



## Research Article

# Formulation for optimizing *Bacillus thuringiensis* production

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**ABSTRACT:** *Bacillus thuringiensis* Berliner, a gram positive aerobic bacterium, produces parasporal crystal (Cry) toxins that are highly specific and effective against insect species. During the course of isolation of native strains, *B. thuringiensis* AUG-5 was found the most effective with a wide range of activity against lepidopterans. Hence, different media were evaluated for its growth and development. Increase in concentration of the Luria Bertani (LB), composed of casein, yeast extract and sodium chloride in 2:1:2 w/w] medium in the fermentation broth from 1 to 2% increased colony forming unit (CFU), spore and also Cry1Ac and Cry2Ab toxin content. However, further increase of LB concentration to 3% adversely affected bacterial growth and development. Addition of 1% Wesson salt in 1% LB broth significantly increased spore, CFU counts, and also that of Cry1Ac but not of Cry2Ab. Spore and CFU counts in media were positively correlated and cell mass negatively correlated with Cry1Ac and Cry2Ab contents. Of all media substituting LB with agro products, medium consisting of 2% wheat flour, 2% soybean meal and 1% Wesson salt could be considered as an alternative to LB medium to achieve economy of large-scale production costs. Spore-crystal complexes of Medium II and III were most toxic to the neonates of cotton bollworm, *Helicoverpa armigera* and tobacco caterpillar, *Spodoptera litura* at 10 µg/g, and differed significantly from those of Medium LB-2X and LB-3X and Cry2Ab2. Cry1Ac was most toxic to *H. armigera* at 1 µg/g and less toxic to *S. litura* than Cry2Ab.

**KEY WORDS:** Agro byproducts, *Bacillus thuringiensis*, endotoxin production, culture media, insecticidal activity, *Helicoverpa armigera*, *Spodoptera litura*

(Article Chronicle: Received: 13-03-2017; Revised: 28-03-2017; Accepted: 31-03-2017)

## INTRODUCTION

The use of synthetic insecticides to control insect pests started with the discovery of insecticidal properties of DDT in 1939. Since then, insecticides belonging to organophosphates, carbamates and many other chemical groups have been developed. In India, about 45,000 tons of pesticides are used annually, of which 50% are insecticides. These insecticides have been in use for a long time because of their stable residual action and toxicity to a wide spectrum of insect pests. However, there is a growing realization that some of these pesticides are disruptive to the environment. Further, their continued use leads to the development of resistance in the target species, thereby leading to their ineffectiveness and higher costs of crop protection by using higher doses and also costlier insecticides (Whalon *et al.*, 2008, 2013).

Hence, the search for alternatives has led to the development of microbial biopesticides. The most promising

microbial bioinsecticide to date is *Bacillus thuringiensis* (Entwistle *et al.*, 1993; Navon, 2000; Bravo *et al.*, 2011). More than 67 subspecies of *B. thuringiensis* have been characterized on the basis of flagellar H serovars (WHO, 1999). *B. thuringiensis* was first used for the field control of European corn borer in 1920s in France. And since then, many formulations have been developed commercially. Besides being effective and safe to the environment, *B. thuringiensis* sprayable formulations have a long shelf life (cf. Entwistle *et al.*, 1993). About 13,000 tons or more of *B. thuringiensis* is produced annually all over the world for controlling insect pests of agricultural, forestry, medical and veterinary importance (WHO, 1999). The biopesticide market accounts for about 600 million US dollars, or 2% of the worldwide crop protection market, in which about 90% of all biopesticide sales involving products based on *B. thuringiensis* (Sanchis and Bourguet, 2008). Ramanujam *et al.* (2014) reported *Bacillus thuringiensis*, as most successfully utilized insect pathogen which is used extensively for management of certain lepidopteran pests.

*Bacillus thuringiensis* is a gram-positive, spore-forming bacterium with entomopathogenic properties, belonging to the group *Bacillus cereus* (Schnepf *et al.*, 1998; Bravo *et al.*, 2011). It was first discovered in 1901 by Shigetane Ishiwata and re-described from the infected larvae of Mediterranean flour beetle in 1911 by Berliner (Nester *et al.*, 2002; Bravo *et al.*, 2011). The most important characteristic of this bacterium is the production of parasporal crystal (Cry) proteins which are insecticidal (Höfte and Whiteley, 1989; Schnepf *et al.*, 1998). These crystals are composed of one or more toxin proteins, each with its specific range of activity against target pests. The Cry toxin proteins are classified on the basis of amino acid identity and homology eliminating need of bioassays, based upon report of the *Bacillus thuringiensis* delta-endotoxin nomenclature committee in 1993 (Crickmore *et al.*, 1998, 2016). More than 318 Cry protein holotypes and approximately 827 Cry proteins have been identified on the basis of above criterion till last updated May 5, 2017 ([http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/)).

*Bacillus thuringiensis* like any aerobic bacterium grows well on the Luria Bertani (LB) medium that contains casein, yeast extract and sodium chloride. Besides, other media, such as mannitol broth, nutrient yeast extract and super broth are also in use for bacterial production. The media for the production of highly effective biomass must contain source for amino acids, proteins, and also energy producing carbohydrates along with essential minerals. Morris *et al.* (1997) reported requirement of inorganic sources that functioned as constituents of macromolecules like protein, nucleic acid, enzymes for optimal growth of *B. thuringiensis*. Similarly, nitrogenous sources were found essential for optimal growth of *B. thuringiensis* (Johnson *et al.*, 1994; Dhingra and Chaudhary, 2011). Carbohydrates, rich sources for energy like starch and molasses were also found to influence endotoxin production (Scherrer *et al.*, 1973). Media containing fish meal, gruel and corn-steep liquor have also been successfully used for production of *B. thuringiensis* and *Bacillus sphaericus* (Salama *et al.*, 1983; Kumar *et al.*, 2000; Zouari *et al.*, 2002). Media containing molasses, corn extract, and mineral salts (Moraes *et al.*, 1981; Dregval *et al.*, 1999), corn steep liquor, glucose, and mineral salts (Dregval *et al.*, 2002), or potato, common sugar and chickpea (Poopathi and Kumar, 2003), cotton seed meal, soya meal, sunflower meal (Dhingra and Chaudhary, 2011) were all used for growth and production of *B. thuringiensis*. Powders of edible leguminous seeds were used as major sources of protein together with different combinations of soluble starch and/or sugarcane molasses as major carbohydrate sources. Similarly, soybean flour, groundnut cake powder and, wheat bran extract were separately used

in large-scale production of *B. thuringiensis* bio-insecticide (Prabakaran and Balaraman, 2006). All these studies were aimed at using locally available cheaper media in place of costly sources, especially those rich in proteins like tryptone, peptone and casein. Most or all of these studies were restricted to biomass production rather than specific estimation of endotoxin production in the final fermentation product.

*Bacillus thuringiensis* strains have been isolated and characterized earlier from soil, phylloplane, dead insects, marshy soils, storage silos (Saravanan and Gujar, 2006; Meena *et al.*, 2012). Of these, the most effective *B. thuringiensis* strain AUG-5 was found to have a wide range of high efficacy against lepidopteran larvae [Saravanan and Gujar (unpublished); Kalia *et al.*, 2013]. It expresses two important toxins, Cry1Ac and Cry2Ab in high concentrations. The objective of this study was to standardize the fermentation of *B. thuringiensis* strain AUG-5 in terms of endotoxin (Cry1Ac and Cry2Ab) content, and also spore and colony forming units with the simple, locally available and cost-effective liquid substrate media.

## MATERIALS AND METHODS

### Site of Experiments

All the experiments in this research work were carried out in the Division of Entomology, Indian Agricultural Research Institute (ICAR), at New Delhi (110012), India in the year 2012- 2013.

### Equipment

A 3-liter vessel bench-top fermenter (Major Science: MS-F1-S-3L, USA) with autoclavable glass vessel was used for fermentation of *B. thuringiensis* in different fermentation media. The glass vessel could be autoclaved at 121 °C for 30 min. The vessel had a condenser to circulate coolant at 10 °C, for cooling the fermentation medium, as per requirement of the fermentation cycle. The fermenter having orifice ring sparger, three adjustable Rushton-type impellers along with removable 316L stainless steel baffles. In the fermentation system, the pre-filtered inlet air was passed through the sparger in to the bottom of the vessel, at a flow rate controlled by air flow controller. A temperature probe (platinum RTD probe: Pt-100) was provided to monitor temperature between 5 and 90 °C. The dissolved oxygen (DO) and the pH of the fermentation medium were measured by a polarographic DO sensor and the pH sensor, respectively. Cell Density Sensor (TrueCell2™; TXN CDS-AOI, Finesse solutions, USA) was used for observing the cell growth during the fermentation. All signals from the sensors were transferred to a RS-485 data acquisition system.

## Bacterial Strain

*Bacillus thuringiensis* isolate named as AUG-5 was isolated from the warehouse stored grain sample collected in 2002 from Delhi region, by using the sodium acetate selection procedure developed by Travers *et al.* (1987) and Saravanan and Gujar (2006). AUG-5 was characterized biochemically and with 16s DNA markers, and submitted to Gene Bank (Accession no. JX674043). It showed presence of *cryIA* and *cry2* genes with specific primers (unpublished). The strain is maintained on nutrient agar slants at 4 °C along with sterile nutrient broth (Hi-Media) containing 20% glycerol at -80 °C for further analysis.

## Media Preparation

*Sources of Agro-byproducts and their Production.* Wheat (*Triticum* spp.) flour (Aashirvad, ITC limited, Mumbai, India), corn flour (Blue Bird Foods India Private Limited, Mumbai, India) and soybean (*Glycine max*) seed powder (Viraki Brothers, Navi Mumbai, India) were purchased from the grocery store, New Delhi, India. Cotton (*Gossypium* spp.) seed powder was obtained by grinding non-transgenic cotton (var. Pusa 8-6, IARI, New Delhi, India) seeds without seed coats.

Chemicals like Luria broth, Wesson salt and yeast autolysate were obtained from Himedia laboratories Pvt. Ltd., Mumbai. All other chemicals were locally procured from the reputed companies. *Medium LB-1X(BOD)* was composed of commercially available LB Broth [containing 1% casein enzymic hydrolysate, 0.5% yeast extract, 1% sodium chloride (NaCl)]. Fermentation was done by the use of incubator shaker (C24; New Brunswick Scientific, Edison, NJ, USA) at 37 °C and 200 rpm. *Medium LB-1X* was composed of LB broth (containing 1% casein enzymic hydrolysate, 0.5% yeast extract, 1% NaCl). For this medium, composition was same as LB-1X(BOD), but the fermentation was carried out in the 3 liter Fermenter with automatic monitoring of DO, pH, temperature and density. *Medium LB-2X* was prepared by doubling the quantity of LB broth (containing 2% casein enzymic hydrolysate, 1% yeast extract, 2% NaCl). *Medium LB-3X* was prepared by tripling the amount of LB broth (containing 3% casein enzymic hydrolysate, 1.5% yeast extract, 3% NaCl). *Medium I* was composed of 2% wheat flour, 2% cotton seed powder, 1% Wesson salt mixture. *Medium II* was composed of 2% soybean seed powder, 2% wheat flour, 1% Wesson salt mixture. *Medium III* was composed of composed LB broth (1% casein enzymic hydrolysate, 1% yeast extract: 0.5% NaCl) and 1% Wesson salt mixture. *Medium IV* was composed of 1% corn flour, 0.2% casein (protein rich), 0.5% yeast autolysate, 0.2% peptone, 0.1% sucrose, 0.5% NaCl and 0.4% Wesson salt mixture. *Medium V* was composed of 1% corn

flour, 1% soybean seed powder, 0.2% casein (protein rich), 0.5% yeast autolysate, 0.2% peptone, 0.1% sucrose, 0.5% NaCl and 0.4% Wesson salt mixture. *Medium VI* was composed 1% corn flour, 1% cotton seed powder, 0.2% casein (protein rich), 0.5% yeast autolysate, 0.2% peptone, 0.1% sucrose, 0.5% NaCl and 0.4% Wesson salt mixture. *Medium VII* was composed 1% wheat flour, 1% Soybean seed powder, 0.2% casein (protein rich), 0.5% yeast autolysate, 0.2% peptone, 0.1% sucrose, 0.5% NaCl and 0.4% Wesson salt mixture.

Ingredients of media I, II, IV, V, VI and VII were soaked in distilled water for overnight at room temperature, and then homogenized in a laboratory grinder (if necessary) and filtered through a plastic strainer (100 mesh size). The clear supernatant was used. All culture media were adjusted 7.5±0.2 pH and autoclaved at 121 °C under 15 lb/in<sup>2</sup> for 30 min (Morris *et al.*, 1997; Shojaaddini *et al.*, 2010).

## Seed Culture Preparation for Inoculation

A loopful of bacteria from the mother culture was streaked onto a sterile nutrient agar plate, incubated at 37 °C for 24 h. Seed culture was prepared by shaken flask technique. A single colony was transferred to each 250 ml conical flask containing 50 ml of LB broth and incubated in a shaker at 200 rpm for 24 h at 37 °C temperature.

## Fermentation

Batch fermentation was carried out under the fully aseptic conditions to avoid cross contaminations during the whole run. A 10% (vol/vol) inoculum was transferred from the Erlenmeyer flask to the fermenter vessel, which contained only 1 liter of the total medium. The fermentation was done at 37 °C, 300 rpm and pH controlled automatically at 6.8-7.0 by the addition of 2N sulphuric acid and 3N sodium hydroxide as per needs for each cycle. The air flow rate was maintained at 0.5-1.5 vol/vol. min (agitated at the 300 rpm) through sterile disk type air filter (Millipore; diameter: 45 mm, pore size: 0.2 µm). It was manually controlled in parallel with agitation speed to maintain the DO level above 20% for the fermentation. No antifoam agent was added in the medium. In all cycles, samples were collected aseptically from the vessel at different intervals up to the end of the whole cycles. In all cycles peristaltic pumps were assigned for acid, base and sample recovery. Peristaltic pumps were connected through the silicon tubing (inner diameter: 3.2 mm) and inlet and outlet speed was adjusted by making the changes in rpm of the pump (Range: 0-100 rpm). For Medium LB-1X(BOD), fermentation was done in incubator shaker at 37 °C and 200 rpm. When the cultures were fully sporulated, they were harvested aseptically to prepare acetone precipitated spore-crystal complex.

### Cell Mass Yield

Fermentation process was stopped after reaching stationary phase in all media. The harvested culture was centrifuged at 8,000 rpm at 4 °C for 10 min. The supernatant was discarded and the pellet collected and re-suspended in 5% lactose solution at 1/10<sup>th</sup> the original volume of the broth (25 ml) and stirred continuously for 30 min, then four volumes (100 ml) of ice-cold acetone were added slowly and stirred for another 30 min. The acetone precipitated powder containing spore-crystal complex was filtered through Whatman filter paper with a vacuum pump and dried overnight in a desiccator, then ground to fine powder, weighed and stored at 4 °C till use (Dulmage *et al.*, 1970). Dry weight was calculated and expressed in grams per liter (g/l).

### Toxin Estimation with ELISA

Cry1Ac and Cry2Ab contents in spore-crystal complex of each isolate were estimated with their respective ELISA kits as per manufacturer's instructions (Quan-T ELISA, DesiGen, Jalna, India). The 2 mg of spore-crystal complex of each product obtained by fermentation was weighed in an eppendorf tube, to which 200 µl of the sample extraction buffer was mixed. In the case of liquid samples, 100 µl of culture broth was mixed with 100 µl of the sample extraction buffer. In both cases, the suspension was sonicated for 30 sec at 50% duty cycle, chilled on ice for 10 min followed by 30 sec sonication again. Then, suspension was vortexed and spinned for 15 min at 8,000 rpm, at 4 °C. The pellet was discarded and the supernatant was proteolyzed (3.5 µl of trypsin was added to each 100 µl sample). The mixture was then incubated for 30 min at 37 °C, after that, 2.5 µl of 50 mM PMSF per 100 µl was added to each sample in the case of Cry1Ac or Cry2Ab estimation and stored at -80 °C.

Positive and negative controls were prepared and divided in two halves. To each well, 150 µl of secondary antibody were added, followed by addition of selected medium samples per well for the quantification of Cry1Ac and Cry2Ab, respectively. Both plates were incubated at 37 °C for one-and-half h in humid chamber. Then the plates were washed for 5 min twice with 1x wash buffer provided with kit, followed by addition of the tertiary antibody (250 µl /well). The plates were incubated at 37 °C for 45 min in humid chamber. After this incubation, 250 µl of freshly prepared *p*-nitrophenyl phosphate solution (0.1 mg/ml) were added to each well and the plates were incubated for 30 min at room temperature. Absorbance of the developed color formed was read at 405 nm after auto zero against control. The quantity of toxin produced by each isolate was calculated from the standard curve.

### Bioassays of *Bacillus thuringiensis* Spore-Crystal Complex from Culturing on Different Media and Cry Toxins

The test insects viz., cotton bollworm *Helicoverpa armigera* and tobacco caterpillar, *Spodoptera litura* were reared in the laboratory on the chickpea based artificial diet. Adult female moths of *H. armigera* and *S. litura* laid eggs on cotton cloth and filter paper, respectively. The eggs were kept at room temperature under moist conditions of about 60% relative humidity (RH) until their hatching. The method of bioassay was essentially as per Gujar *et al.* (2000). The spore-crystal complex of each selected medium was dissolved in the water to prepare stock solution of 1000 µg/ml on the basis of total Cry toxin content. The final concentration of spore-crystal complex of *B. thuringiensis* of 10 µg/g was made in 10 gm artificial diet. An aliquot of about 3 gm diet was offered to 10 neonates of each test insect in a Petri plate for the period of 96 h. Four treatments viz., Medium LB-2X, LB-3X, Medium II and III were used as their Cry2Ab/Cry1Ac ratios were 41, 22, 8.7 and 15.0, respectively. Besides, MVP II (19.7% Cry1Ac) and the Bt corn leaf powder (6 mg Cry2Ab2/g) meant for resistance monitoring (from Monsanto Research Centre, Bangalore, India) were used as standards at final respective concentrations of 1 µg/g and 10 µg/g, while control did not contain any toxin in the artificial diet. As many as 6 replicates for each treatment were kept. The mortality was recorded at 24 h interval until 7 days after treatment and analyzed for significant differences with analysis of variance using SAS software. The data of mortality on the 4<sup>th</sup> and 7<sup>th</sup> day of treatment were subjected to Statistical Analysis System (SAS) version 4.2 (SAS Institute Inc. Cary, USA) to determine significant differences with one-way analysis of variance (ANOVA). The significantly different means (<0.05) were separated using Tukey's Studentized Range Test.

### Colony Forming Units (CFU)

The samples were drawn at different intervals and analyzed for CFU to indicate presence of the viable cells. Total CFU was determined by spread plate method using nutrient agar plate. Samples were withdrawn at an interval of 24 h, serially diluted with autoclaved distilled water, mixed on a vortex and 0.1 ml of the each dilution above than 10<sup>-3</sup> plated on nutrient agar plates in five replicates. Bacterial colonies were counted after overnight incubation at 37 °C using a digital colony counter, and then total CFU were calculated.

### Total Spore Counts

The culture samples were collected in triplicates from each fermentation cycle. The number of individual spore was counted directly in a Neubauer counting chamber (Fein-Optik, Blankenburg, Germany) under a phase-contrast microscope at 400 x magnification.

### Growth of *Bacillus thuringiensis* in Selected Medium

The growth of *B. thuringiensis* in the eleven different fermentation media was monitored with cell density sensor throughout fermentation at 650 nm. Fermentation was terminated as soon as absorbance reached constant over 2-3 h.

### Microscopic Studies

The presence of spore-crystal complex was recorded at various growth stages by phase contrast microscopy (DM 2500; LEICA Microsystems, Germany). The bacteria in the form of cells, spore-crystal complex in different fermentation media were harvested and suspended in water, and then smeared over the glass slide and viewed at 1000x magnification under oil emulsion in phase contrast microscopy to determine completion of spore formation.

### SDS Gel Electrophoresis of Toxin Proteins

*Bacillus thuringiensis* spore-crystal complex obtained from the selected fermentation media was characterized using the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as per Laemmli (1970). Four mg spore-crystal complex of the selected isolates as well as standard (reference medium) were solubilized in 400 µl of solubilization buffer (50 mM sodium carbonate buffer, 10 mM dithiothreitol, pH 10.5) and sonication process was carried out twice at output 2 sec pulse, 50% duty cycle, output control setting 8; timer on 2 min (LABSONIC L, Braun Biotech International, Germany). Solubilized samples were incubated at 37 °C for 3-4 h with slow shaking and after that centrifuged at 10,000 rpm for 10 min; supernatant containing solubilized protoxin samples were transferred to new sterilized tubes. Solubilized protoxin were resuspended in equal amount of sample buffer (0.6 ml of 1 M Tris buffer (pH 6.8), 5 ml 50% glycerol, 2 ml 10% SDS, 1 ml 1% bromophenol blue, 50 µl -mercaptoethanol, 0.9 ml water) and boiled at 95-100 °C for 5 min. Ten µl of sample was then loaded and separated by SDS-PAGE using a continuous gel on 8% resolving gel and 5% stacking gel. The gel was stained in staining solution (0.25% Coomassie brilliant blue R250 in methanol: distilled water: acetic acid; 40: 50: 10) for 1 h and destained in destaining solution (methanol: distilled water: acetic acid; 40: 50: 10 (v/v)) for 2 h with gentle shaking. The protein bands were visualized and photographed in a Gel Doc system (Alphaimager™ Documentation and analysis system).

### Statistical Data Analysis

All data obtained from the determination of cell mass, delta-endotoxin production, CFU and spore counts were statistically analyzed by the SAS software using the one-way ANOVA test to compare the means. Correlation coef-

ficients of cell mass, CFU and spore counts vis-à-vis Cry toxin contents were calculated to determine relationship using SAS software. The significantly different means (<0.05) were separated using Tukey's Studentized Range Test.

## RESULTS AND DISCUSSION

### Fermentation

During fermentation, pH of media ranged from 6.8 to 7.2, DO levels remained constant at 20%, temperature at 37 °C and absorbance at 650 nm varied from 0.75 to 2.80 at the time of harvest depending upon medium and duration of fermentation. The sample culture collected from different fermentation medium was observed for vegetative cells, spores and crystal. Initially, until 24 h of culture growth, highest numbers of vegetative cells were recorded. Depending upon the fermentation medium, the spore-crystal complex occurred. Presence of maximal sporulation and also of the high absorbance at 650 nm was considered criteria of completion of run. Media LB-1X and LB-1X(BOD) were harvested after 72 h, whereas, medium LB-2X, medium I, II, III, VII were harvested after 48 h. In media LB-3X and medium V stationary phase achieved after 60 and 45 h, respectively. The bacterial growth in Medium IV and medium VI reached stationary phase after 29 and 26 h, respectively.

### Cell Mass (Spore-Crystal Complex)

The dry cell mass of AUG-5 obtained by different fermentation media varied. Out of all examined media, only the medium VI had the highest biomass production (5.44 g/l), as compared to the other media including the reference LB (Table 1). The biomass produced from medium V and VI was at par, which was followed by medium I, III and IV. Fermentation done in incubator shaker LB-1X(BOD) yielded the lowest biomass of 1.58±0.17 g/l.

### Cry1Ac and Cry2Ab Toxin Quantification

In all fermentation media, Cry1Ac content of the harvested spore-crystal complex ranged from 5.6 to 139.3 ng/mg powder. Similarly, Cry2Ab content ranged from 0 to 4235 ng/mg powder (Table 2). Cry1Ac estimation was also done for the liquid samples from the fermenter collected at 24 h. It ranged from 28.2 to 1120 ng/ml at 24 h, whereas the range was from 10.4 to 1500.6 ng/ml at 48 h of fermentation in different media. Cry2Ab toxin also varied from 0 to 5778.0 ng/ml at 24 h and 0 to 7205.2 ng/ml at 48 h of fermentation in different media (data not presented). Ratio of Cry2Ab/Cry1Ac differed from less than 0 to as high as 41.14 depending upon the medium used for culturing bacterium. In spore-crystal complexes from medium VI and VII, only Cry1Ac was found to be 12.92 ng/mg and 5.62 ng/mg, respectively.

**Table 1. Details of fermentation process and production of spore-crystal complex of *Bacillus thuringiensis* in different fermentation media**

Medium Annotation	Fermentation method	Incubation Time (h)	Medium Composition	Mass (g/l) <sup>1</sup> ± SE
LB-1X(BOD)	Incubator shaker	72 h	1% Casein enzymatic hydrolysate, 0.5% Yeast extract, 1% NaCl	1.58±0.17 <sup>f</sup>
LB-1X	Fermenter	72 h	1% Casein enzymatic hydrolysate, 0.5% Yeast extract, 1% NaCl	2.52±0.13 <sup>e</sup>
LB-2X	Fermenter	48 h	2% Casein enzymatic hydrolysate, 1% Yeast extract, 2% NaCl	2.48±0.03 <sup>e</sup>
LB-3X	Fermenter	60 h	3% Casein enzymatic hydrolysate, 1.5% Yeast extract, 3% NaCl	2.66±0.04 <sup>e</sup>
Medium I	Fermenter	48 h	2% Wheat flour, 2% Cotton seed powder, 1% Wesson salt mixture	4.16±0.04 <sup>e</sup>
Medium II	Fermenter	48 h	2% Wheat flour, 2% Soybean meal, 1% Wesson salt mixture	3.14±0.01 <sup>d</sup>
Medium III	Fermenter	48 h	1% Casein enzymatic hydrolysate, 0.5% Yeast extract, 0.5% NaCl) and 1% Wesson salt mixture	4.00±0.01 <sup>e</sup>
Medium IV	Fermenter	29 h	1% Corn flour, 0.2% Casein (protein rich), 0.5% Yeast autolysate, 0.2% Peptone, 0.1% Sucrose, 0.5% NaCl and 0.4% Wesson salt mixture	4.02±0.01 <sup>e</sup>
Medium V	Fermenter	45 h	1% Corn flour, 1% Soybean meal, 0.2% Casein (protein rich), 0.5% Yeast autolysate, 0.2% Peptone, 0.1% Sucrose, 0.5% NaCl and 0.4% Wesson salt mixture	4.60±0.06 <sup>b</sup>
Medium VI	Fermenter	26 h	1% Corn flour, 1% Cotton seed powder, 0.2% Casein (protein rich), 0.5% Yeast autolysate, 0.2% Peptone, 0.1% Sucrose, 0.5% NaCl and 0.4% Wesson salt mixture	5.44±0.04 <sup>a</sup>
Medium VII	Fermenter	48 h	1% Wheat flour, 1% Soybean meal, 0.2% Casein (protein rich), 0.5% Yeast autolysate, 0.2% Peptone, 0.1% Sucrose, 0.5% NaCl and 0.4% Wesson salt mixture	4.66±0.11 <sup>b</sup>

<sup>1</sup>Mean and standard error (SE) were generated by the SAS System; using the one-way analysis of variance (ANOVA), Tukey's Studentized Range (HSD) Test ( Means followed by same letters within a column are not significantly different [F=229.57; df= 10; P <0.0001]

**Table 2. Spore, colony forming unit (CFU) count and quantitative estimation of Cry1Ac and Cry2Ab toxin produced by *Bacillus thuringiensis* in different fermentation media**

Medium Annotation	Fermentation equipment	Incubation Time (h)	Spore/ml <sup>1</sup> ± SE	CFU/ml <sup>1</sup> ± SE	Cry1Ac (ng/mg) <sup>1</sup> ± SE	Cry2Ab (ng/mg) <sup>1</sup> ± SE	Cry2Ab/ Cry1Ac Ratio (ng/mg)
LB-1X (BOD)	Incubator shaker	72 h	9.55±0.20 x10 <sup>8(f)</sup>	7.33±0.33 x10 <sup>8(f)</sup>	83.69±1.25 <sup>c</sup>	2607.50±1.23 <sup>c</sup>	31.16
LB-1X	Fermenter	72 h	13.53±0.49 x10 <sup>8(e)</sup>	12.67±0.33 x10 <sup>8(e)</sup>	108.88±0.99 <sup>b</sup>	3610.00±0.99 <sup>b</sup>	33.16
LB-2X	Fermenter	48 h	27.67±0.60 x10 <sup>8(c)</sup>	24.33±0.33 x10 <sup>8(c)</sup>	102.93±2.99 <sup>b</sup>	4235.00±175.99 <sup>a</sup>	41.14
LB-3X	Fermenter	60 h	9.98±0.02 x10 <sup>8(f)</sup>	8.67±0.67 x10 <sup>8(f)</sup>	60.11±1.02 <sup>c</sup>	1340.00±130 <sup>c</sup>	22.29
Medium I	Fermenter	48 h	4.03±0.04 x10 <sup>8(hi)</sup>	3.43±0.08 x10 <sup>8(g)</sup>	32.99±0.38 <sup>f</sup>	565.34±35.83 <sup>f</sup>	17.14
Medium II	Fermenter	48 h	6.37±0.04 x10 <sup>8(g)</sup>	4.93±0.23 x10 <sup>8(g)</sup>	83.34±1.82 <sup>c</sup>	726.49±15.07 <sup>f</sup>	8.72
Medium III	Fermenter	48 h	87.33±0.17 x10 <sup>8(a)</sup>	84.67±0.88 x10 <sup>8(a)</sup>	139.31±0.71 <sup>a</sup>	2082.09±5.79 <sup>d</sup>	14.95
Medium IV	Fermenter	29 h	17.75±0.03 x10 <sup>8(d)</sup>	16.00±0.58 x10 <sup>8(d)</sup>	69.11±1.98 <sup>d</sup>	1545.58±40.95 <sup>e</sup>	22.36
Medium V	Fermenter	45 h	52.67±0.44 x10 <sup>8(b)</sup>	52.00±0.58 x10 <sup>8(b)</sup>	90.52±1.25 <sup>c</sup>	1556.38±68.74 <sup>e</sup>	17.19
Medium VI	Fermenter	26 h	3.20±0.03 x10 <sup>8(i)</sup>	2.77±0.12 x10 <sup>8(g)</sup>	12.92±0.87 <sup>g</sup>	0.00±0.52 <sup>g</sup>	0.00
Medium VII	Fermenter	48 h	5.28±0.18 x10 <sup>8(gh)</sup>	4.30±0.30 x10 <sup>8(g)</sup>	5.62±1.37 <sup>g</sup>	0.00±0.80 <sup>g</sup>	0.00
<i>p</i> value	--	--	<0.0001	<0.0001	<0.0001	<0.0001	--
<i>F</i>	--	--	8325.13	3047.73	761.18	378.4	--
<i>df</i>	--	--	10	10	10	10	--

<sup>1</sup>Mean and Standard error (SE); Figures followed by different letters within a column are significantly different as per Tukey's Studentized Range (HSD) test.

### Efficacy of Spore-Crystal Complex of *Bacillus thuringiensis* against *Helicoverpa armigera* and *Spodoptera litura*

MVP11 Cry1Ac was highly effective against *H. armigera* (100% mortality) even at 1 µg/g, while Cry2Ab caused a maximum mortality of 46.7 %, 4 days after treatment. All other treatments at 10 µg/g caused 100% mortality within 2 days. It seemed that low Cry2Ab/Cry1Ac ratio (8.7 in Medium II) caused the highest mortality (ca. 63.3%) of *H. armigera* larvae one day after treatment as compared to other treatments arising out of other media. In the case of *S. litura*, Cry2Ab2 caused maximal mortality of 60%, while the spore-crystal complex of Media LB-2X and LB-3X caused about 80% at par, and Media II and III 100% mortality each at 10 µg/g concentration, 7 days after treatment. Further, *S. litura* was less susceptible to the spore-crystal complex than *H. armigera* (Table 3).

### Colony Forming Units (CFU)

The cell growth of AUG-5 isolate in all fermentation media was determined by CFU count method (Table 2). A maximum viable cell count of  $84.67 \pm 0.88 \times 10^8$  CFU/ml was observed in Medium III, followed by medium V ( $52.00 \pm 0.58 \times 10^8$  CFU/ml), then Medium LB-1X ( $12.67 \pm 0.33 \times 10^8$  CFU/ml), and finally the Medium LB-1X(BOD) ( $7.33 \pm 0.33 \times 10^8$  CFU/ml). In the Medium VI, lowest CFU count  $2.77 \pm 0.12 \times 10^8$  CFU/ml was observed. Similarly, in Medium I, it was  $3.43 \pm 0.08 \times 10^8$  CFU/ml, and in Medium II,  $4.93 \pm 0.23 \times 10^8$  CFU/ml.

### Microscopic Examination and Total Spore Counts

Microscopic examination of cells, spore and crystals under oil at 1000x magnification revealed presence of spore and crystals as cells crossed stationary phase. Later, spores-crystal complexes burst out and few cells were seen

at the fag end of sporulation. Spore count produced by the different fermentation media varied. The spore count of Medium III was the highest ( $87.33 \pm 0.17 \times 10^8$  /ml) than the Medium VI ( $3.20 \pm 0.03 \times 10^8$  /ml). In media LB-1X(BOD), LB-1X, LB-2X, and LB-3X, observed spore counts were  $9.55 \pm 0.20 \times 10^8$  /ml,  $13.53 \pm 0.49 \times 10^8$  /ml,  $27.67 \pm 0.60 \times 10^8$  /ml,  $9.98 \pm 0.02 \times 10^8$  /ml, respectively (Table 2).

Cry1Ac contents in the spore-crystal complexes were positively correlated with CFU counts ( $r^2$ , 0.46) and spore counts ( $r^2$ , 0.47) of nine media, and negatively correlated with cell mass ( $r^2$ , 0.26) of eleven media (Fig. 1). Similarly, Cry2Ab contents in the spore-crystal complexes were positively correlated with the CFU counts ( $r^2$ , 0.68) and spore counts ( $r^2$ , 0.68) of nine media, and negatively correlated with cell mass ( $r^2$ , 0.51) of eleven media (Fig.1).

### SDS-PAGE of Cry Toxin

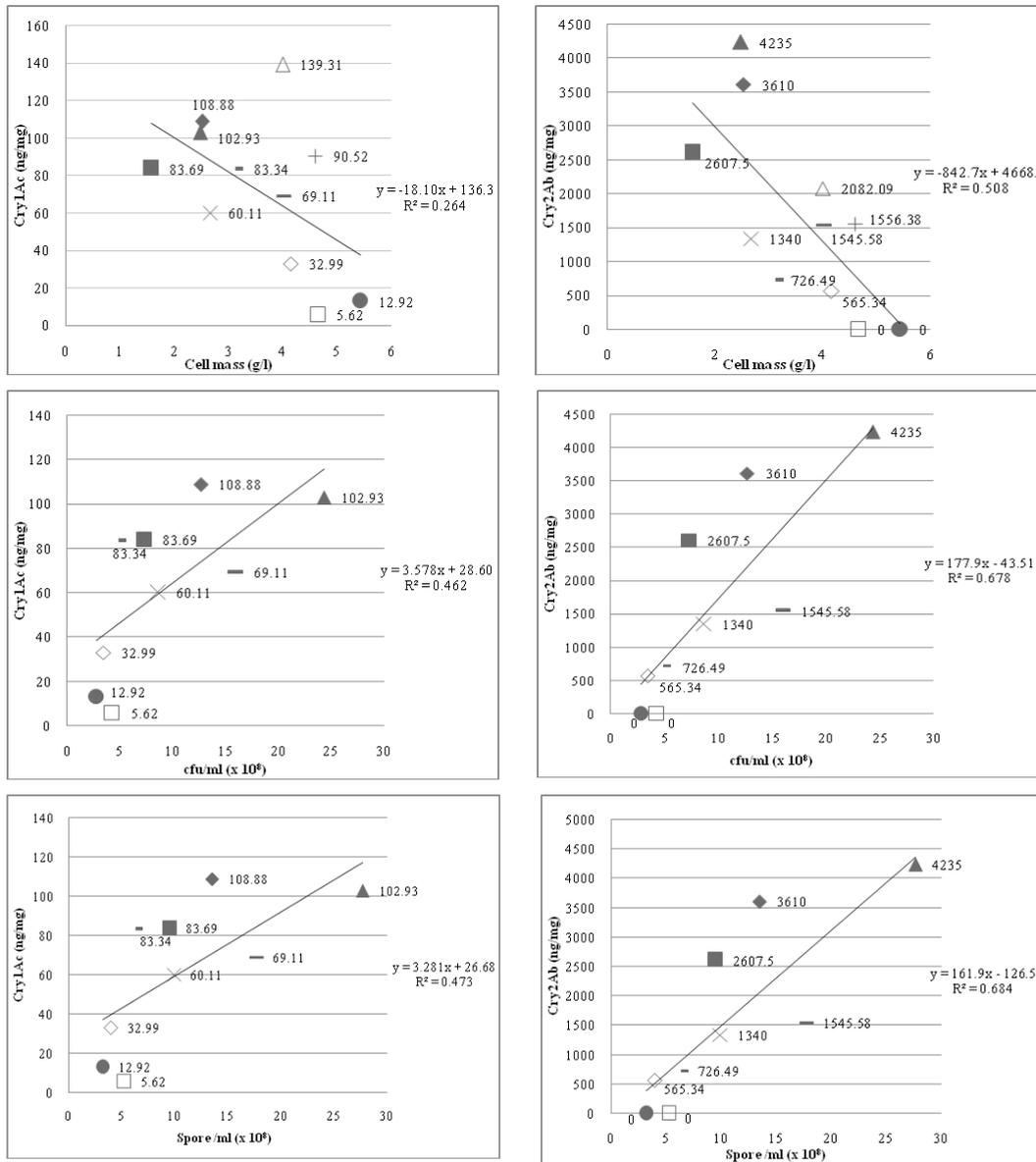
The protein profile of endotoxin complex of the bacterial isolate from selected fermentation media revealed presence of major protein bands of about 130, 63-75 and 48 kDa (Fig. 2). These proteins may belong to Cry1 and Cry2 protoxins and their activation products. The protein of 29 kDa may correspond to the cytolytic toxin.

One of the most important aspects of fermentation is sourcing for readily available relatively cheap agro byproducts. These sources provide necessary carbon, nitrogen, and other essential minerals for the vegetative cells, spores and crystal toxin growth. Various agricultural nitrogen sources like defatted and expeller cakes of mustard *Brassica*, cottonseed, groundnut, corn steep liquor have been used to develop low-cost fermentation media in batch culture process (Johnson *et al.*, 1994). High spore yield was reported with a medium containing cheese whey, soybean milk and mo-

**Table 3. Toxicity of spore-crystal complex of *Bacillus thuringiensis* in different fermentation media at 10 µg/g except Cry1Ac at 1 µg/g to the neonates of *Helicoverpa armigera* and *Spodoptera litura***

Fermentation product	Mean mortality (%) <sup>1</sup> ± SE						
	<i>H. armigera</i>				<i>S. litura</i>		
	1 day	2 day	3 day	4 day	1 day	4 day	7 day
LB-2X	48.33±4.77 <sup>b</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>	25.00±2.24 <sup>a</sup>	75.00±4.28 <sup>b</sup>	81.67±4.01 <sup>b</sup>
LB-3X	43.33±4.22 <sup>b</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>	25.00±2.24 <sup>a</sup>	71.67±3.07 <sup>b</sup>	80.00±2.58 <sup>b</sup>
Medium-II	63.33±3.33 <sup>a</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>	36.67±4.94 <sup>a</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>
Medium-III	55.00±4.28 <sup>ab</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>	26.67±4.94 <sup>a</sup>	100.00±0.00 <sup>a</sup>	100.00±0.00 <sup>a</sup>
Cry1Ac	13.33±2.11 <sup>cd</sup>	33.33±4.22 <sup>b</sup>	68.33±3.07 <sup>b</sup>	100.00±0.00 <sup>a</sup>	5.00±2.24 <sup>b</sup>	26.67±2.11 <sup>d</sup>	31.67±1.67 <sup>d</sup>
Cry2Ab2	25.00±2.24 <sup>c</sup>	40.00±3.65 <sup>b</sup>	46.67±4.22 <sup>c</sup>	46.67±4.22 <sup>b</sup>	6.67±4.94 <sup>b</sup>	51.67±4.01 <sup>c</sup>	60.00±3.65 <sup>c</sup>
<i>p</i> value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>F</i>	47.62	401.43	383.91	622.50	15.09	201.26	249.40
<i>df</i>	6	6	6	6	6	6	6

<sup>1</sup>Mean and standard error (SE) were generated by the SAS System; using the one-way analysis of variance (ANOVA), Tukey's Studentized Range (HSD) Test (Means followed by same letters within a column are not significantly different).



**Fig. 1.** Analysis of cell mass, CFU and spore count estimated in crystal-spore complex of *Bacillus thuringiensis* strain AUG-5 in different fermentation media ■-LB-1X(BOD),◆-LB-1X, ▲-LB-2X, ×-LB-3X, ◇-Medium I, -Medium II, Δ-Medium III, —Medium IV, +-Medium V, ●-Medium VI, □-Medium VII (data with extremely high values of spore and CFU counts as in Media III and V removed).

lasses (Alves *et al.*, 1997). *B. thuringiensis* also requires, for optimal growth, a variety of macro and microelements which function as co-factors of numerous enzyme reactions (Sarrafzadeh *et al.*, 2005). *B. thuringiensis* isolates showed maximum growth after 48 h ( $1.3 \times 10^{10}$  cells/ml) when 10 g/l of cottonseed meal was added in the production media (Harish, 2006). Valicente *et al.* (2010) developed a medium with  $1.4 \times 10^9$  spores/ml after 96 h of fermentation and was toxic to the *Spodoptera frugiperda* first instar larvae.

Cotton seed meal, chickpea, defatted groundnut cake, gram flour, soybean, defatted soya flour and corn gluten

meal were found to be the most efficient substrates for the production of *B. thuringiensis* biomass (Morris *et al.*, 1997; Poopathi and Kumar, 2003), whereas for the production of *B. sphaericus* strain egg yolk provided a low cost yet efficient medium (Yadav *et al.*, 2011).

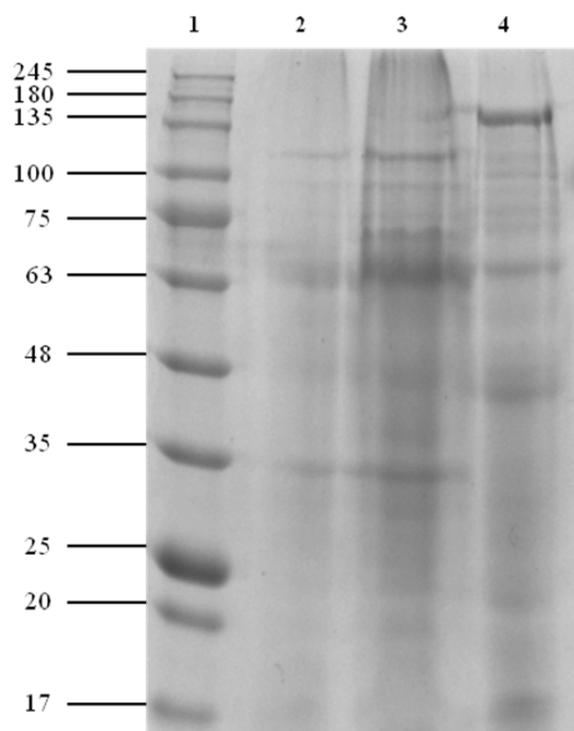
Bacteria in the incubator shaker grew at a slower rate than those in the automated fermenter, as the former was without pH and DO controls. Increase in oxygen concentration was shown to enhance bacterial growth in the medium containing molasses and corn steep liquor (Moraes *et al.*, 1981).

Usually, pH pattern of fermentation medium of *B. thuringiensis* in the incubator-shaker (without automatic pH control) showed a definite trend. Initially, pH dropped slightly followed by increase even up to 9.0 on the completion of fermentation. In the case of fermenter, the pH of medium was auto-controlled and maintained at 6.8 to 7.0 with a little quantity of alkali and acid to ensure fast bacterial growth as reflected by spore-crystal mass in fermenter rather than in the incubator-shaker. Similar observations were also recorded by Abdel-Hameed *et al.* (1991).

Increase in concentration of LB medium from 1 to 2% in fermentation broth enhanced spore and CFU counts; and also of Cry2Ab content; but not of Cry1Ac content, but further increase of LB concentration to 3% adversely affected spore, CFU and toxin contents. Addition of Wesson salt was found to enhance cell mass as well as endotoxin (Cry1Ac and Cry2Ab) production as seen from AUG-5(6) vis-à-vis Medium III. Dulmage (1971) reported that endotoxin production by *B. thuringiensis* varied over the medium in which was grown. Khodair *et al.* (2008) reported differences in the production of cell mass based upon the media used for culturing *B. thuringiensis* and also showed wide variation in the mortality of cotton leafworm, *Spodoptera littoralis* on the basis of differences in media used. Positive relationship between Cry toxin contents and CFU or spore counts and the negative relationship with cell mass suggest that the CFU and spore counts are also useful criteria. It is also likely that negative relationship with cell mass is indicative of presence of unutilizable nutrients in the cell mass at the end of fermentation. This is in contrast with cell mass criterion for production used earlier. All other agrobased media except Medium VI and VII were found to support growth and development of *B. thuringiensis*, but not as efficiently as LB medium. Further, Medium VI and VII were quite deficient in supporting bacterial growth and Cry toxin production. These studies show that nutritional constituents should be qualitatively as well as quantitatively balanced for optimal bacterial growth. Further, fermentation media seemed to influence the relative proportion of two Cry toxins in the final product. The toxicity of spore-crystal complex from different media caused the fastest and the highest mortality of *H. armigera* at 10 µg/g which suggested innate synergistic susceptibility of test insect to the mixture of Cry toxins. It is well known that Cry1Ac is highly toxic to *H. armigera* than Cry2Ab. Although initial response of larvae of *S. litura* over first four days to the toxicity of Cry2Ab and spore-crystal complex of various media was slow and varied, Media II and III caused 100% mortality of larvae of *S. litura* 7 days after treatment. As reported in the present study, Cry2Ab is known to be more toxic to *S. litura* than Cry1Ac while Cry1Ac is more toxic to *H. armigera* than Cry2Ab (Lu *et al.*, 2013). The spore-crystal complexes of

media with higher ratios of Cry2Ab/Cry1Ac were expected to be more toxic to *S. litura* than others, but were not discriminatory in the present case. However, spore-crystal complexes of Media II and III with Cry2Ab/Cry1Ac ratio of 15 or less were more toxic to *H. armigera* than those with higher ratios. It appeared that hereto, mixtures of Cry1Ac and Cry2Ab was more toxic than Cry1Ac or Cry2Ab to neonates of *S. litura*. The higher toxicity of Medium II may also be attributed to the presence of toxin proteins as these are reported in soybean (Hwang *et al.*, 1978; Oliveira *et al.*, 2010).

These studies showed that Medium II consisting of 2% wheat flour, 2% soybean meal and 1% Wesson salt could prove to be good alternative to LB medium in terms of economy of input cost if production is taken upon the large scale.



**Fig. 2.** SDS-PAGE analysis of acetone precipitated spore-crystal complex of best selected medium and reference *Bacillus thuringiensis* strain. From left to right: (1) BLUeye prestained protein marker (GeneDirex®): 245, 180, 135, 100, 75, 63, 48, 35, 25, 20, 17, 11 kDa, (2) Crude protein of *Bacillus thuringiensis*-AUG-5 in LB-2X medium, (3) Crude protein of *Bacillus thuringiensis*-AUG-5 in Medium-III, (4) HD-1 (*Bacillus thuringiensis* spp. *kurstaki*).

#### ACKNOWLEDGEMENT

Thanks are due to the Director, Indian Agricultural Research Institute, New Delhi for providing infrastructural facilities. We are also thankful to National Agricultural In-

novation Project (NAIP), Indian Council of Agricultural Research, New Delhi, for providing funding and fellowship to fulfill all necessity while research work. Author is also thankful to Prof. Aditya Shastri, Vice-Chancellor, Banasthali University and Prof. Vinay Sharma, Head, Department Bioscience & Biotechnology, Banasthali University, Banasthali, Rajasthan.

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