. Gram staining was done as per the standard methodology given by Gram [12] to differentiate and characterize bacteria as gram positive and gram negative. Cell shape and cell arrangement were also observed.

3. Biochemical Characterization

The bacterial isolates showed Gram positive and bacillus shape under microscopic examinations were selected for the biochemical characterization.

3.1 Indole test

Tryptone broth (Tryptone 10g/L) tubes were inoculated with the pure culture of bacterial isolates and incubated at 30 ± 1^0 C for 2 days along with control. After incubation 0.5 ml of the Kovac's reagent was added. Development of colored ring indicated the positive result.

3.2 Methyl red test

Glucose phosphate peptone broth tubes were inoculated with the pure culture of test organisms and incubated at 30 ± 1^0 C for 2-3 days. Few drops of methyl red reagent was added to the broth and the appearance of red color indicated positive test.

3.3 Voges –proskauer test

Glucose phosphate peptone broth tubes were inoculated with a pure culture of the test organism and incubated at 30 ± 1^0C for 2-4 days. After incubation 0.6 ml of alpha-naphthol was added followed by 0.2 ml of 40% KOH. The tube was shaken gently to expose the medium to atmospheric oxygen and allowed to remain undisturbed for 10-20 minutes. A positive test was indicated by the development of a pink to red color

3.4 Citrate utilization test

Simmons citrate agar slants were inoculated with pure culture of test organism and incubated at 30 ± 1^0 C for 2 to 3 days. Change of agar slant color from green to blue was taken as positive [13].

3.5 Starch hydrolysis

Starch agar (1gm soluble starch powder suspended in 10 ml of distilled water and mixed with 90 ml of N.A.) plates were inoculated with test organism and incubated at 30 ± 1^0 C for 2-3 days. After incubation, the plates were flooded with Lugol's iodine solution, allowed to stand for 15-20 minutes. Appearance of clear zone around the colony indicates the hydrolysis of starch and taken as positive result.

3.6 Gelatin liquefaction

Nutrient gelatin agar test tubes were inoculated with liquid cultures of test organism by stabbing. Tubes were incubated at 30 ± 1^0 C for 2 days; after the incubation tubes were refrigerated for 30 minutes. The bacteria positive for this test, produced sufficient gelatinase that liquefied the medium which did not solidify even at refrigeration [14].

3.7 Casein hydrolysis

This test was performed as per the methodology of Seeley and Vandemark [15]. Milk agar plate was streaked with the bacterial isolate and incubated at 30 ± 1^0 C for 5-7 days. Presence of clear zone around the colony and underneath the growth indicates the hydrolysis of casein and considered as positive result.

3.8 Carbohydrate fermentation test

Fermentation of various carbohydrates viz., glucose, sucrose, fructose, lactose and xylose was performed by incorporating these sugars in the peptone water. One percent of each sugar and phenol red as an indicator (0.5%) was added to peptone water tubes [16]. These tubes were inoculated with 0.1 ml of 24 hours culture broth of test organism and incubated at 30 ± 1^0 C for 2 days. Change in color from yellow to pink is taken as positive.

4. Optimization of toxin production

4.1. Bacterial Isolate and Inoculum preparation

Bacterial isolate (B7) was selected to study the effectiveness of low cost materials for the production of Bt toxin as, it showed the fermentation of maximum number of sugars viz., Glucose, Fructose, Lactose and Sucrose. Bacterial isolate was maintained on N.A. slants at 4^0 C for further studies. Inoculum was prepared by inoculating one loop full of culture from slant into 100 ml of L. B. broth. Inoculated broth was incubated at 30 ± 1^0 C for overnight with shaking. 1 ml culture was drawn out from the broth and CFU/ml was measured by using serial dilution method. Inoculum size of 10^6 CFU/ml was prepared for inoculating different substrates based media.

4.2. Media preparation for toxin production

The toxin production in various low cost based mediums was evaluated as described by Devidas et al [8] with modifications. The Basal medium of composition (g/L): NaCl, 2.5; Na_2HPO_4, 1; $MgSO_4$, 0.2; $MnCl_2$, 0.05 and Glucose, 10 was incorporated with various agricultural waste products. Six different mediums named as M1-M6 based on agricultural waste products were prepared and evaluated for the production of Bt toxin along with L.B. broth. Mediums (pH 7.2) were autoclaved for 30 minutes at 121^0 C.

4.2a **M1 (Banana Peels**) Fresh banana peels were crushed in a motor pestle and blended in a mixer and reduced to the puree. The puree was used at a concentration of 100ml/L of the basal media.

4.2b **M2 (Orange Pulp waste)** Fresh fruit pulp waste after harvesting juice from oranges was collected from the local market and added into the basal media at concentration of 50g/L

4.2c **M3 (Sugarcane baggase)** Sugarcane baggase was collected from the local market and crushed in a motor pestle and reduced to small fibers. Finely crushed sugarcane baggase was amended in basal media at concentration of 50g/L.

4.2d **M4, M5 and M6 (wheat bran, oats flour and corn flour respectively)** Wheatbran, oats flour, and corn flour were purchased from the market in finely powdered form. These were amended into basal media at the concentration of 10g/L. 1 ml of bacterial culture at a concentration of 10^6 CFU/ml were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of basal media amended with the agricultural waste product. The flasks were incubated on the rotary shaker at 150 rpm for five days at 30 ± 1^0 C.

4.3 Protein Determination

2 ml of culture medium was withdrawn from the incubated flask at successive days of incubationand centrifuged at 4,000g for 4 minutes to get clear supernatant. Then, 1 ml of supernatant was centrifuged for 10 minutes at 10,000g and the resulting pellets were washed twice with NaCl (1mL) and twice with distilled water. Then, pellets were suspended in 1 mL of NaOH (50mM/L, pH12.5) in order to solubilize protein crystals. After 2hours of incubation at 37^0C, total proteins in the supernatant were measured by using the method of Bradford [17].

4.4 CFU Count

Culture samples were withdrawn from each flask at successive days and serially diluted, and plated onto N.A. plates. Plates were then incubated at 30 ± 1^0 C for 2 days and the developed B. thuringiensis colonies were counted and expressed in CFU (Colony Forming Units) per ml.

Data Analysis

One-way analysis of variance (ANOVA) was used to compare the results by using CPCS1 software. Means with $P < 0.05$ were considered to be significantly different.

3. RESULTS AND DISCUSSION

1.Isolation of *Bacillus thuringiensis*

Twelve different soil samples from different field crops of Punjab, India were analyzed and fifteen native isolates were selected on basis of their color, shape, texture and surface. These fifteen isolates were named as B1-15 (Table 1).

2.Morphological and cultural characteristics

All bacterial isolates showed circular colonies with irregular margins. Variations in the colony color were observed among the bacterial isolates. White color colonies were observed in B1, B3, B6, B7, B9, B10, B11, B13, and B15 bacterial isolates and Off-white color colonies were observed in B2 and B14 bacterial isolates. Whereas, creamish color colonies were observed in B4, B5, B8, and B12 bacterial isolates. However, there was not much variations were observed in colony surface of bacterial isolates. Maximum number of bacterial isolates showed wavy colony surface except B1, B4, B5, B11 and B13 isolates showed colonies with wrinkled surface (Table No. 1). All bacterial

isolates were gram positive except B8 and B11. Variation was observed in cell arrangement and size of the cells among the bacterial isolates such as B1, B3, B5, B7, B10, B12 and B13 showed rod shape and small sized cells. Whereas, B2, B4, B9, B14 and B15 isolates showed large sized and rod shape cells. However, B6 showed large sized and curved shape and B8 isolates showed small sized and round shape cells. Moreover, B2, B5, B6, B7, B8, B9, B11, B13 and B14 isolates showed single cell arrangement (Figure No.1). Cells are present in pairs in case of B3, B12 and B15 bacterial isolates. Whereas, cells are present in the long chains in case of B1, B4, and B10 isolates (Table No.2).

3.Biochemical Characterization

Thirteen bacterial isolates were selected for further biochemical characterization as, B8 and B11 were found as gram negative. All bacterial isolates were found as negative for indole and citrate utilization test. Whereas, all bacterial isolates were shown positive test for methyl red except B2 and B14 isolates. Maximum number of bacterial isolates was found as positive for VP test except B2 and B15. Starch hydrolysis was shown by maximum number of bacterial isolates, whereas; B4, B6, B14, and B15 were found as negative for starch hydrolysis. Gelatin hydrolysis was observed in all bacterial isolates except B6. Maximum number of bacterial isolates showed casein hydrolysis except B6 and B15. Delayed positive reaction for casein hydrolysis was observed in B3 and B14. All bacterial isolates were found as positive for catalase test (Table No.3).

4.Carbohydrate fermentation

All bacterial isolates showed glucose fermentation, however; B4 and B15 showed delayed reaction. Fructose was fermented by all bacterial isolates (B4, B9 and B15 showed delayed reaction). Sucrose fermentation was observed in maximum number of bacterial isolates except B5 and B10 bacterial isolates. Lactose fermentation was observed only in B7 and B10, whereas; B4 and B9 showed delayed positive reaction. All bacterial isolates showed negative reaction for xylose fermentation (Table No. 4).

5.Toxin Production by using agricultural wastes

A significant difference was observed between the protein (toxin) content in different media. The maximum mean protein content (47.85 mg/L) was recorded in M7 medium which was followed by the mean protein content (36.84 mg/L) recorded in M6 medium. The minimum protein content (4.53 mg/L) was observed in M3 medium which was followed by the mean protein content (9.93 mg/L) recorded in M1 media. A significant difference was recorded in the protein content at successive days of incubation. The maximum mean protein content (31.32 mg/L) was recorded on 3rd day of incubation which was significantly higher from mean protein content (30.22 mg/L) recorded on 2nd day of incubation. The minimum mean protein content (15.52 mg/L) was recorded on 5th day of incubation (Table No.5). Protein content increased from 1st to 3rd day and decreased from 4th to 5th day of incubation (Figure No.2).A significant difference was observed between the CFU/ml counts

in different toxin production media. The maximum mean CFU/ml ($157.1x10^6$) count was observed in the M7 medium. This was followed by mean CFU/ml ($112.78x10^6$) count observed in the M6 medium. The minimum mean CFU/ml ($15.94x10^6$) count was observed in the M3 medium and followed by mean CFU/ml ($21.28x10^6$) count observed in M1 media (Table No.6).The significant difference was observed between CFU/ml counts at successive days of incubation. The maximum mean CFU/ml ($107.4x10^6$) count was observed at 3rd day of incubation which was followed by mean CFU/ml ($96.6x10^6$) count observed at 2nd day of incubation. Minimum mean CFU/ml ($23.74x10^6$) was observed at 1st day of incubation. Increase in CFU count was observed from 1st to 3rd day of incubation. There was a steep decrease in CFU count after 4th day of incubation (Figure No. 3).

Discussion:

Soil is the natural reservoir of Bt spores and is currently preferred source for the isolation of *Bacillus*species [18-19]. Several methodologies with some modifications have been employed for the isolation of native strains of *B. thuringiensis* from soil [20-22]. Preparation of T3 medium, which most commonly used media for the isolation of *B. thuringiensis,* is a tedious task. Therefore, in our study T3 media was replaced by N.A. which is easily available media. However, heat treatment was extended to eight minutes to select only spore forming bacteria since N.A. is not a selective medium unlike T3 media. Fifteen native bacterial isolates were isolated from twelve different soil samples. Out of these fifteen bacterial isolates, thirteen isolates were screened as *Bacillus* strains according to their biochemical characters. Hence, this modified method can be exploited for the isolation of *B. thuringiensis*.However, biochemical tests are widely used for the characterization of bacterial isolates which leads to presumptive identification of bacterial isolate. All *Bacillus* isolates were found negative for indole and citrate utilization test this is in corroboration with the Padole et al [23] who isolated and biochemically characterized nineteen native isolates of *Bacillus* and compared results with one procured isolate of *B. thuringiensis*. They have reported most of the *Bacillus* isolates negative for indole and citrate utilization. Further, most of *Bacillus* isolates were found as positive for MR, VP and catalase test. This is also in accordance with Padole et al [23], they have found most isolates were positive for MR, VP and catalase test. Moreover, gelatinase and caseinase are the important enzymes of *Bacillus* species and required for the hydrolysis of gelatin and casein, respectively. Most of the native *Bacillus* isolates were found as positive for gelatin and casein hydrolysis. Carbohydrate metabolism profile of bacterial isolate also gives preliminary identification about the native strain. Glucose and fructose are monosaccharide sugars and their metabolism was observed in all bacterial isolates. Whereas, Lactose is a disaccharide and its metabolism was observed only in four bacterial isolates. Lack of lactose metabolism may be due to its disaccharide nature or the ability of bacterial isolate to produce enzymes to degrade lactose. However, Xylose is also a monosaccharide sugar with free aldehyde group but all bacterial isolates showed negative results for its metabolism. This may suggest that xylose fermentation test can be

used to detect the *Bacillus* species. Keshavarzi [24] biochemically characterized *B. thuringiensis* isolates and found that most of isolates were able to hydrolyze starch, gelatin and ferment glucose and fructose. He further reported that *B. thuringiensis* isolates did not ferment galactose and lactose. Media components play an important role in the production of Bt toxin and variability in the toxin production was observed among the different media. This is in accordance with the Devidas et al [8] who also reported variable toxin content in different media. They have suggested that toxin production is substrate specific. L.B. media showed highest production of the toxin and this can be related with the presence of adequate amount of carbohydrate and protein components. However, Corn flour and Oat flour based medium also showed significant production of Bt toxin other then all waste product based mediums and this can be linked to the presence of higher amounts of the carbohydrates and proteins. Whereas, corn flour is a good source of starch and fibers due to which higher content of toxin production has been observed. Banana peels, fruit pulp and sugarcane baggase based mediums showed significantly lower production of Bt toxin. This may be due to the lower amounts of sugars, proteins and fibers present in them. Further, the highest toxin production has been observed on third day of incubation and then a steep decrease was recorded. The toxin production has been increased from 1st day to the 3rd day of incubation due to the availability of nutrients on the initial days. This suggests that Bt toxin can be harvested during 2nd and 3rd day of the fermentation. Yield of toxin also varies according to the strain of *B. thuringiensis*. Bt toxin production varies with strain as; Shojaaddinni et al [25] evaluated two different species of *B. thuringiensis* and reported significant difference in toxin production on the similar media. This may be due to the physiological attributes of the strain. So, optimization of media components may vary according to the strain. These ambiguities may be solved by genetic dissection of the strain. CFU count varies among the media due to the different chemical constituents of each media. However, CFU/ml has been observed highest in L.B media and followed by Corn flour and oat flour media respectively. CFU count showed variable results among the days due to the availability of nutrients at successive days. A positive correlation has been found in CFU count and toxin at successive days of incubation during initial days of incubation.

4. CONCLUSION

This modified approach for the isolation of new native strains of *B. thuringiensis* may be proved as an effective methodology and further it can be improved. Novel isolates of *B. thuringiensis* are of great importance for the development of new microbial insecticides for the control of several agriculturally important insect pests. Moreover, low cost production of Bt toxin is of great commercial importance. Present study suggests the use of corn flour for the formulation of fermentation media for Bt toxin production. This study helps in the efficient production of Bt based biopesticides on the commercial level

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

7. AUTHORS' CONTRIBUTIONS

MD, SK^2 and RK designed the experiments. MD, MS, HC, SK^1 and GK performed the experiments. MD, MS and SK^2 analyzed and interpreted data. SK^2 carried out statistical analysis. MD, SK^2, MS wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLIMENTARY FILES

Table No. 1: Colony characteristics of Bacterial isolates

Sr.No.	Isolate	Colony Color	Colony Shape	Colony Margin	Colony Surface
1.	B1	White	Circular	Irregular	Wrinkled
2.	B2	Off white	Circular	Irregular	Wavy
3.	B3	White	Circular	Irregular	Wavy
4	B4	Creamish	Circular	Irregular	Wrinkled
5.	B5	Creamish	Circular	Irregular	Wrinkled
6.	B6	White	Circular	Irregular	Wavy
7.	B7	White	Circular	Irregular	Wavy
8.	B8	Creamish	Circular	Irregular	Wavy
9.	B9	White	Circular	Irregular	Wavy
10.	B10	White	Circular	Irregular	Wavy
11.	B11	White	Circular	Irregular	Wrinkled
12.	B12	Creamish	Circular	Irregular	Wavy
13.	B13	White	Circular	Irregular	Wrinkled
14.	B14	Off white	Circular	Irregular	Wavy
15.	B15	White	Circular	Irregular	Wavy

Table No. 2: Morphological characteristics of Bacterial Isolates

S.N0.	Isolate	Gram Reaction	Shape and size	Cells arrangement
1.	B1	Gram Positive	Small sized, Rod Shape	Present in chains
2.	B2	Gram Positive	Large sized, Rod shape	Single celled
3.	B3	Gram Positive	Small sized, Rod Shape	Present in Pairs
4	B4	Gram Positive	Large Sized, Rod shape	Present in chains
5.	B5	Gram Positive	Small Sized, Rod shape	Single celled
6.	B6	Gram Positive	Large Sized, Curved Shape	Single celled
7.	B7	Gram Positive	Small Sized, Rod Shape	Single celled
8.	B8	Gram Negative	Small sized, Round shape	Single celled
9.	B9	Gram Positive	Large Sized, Rod Shape	Single celled
10.	B10	Gram Positive	Small Sized, Rod shape	Present in chains
11.	B11	Gram Negative	Small Sized, Round shape	Single celled
12.	B12	Gram Positive	Small sized, Rod Shape	Present in Pairs
13.	B13	Gram Positive	Small sized, Rod shape	Single celled
14.	B14	Gram Positive	Large Sized, Rod Shape	Single celled
15.	B15	Gram Positive	Large Sized, Rod shape	Present in pairs

Table No. 3: Biochemical Characteristics of Bacterial Isolates

Sr.No.	Isolate	Indole	Methyl Red test (MR Test)	Voges-proskeur test(VP Test)	Citrate Utilization	Starch Hydrolysis	Gelatin Hydrolyis	Casein Hydrolyis	Catalase Test
1	B1	-	+	+	-	++	++	+	+
2	B2	-	-	-	-	+	++	+	+
3	B3	-	+	++	-	++	++	d	+
4	B4	-	++	+	-	-	++	++	+
5	B5	-	+	+	-	++	+	++	+
6	B6	-	+	+	-	-	-	-	+
7	B7	-	+	+	-	++	++	+	+
8	B9	-	+	++	-	+	++	+	+
9	B10	-	+	+	-	++	+	+	+
10	B12	-	++	+	-	+	++	+	+
11	B13	-	+	++	-	+	++	+	+
12	B14	-	-	+	-	-	+	d	+
13	B15	-	+	-	-	-	+	-	+

+ = positive reaction, ++ = strong positive reaction, - = negative reaction, d= delayed reaction

Table No. 4: Carbohydrates Metabolism Profile of Bacterial Isolates

Sr. No.	Isolate	Glucose	Sucrose	Fructose	Lactose	Xylose
1	B1	+	+	+	-	-
2	B2	+	+	+	-	-
3	B3	+	d	+	-	-
4	B4	d	+	d	d	-
5	B5	+	-	+	-	-
6	B6	+	d	+	-	-
7	B7	+	+	+	+	-
8	B9	+	+	+	d	-
9	B10	+	-	+	+	-
10	B12	+	+	+	-	-
11	B13	+	+	d	-	-
12	B14	+	+	+	-	-
13	B15	d	+	d	-	-

+ = positive reaction, ++ = strong positive reaction, - = negative reaction, d= delayed reaction

Table No. 5: Toxin content (mg/litre) in different production media at successive days

S. No.	**Medium**	**1st day**	**2nd day**	**3rd day**	**4th day**	**5th day**	**Mean**
1	M1	22.43±0.524	15.32±0.993	6.31±0.971	3.83±0.227	1.74±0.479	9.93
2	M2	13.23±0.848	24.12±0.765	14.47±0.538	14.2±0.310	9.10±0.746	15.03
3	M3	4.527±0.524	5.671±0.538	6.21±0.565	4.27±0.751	1.99±0.375	4.53
4	M4	15.42±0.455	23.48±0.911	34.47±0.932	16.56±0.447	14.42±0.751	20.87
5	M5	18.95±0.447	39.25±0.446	46.26±0.597	39.75±1.12	32.83±0.597	35.41
6	M6	39.45±0.822	49.25±0.895	50.34±0.344	28.90±0.524	16.26±1.07	36.84
7	M7	41.39±1.12	54.42±0.911	61.19±0.597	49.95±0.751	32.28±0.375	47.85
	Mean	22.20	30.22	31.32	22.50	15.52	
CD (5%) Media= 0.51 CD (5%) Days= 0.43 CD (5%) Media x Days= 1.15							

Values are mean ± standard deviation of three replicates.

Table No. 6: CFU count at successive days of Incubation in different toxin production media

Sr. No.	Medium	$1x10^6$ CFU/ml					
		1st day	2nd day	3rd day	4th day	5th day	Mean
1	M1	8.1±3.00	39±5.00	34.4±4.04	15.4±4.03	9.56±5.50	21.28
2	M2	9.7±6.11	58.1±4.16	81.5±5.50	34.8±2.64	13.2±4.50	39.46
3	M3	5.93±4.16	15.8±3.05	25.5±4.00	20.4±5.50	12.1±3.05	15.94
4	M4	9.6±6.00	90.9±3.60	100±5.85	91.9±6.55	51±5.00	68.68
5	M5	14.1±6.24	120.5±5.00	140.5±6.02	110.5±5.13	85.8±3.21	94.28
6	M6	25.8±3.51	160.9±7.21	165.9±3.60	120.5±4.50	90.8±2.64	112.78
7	M7	93±5.13	191±6.02	204.2±6.42	185.5±5.50	111.8±3.05	157.1
	Mean	23.74	96.60	107.52	82.71	53.45	
CD (5%) Media= 3.38 CD (5%) Days= 2.85 CD (5%) Media x Days= 7.56							

Values are mean ± standard deviation of three replicates

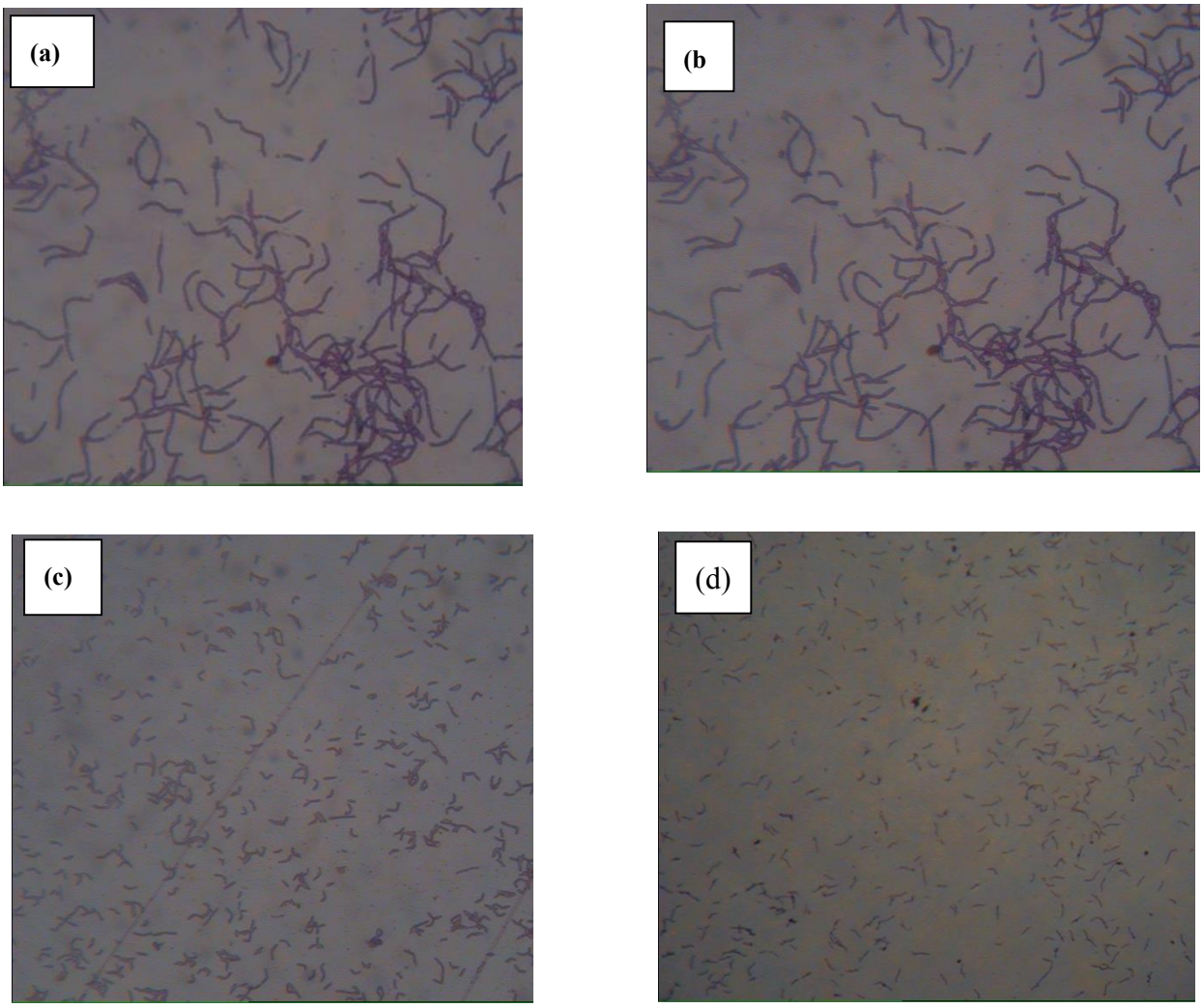

Fig 1: Morphological variation among the bacterial isolates a) B4 b) B7 c) B12 d) B13 bacterial isolates

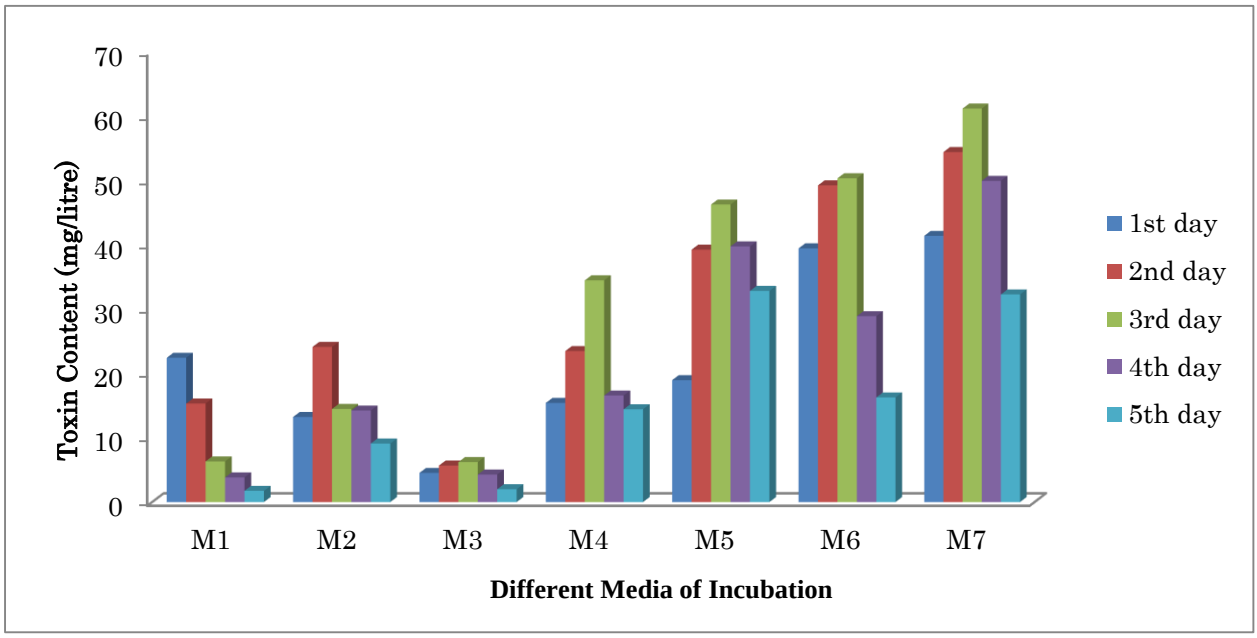

Fig. 2: Toxin content at various days of incubation in different toxin production media

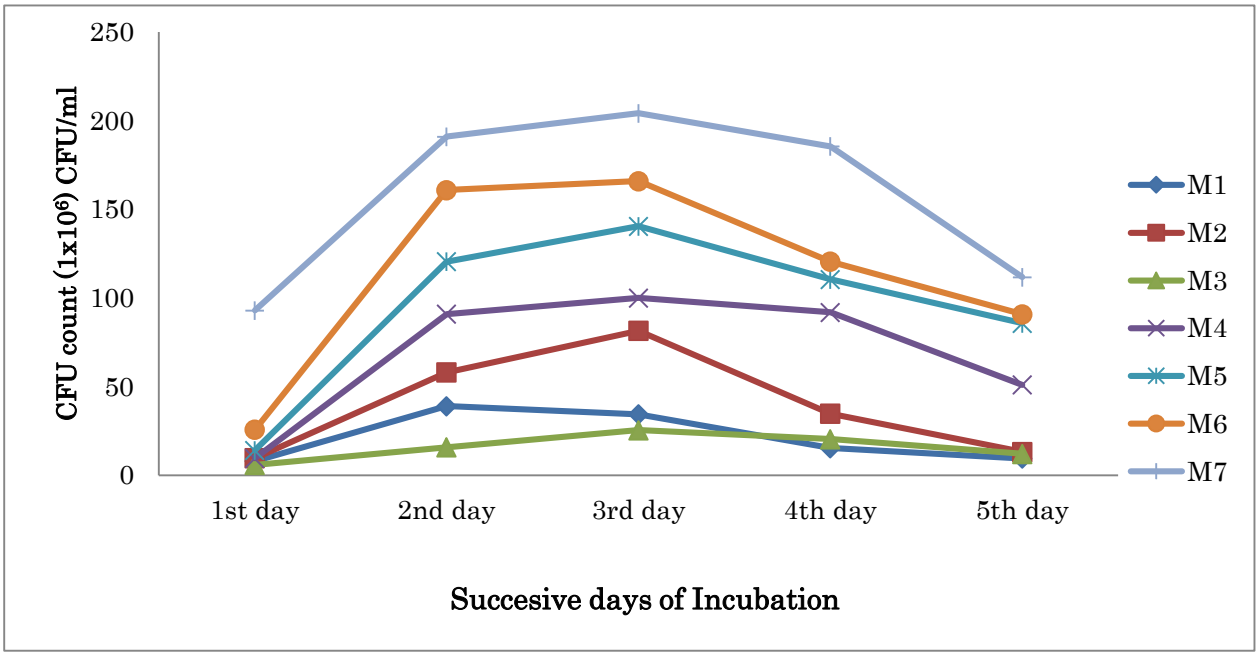

Fig 3: Change in the CFU count at successive days of incubation in different toxin production media