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*Full Length Research Paper*

# Enhanced sporulation and toxin production by a mutant derivative of *Bacillus thuringiensis*

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***Bacillus thuringiensis* (*Bt*) strains along with insecticidal crystal proteins (ICP) also produce proteases, and chitinases during growth and sporulation. These both potentate the activity of ICPs. This work aimed to obtain bioinsecticide over-production and thermotolerant mutant through classical mutagenesis of vegetative cells of *Bt* by using UV-mutation multiple times. The isolated survivors were screened on the basis of their production of ß-glucosidase, delta-endotoxins and biomass in glucose-based medium. Maximum spore and crystal proteins were produced at 40°C with corn steep liquor as nitrogen source and hydrol as a carbon source. The best mutant MUV7 supported significantly (*P* = 0.0001) higher values of all kinetic parameters than those supported by the wild culture. Lower demand of activation energy (47.4 kJ mol$^{-1}$) for crystal protein formation revealed that the best mutant was comparable with those of thermophilic group of organisms.**

**Key words:** Bioinsecticide, biomass, delta-endotoxin, insecticidal crystal proteins, kinetics, spores.

## INTRODUCTION

The unique ability of *Bacillus thuringiensis* to produce insecticidal crystal proteins (ICP) or δ-endotoxin (Ghribi et al., 2004) has made this organism the most successful commercial biopesticide (Içgen et al., 2002). These ICPs are produced during sporulation. After ingestion, the ICPs contained within the parasporal crystals are released into the insect mid-gut and get solubilized there. Intoxicated insect larvae stop feeding within few minutes and eventually die (Chang et al., 2001). ICPs produced by *Bt* strains are toxic to the larvae of diptera, lepidoptera, coleoptera, hymenoptera, homoptera, and mallophaga insects. They are also toxic to many of the nematodes, flatworms and sarcomastigophora (Duan and Wu, 2002; Zi-Quan et al., 2007).

Currently, there is interest in the improvement of bioinsecticide production by using an adequate fermentation technology, overcoming the problem of metabolic regulation by carbon and nitrogen sources, application of classical mutagenesis for improvement of *Bt* strains and induction of abiotic tolerance (Ghribi et al., 2004; Thamthiankul et al., 2004). Brar et al. (2007) reported that entomotoxicity of *Bt* preparations is due to the toxin crystals, cell spore complex of *Bt* strains, ICPs, and vegetative insecticidal proteins (VIPs). Therefore, both extra-cellular and intracellular components of *Bt* strains must be admixed to enhance the toxicity of *Bt* preparations (Vu et al., 2009).

Like other industrial strains, genetic modification of candidate organisms has been achieved by genetic recombination, mutation and using appropriate selection techniques. In such studies, major problem is the selection technique for putative mutant strains. Currently, it has been reported that ß-glucosidase is resident in the crystals of *B. thuringiensis* strain 1.1 (Papalazaridou et al., 2003). The enzymatic properties of commercial β-glucosidase and those residents in the insecticidal

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crystals of *B. thuringiensis* strain 1.1 are very identical and both exhibit almost equal levels of entomogenic activity against *Drosophila melanogaster* larvae and, additionally, bring about reduction in survival of adult flies after their treatment. Thus, we hypothesized that the selection strategy may be based on the level of ß-glucosidase secretion in plate selection media as reported earlier (Papalazaridou et al., 2003). This approach may save extensive effort done earlier by other workers by studying each putative mutant colony in independent submerged fermentation and select out the most potential strain for mass production of biopesticide.

## MATERIALS AND METHODS

### Organisms

Strain of *B. thuringiensis Bt*JR7 was procured from NIBGE and *B. thuringiensis* subsp *kurstaki* NSTD1 (a gift of National Agricultural Research Centre, Islamabad, Pakistan) was used as a control strain. The Bt7JR cry$^{-1}$ and cry mutant strain lacking toxin crystal were used as a negative control and to determine final toxin amounts.

### Chemicals and media

All chemicals and storage media used were purchased from M/S Roth Company Germany. All chemicals were of analytical grade.

### Improving *Bacillus thuringiensis Bt* JR7 for bioinsecticide production

UV mutagenesis was carried out according to earlier workers (Ghribi et al., 2004; Saxena et al., 2002). An inoculum (50 ml) was prepared in LB medium up to an OD of 0.4 and exposed to UV-rays using a 30 W tube lamp emitting UV light at a wavelength of 240 nm and kept at a distance of 10 cm away for 30, 60, 90 and 120 min. After each exposure, serial dilutions were used to calculate the lethality percentage as described earlier (Ghribi et al., 2004). Following UV irradiation, the suspension was transferred to Dubos salt medium (pH 7) and grown for 24 h at 37°C. Cells which had survived irradiation were collected and retreated with UV irradiation. This cycle was repeated once more. The treated cells were centrifuged and transferred to liquid Dubos Salts Medium (DSM) and were grown for 24 h at 55°C. At the end of the second cycle of treatment, the cells were plated on nutrient agar plates containing esculin (0.2%) and ferric ammonium citrate (0.05%) and incubated for 48 h at 50°C as described previously (Ghribi et al., 2004). The colonies that produced bigger black zones were screened and one colony (UVM7) was over-producing mutant and was selected for characterization for overproduction of bioinsecticide in 23 L fermenter (working volume 10 L).

### Hydrol and corn steep liquor (CSL)

Both hydrol (corn molasses) and corn steep liquor are cheap carbon and nitrogen sources respectively and were obtained from Rafhan Maize Products, Faisalabad. In production medium when hydrol or CSL were used as carbon and nitrogen source in Dubos salt medium, no mineral supplements were added because both of them have sufficient minerals to support microbial growth. When glucose was used with other nitrogen sources, only then Dubos salts (DSMG) (Rajoka, 2005) were used. We earlier optimized 55 ml hydrol (50 g sugars L$^{-1}$ medium), CSL (45 g L$^{-1}$), yeast extract (5 g L$^{-1}$) and ammonium sulphate (6.6 g L$^{-1}$) to maintain C:N ratio at 7.0. Tween 80 was added at 1.0 g L$^{-1}$.

### Intracellular spore and endotoxin production screening

All cultures were processed for the production of spores and δ-endotoxins in hydrol medium at different temperatures ranging from 25 to 55°C in a microprocessor controlled 23-L stainless steel fermenter (Biostat C5, Braun Biotechnology, Melsungen, Germany) containing 10 L of culture medium as described earlier (Ghribi et al., 2004). The medium pH = 7 (found optimum) was adjusted with $H_3PO_4$ and was steam-sterilized *in situ* for 1 h. The inoculum was prepared in 1000-mL conical flasks containing glucose (10 g L$^{-1}$) in DSM (pH = 7) grown at 30°C on an orbital shaker (150 rpm for 24 h). Ten percent of this freshly prepared inoculum was aseptically transferred to the fermentation medium (pH = 7). This medium pH was controlled automatically using $NH_4OH$ during all fermentation experiments at 40°C or as described. Aeration was carried out through a sparger at 0.5 vvm. Foaming was controlled by adding pre-autoclaved silicone oil. Time course measurements were done in triplicate and repeated twice.

### Sample harvesting

Samples were collected after different time intervals namely; 8, 12, 20, 24, 32, 40, 48, 60 and 72 h. These samples were centrifuged at 10,000 rpm for 15 min and supernatant was used to measure free sugars and extra-cellular protein while cell pellet was used to determine cell density, and intra-cellular protein (toxin) as described earlier (Ghribi et al., 2004). Spores were estimated by counting colony forming units (CFU) on samples heated at 80°C for 10 min and plating appropriate dilutions on solid LB medium and incubating at 37°C overnight. Delta-endotoxin concentration in each sample was determined in the solubilized crystal preparation from each treatment. For this purpose, 10 mL of culture medium was centrifuged at 10,000 rpm for 10 min and the pellet was washed twice with 1 M NaCl and twice with distilled water. This pellet was suspended in 10 ml of 50 mM NaOH (pH 12.5) to solubilize δ-endo-toxin crystals. After 2 h incubation at 37°C, total proteins in the supernatant were determined according to the method of Bradford (1976) and reducing sugars by DNS method (Miller, 1959). A acrystalliferous strain, Bt7JR cry$^{-1}$ was applied as a negative control, to consider possible contribution of dissolved proteins from spore coat, intracellular proteins, cell debris and insoluble materials. This negative control was applied in all experiments and all cultural conditions. We calculated toxin contents as the result of subtracting the total proteins determined using the *Bt* 7JR cry$^{-1}$ strain from the total proteins determined with the toxin producing organism. All values are the mean of three independent determinations of triplicate experiments for each cultural condition. The toxin synthesis specific yield was the ratio of δ-endo-toxin (mg L$^{-1}$) divided by CFU (spores L$^{-1}$), while the toxin product yield was the ratio of δ-endo-toxin (mg L$^{-1}$)/ glucose consumed (g L$^{-1}$).

### Cell, spore count and bioassay

Total cell counts (TC) comprising vegetative cells and spores were determined as described earlier (Ghribi et al., 2004). Viable spore (VS) concentration was determined by heat shock treatment at 80°C for 10 min in a shaking water bath. The samples were cooled for 5 min before spreading appropriately diluted samples onto nutrient agar plates.

The entomotoxicity (Tx) was determined against *Helicoverpa almigera* larvae by using the diet incorporation method (Nonklang, 2003). About 1.5 mL of appropriately diluted *Bt* samples (2.5 to 8.5 mg protein $mL^{-1}$) of fermented broth were incorporated into 30 mL of molten agar based diet at 60 ± 1°C. Afterwards, the mixture was distributed in aliquots of 1 mL in twenty 15 × 45 mm glass vials with perforated plastic caps. For each sample, at least three dilutions were used, and hence sixty glass vials were used for each sample. Tx was expressed in relative spruce budworm units (SBU) and was compared with commercial formulation Foray 76B (Abbott Labs, Chicago, USA) at a potency of $20.1 \times 10^9$ IU $L^{-1}$ (International Units per L) measured against *H. armigera.* SBU in this study was equivalent to 75 to 80% of international units.

### Analytical methods

#### *Intracellular proteases and δ-endotoxin determination*

To determine the intracellular proteases (PA) and δ-endotoxins, cell pellet was treated with Tris-HCl (100 mM) and NaOH (1N) at 90°C for 2 min and centrifuged at 10,000 rpm for 15 min. Proteolytic activity was checked by adding properly diluted sample (1 mL) to 0.65% casein solution. After incubation at 37°C, the reaction was terminated by adding 1 mL of sodium nitrite (0.2%). In this mixture, 1 mL of mercuric sulphate reagent (15 g of mercuric sulphate in 100 mL of 5 N $H_2SO_4$) and incubated in boiling water for 10 min. Volume was made up to 5 mL with distilled water, solution became turbid because of reddish pink precipitate formation. Clear solution was obtained by centrifugation. Optical density was measured at 590 against standard tyrosine of different concentrations (Ghribi et al., 2004). One unit of PA was that amount which supported formation of 1 micro-mol (181 microgram) tyrosine per mL per min incubation. Crystal proteins were counted with the help of cytometer and δ-endotoxin protein was measured by method of Bradford (1976).

### Determination of kinetic parameters

All kinetic parameters namely rate of *Bt* production ($Q_{PBt}$ ,g $L^{-1}$ $h^{-1}$), cell mass formation ($Q_X$, g cell $L^{-1}$ $h^{-1}$), rate of substrate consumption ($Q_S$, g sugar $L^{-1}$ $h^{-1}$), product yield ($Y_{P/S}$ , $g.g^{-1}$) and toxin specific yield ($Y_{P/X}$, g g $cells^{-1}$) were determined according to the method of Aiba et al. (1973). Activation energy ($E_a$) for toxin production was calculated using equation:

$$E_a = -\ln (q_p)/RT$$

Where, R is the gas constant and is the specific rate of toxin formation.

### Statistical analysis

Treatment effects were compared by the protected least significant difference method using software GraphPad Instat 3.0. Significance difference was presented using one way ANOVA as *P* values using Tukey-Kramer test. *P* values less than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

### Shake flask studies for production of toxin

*B. thuringenesis* is known to possess entomopathogenic activity. Previously *Bt* preparations from different strains usually comprised a mixture of cells, spores and parasporal crystals and were practiced as microbial insecticides for decades and no untoward entomotoxicity has been reported against untargeted subjects (Molva et al., 2009). We performed initial entomotoxicity testing using 72 h grown cells in DSMG medium in shake flasks. Strain *Bt* NSTD1 was used as a positive control. These strains were assayed with serial dilutions of spore-crystal suspensions to estimate their $LD_{50}$ (Table 1). As expected, strain *Bt*RJ7 showed good toxicity against *H. armigera* first instar larvae producing mortalities between 83 and 100%.

According to this parameter, *Bt* JR7 was the more toxic showing potency similar to the reference strain *Bt* NSTD1. In addition, the crystalliferous strains *Bt* JR7 displayed $LD_{50}$ several times higher than that of the positive control. *Bt* strain JR7 was specifically interesting because of its entomotoxicity against *H. armigera* (Table 1). Thus prospects of using new *Bt* strain JR7 with improved potential and specificity may be beneficial in developing new control strategy against *H. armigera*. This new isolate comparable with control strain might be a good candidate for harbouring novel cry genes, thus may be the first step in developing a novel indigenous strain with improved toxicity for new insect targets.

### Fermenter studies for production of toxin

*Bt* δ-endotoxins are very toxic to insects and up to some extent to nematodes. These crystal proteins (δ-endotoxins) are of great importance because of their environmental impact and crop yield improvement. Enhanced results with mutant strain (Table 2) in 10 L working volume fermenter were most probably because of the improvement in cry genes expression and/or genes involved in enhanced amount of sporulating cells leading subsequently to enhanced delta-endotoxin yield. These findings were significantly improved over those reported earlier (Ghribi et al., 2004; Saxena et al., 2002). The colony morphology and phenotypic expression of putative mutants remained unchanged but the crystals present in the putative mutant strains were significantly bigger than those of the parental strain (results not shown). Five variants were recognized amongst the 30 pre-selected variants from the UV irradiation.

### Specific yield of toxin in fermenter

In order to demonstrate the impact of random mutations on the metabolic network, total spores were counted and yields of delta-endotoxin synthesis per CFU were determined (Table 3). As the wild strain *Bt* JR7 produced 22x $10^8$ spores $mL^{-1}$ corresponding to 31.8 mg $10^8$ $spore^{-1}$ delta-endotoxins, it became apparent that

**Table 1.** Determination of 50% lethal dose of insecticidal spores and biomass-bound protease activity of native and standard strains of *Bt* in shake flask studies in DSM basic medium.

| Strain | Protein (mg $mL^{-1}$) | Toxin (mg $L^{-1}$) | LD50 (x $10^6$ CFU $mL^{-1}$) | Protease activity (U $g^{-1}$ cells) |
|---|---|---|---|---|
| Bt JR 7 | 5.4[a] | 700[a] | 3.8[a] | 2015[a] |
| Bt NSTD1 | 4.5[b] | 650[a] | 4.1[a] | 1850[b] |
| *P* | 0.0002 | 0.0008 | 0.8715 | 0.0004 |

Values followed by different letters differ significantly at $p \leq 0.05$. [a]Values of specific biomass-bound protease activity sharing the same letter in each column were non- significantly different according to Tukey-Kramer test.

**Table 2.** Comparative study of two different strains for production of toxin in Dubos salt medium containing glucose (50 g $L^{-1}$) (DSMG), corn steep liquor (45 g $L^{-1}$), yeast extract (5 g $L^{-1}$) and ammonium sulphate (5 g $L^{-1}$) at 30°C in shake flask cultures

| Parameter | *Bt* JR7 | *Bt* NSTD1 | *P* |
|---|---|---|---|
| Toxin (mg/L) | 700[a] | 658[b] | 0.0014 |
| Protein (mg $mL^{-1}$) | 3.8[a] | 3.6[a] | 0.9999 |

Values followed by different letters differ significantly at $P \leq 0.05$. [a]Values of protein sharing the same letter in respective rows were non-significantly different according to Tukey- Kramer test.

**Table 3.** Production of toxins and spores by different UV multiple mutant in 50 g sugars in hydrol $L^{-1}$ medium with ammonium sulphate (5.4 g $L^{-1}$) and yeast extract (5 g $L^{-1}$) in DSM at 40°C in 23 L fermenter.

| Putative mutants | Toxin (mg $L^{-1}$) | Spore ($10^8$) $mL^{-1}$ | Yield toxin (mg $10^{-8}$ $spores^{-1}$) |
|---|---|---|---|
| JR7 | 700[c] | 22[c] | 31.8[b] |
| UVM1 | 1000[b] | 28[b] | 35.7[b] |
| UVM2 | 850[c] | 25[b] | 34.0[b] |
| UVM3 | 950[b] | 27[b] | 35.2[b] |
| UVM4 | 734 | 23[c] | 31.9[b] |
| UVM5 | 876[b] | 26[b] | 33.7[b] |
| UVM6 | 1025[a] | 30[a] | 34.2[b] |
| UVM7 | 1200[a] | 32[a] | 39.5[a] |
| UVM8 | 912[b] | 26[b] | 35.1[b] |
| *P* | 0.0001 | 0.0014 | 0.0133 |

Values followed by different letters in different columns differ significantly at $P \leq 0.05$. [a]Values of specific toxin yields sharing the same letter were non- significantly different according to Tukey-Kramer test.

two amongst the 8 UV mutants, produced CFU similar to their parental strains. Therefore, their corresponding delta-endotoxin yields per CFU were improved over the wild strain Improvement in mutant UVM7 (1200 mg $L^{-1}$) for delta-endotoxin production was 1.75-fold higher compared with *Bt* JR7 (650-700 mg $L^{-1}$).

In order to confirm and elucidate the effect of mutations involved in the metabolism, leading to the increase of spores counts and/or delta-endotoxin yields by the mutants, complex substrates namely hydrol and CSL were used. The 7 over-producing mutants selected from the DSMG grown cultures, some mutants showing delta-endotoxin increased production lower than 50% in glucose medium, were cultured under such conditions. In the first group, mutants were characterized by their toxin yields per spore which were improved not only in the DSMG medium but also in the hydrol-based medium (Table 3). The other group consisted of mutants characterized by improvement of delta- endotoxin production which was much higher in the hydrol-based medium than in the glucose based one. However, these mutants were improved either in biomass production or delta-endotoxin synthesis depending on the medium as well as cultural conditions. Concerning the stability of the mutants, preliminary results indicated that several of them retained the improved activity and were stable.

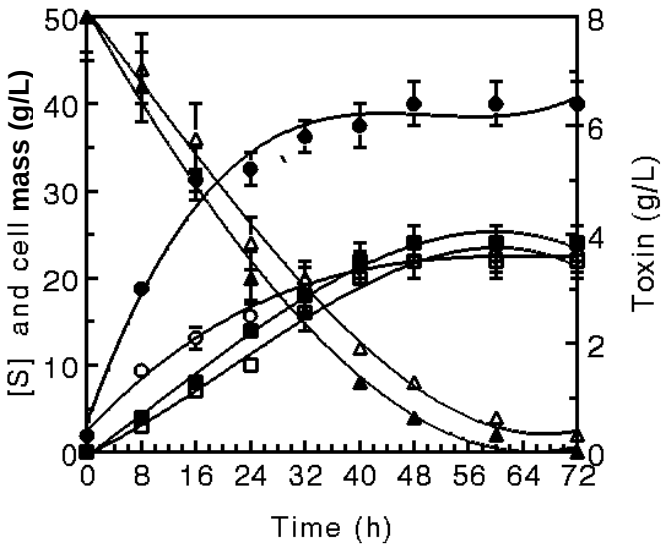


**Figure 1.** Time course of toxin ($g.L^{-1}$) production (○) cell mass (□) formation and substrate (Δ) present in the fermentation broth of parental (open symbols) and mutant (closed symbols) strains respectively in 10 L medium in fully controlled fermenter at 40°C as described in materials and methods.

## Fermenter studies with hydrol as carbon source

Hydrol contains glucose (56%), maltose (13%), maltotriose (2%), oligosaccharides (10%) and ash (1.56%). Corn steep liquor had 25% protein (dry weight basis) with 4% total nitrogen. Additionally, it had carbohydrates (5.8%), arginine (0.4%), cystine (0.5%), glycine (1.1%), histidine (0.3%), isoleucine (0.9%), leucine (0.1%), lysine (0.2%), methionine (0.5%), phenylalanine (0.3%), tyrosine (0.1%) and valine (0.5%). Among vitamins, inositol (602 mg/100 g) and choline (351 mg $100\ g^{-1}$) were dominant. Total ash content was 8.8%. Therefore production of toxin (mg $L^{-1}$), spores and yield of toxin were higher in the case of mutant derivatives.

For the production of *Bt* δ-endotoxins, hydrol and CSL and ammonium sulphate as optimized previously (Ghribi et al., 2004; Yezza et al., 2004; 2006) were applied in time course production of cell mass, toxin and substrate consumption (Figure 1). Kinetic results are shown in Table 4. Mutant was significantly ($P < 0.008$ to 0.0002) improved over its wild parent for all kinetic parameters.

## Effect of corn steep liquor (CSL) on toxin production

Nitrogen sources, including inorganic and organic nitrogen sources, play a pivotal role in regulating synthesis of proteins of the metabolic network. Inorganic nitrogen sources are consumed quickly and normally cause repression of enzyme synthesis due to the formation of protein (Awan et al., 2008), while complex organic nitrogen sources possess amino acids, and many growth factors, which are needed for the growth of the organism during metabolism and protein synthesis. Our earlier studies indicated that CSL was the best nitrogen source among all nitrogen sources for *Bt* strains (results not shown). Therefore, we applied only CSL to observe its role in toxin fermentation in submerged cultures. Time course of toxin production by both parental and UVM7 mutant cultures was performed at 40°C in fermenter (Figure 1). Yield of toxin $g^{-1}$ sugar were 0.22 and 4.0 respectively (Table 4). This parameter and other parameters of both organisms are superior to those described by other authors (Amin et al., 2008; Ghribi et al., 2004; Içgen et al., 2002).

## Effect of pH on toxin production

The pH of the culture medium is important in the regulation of product formation. Initial pH controlled conditions were optimized for maximum production of toxin. Maximum activity was observed at pH = 7.0 by *Bt* over-producing mutant (Table 5) in 48 h at 45°C with inoculum size of 10%. Some acidic compounds may accumulate in the fermentation medium and lead to decrease in medium pH (to pH = 5.5) in pH uncontrolled condition) but since pH was controlled, this pH decreasing ability of the system was not observed. But when medium pH was regulated at 8.0, there was delayed production of toxin. Alkaline extra-cellular pH could enhance intracellular pH of the cells and destabilize the enzyme synthesis network. This happens because the equilibrium in enzyme catalyzed reactions is normally reached at a specified intracellular pH value (Yezza et al., 2006), and ionic strength of the biological systems. For other toxin-producing organisms different media of similar pH supported maximum production of toxin (Yezza et al., 2006).

## Effect of temperature on toxin production

It is well documented that the most desirable property of new developed industrial organisms is their genetic stability and physiological reproducibility at various temperatures, particularly in case of highly mutated organisms. To assess the mutational effect on the prolonged thermostability of the genetic makeup of the selected UV mutant organism, the inoculated fermentation medium was incubated at 25, 30, 35, 40, 45 50 and 55°C (Figure 2a*)* keeping all other fermentation variables constant. Normally higher temperature than optimum one can cause inactivation of enzymes regulating the metabolic pathway while low temperature may not be suitable to permit the flow of nutrients across the cell membrane. Under such conditions, it may result in high demand for maintenance energy (Aiba et al., 1973) for toxin production by the organism (Figure 2b). There was a progressive increase in the average specific

**Table 4.** Kinetic parameters for parental and mutant (MUV7) cultures under controlled pH of the medium (7) containing 50 g sugars from hydrol $L^{-1}$ and corn steep liquor (45 g $L^{-1}$) on *Bt* toxin formation parameters in 10 L fermentation medium at 40°C.

| Kinetic parameters | Parental strain | UVM7 mutant strain | *P* |
|---|---|---|---|
| $Q_{PBt}$ (g $L^{-1}$ $h^{-1}$) | 0.135 ± 0.02 | 0.35 ± 0.02 | 0.0002 |
| $Q_X$ (g cell $L^{-1}$ $h^{-1}$) | 0.37 ± 0.02 | 0.576 ± 0.03 | 0.0005 |
| $Q_S$ (g sugar $L^{-1}$ $h^{-1}$) | 1.03 ± 0.1 | 2.1 ± 0.2 | 0.0008 |
| $Y_{P/S}$ (g.$g^{-1}$) | 0.22 ± 0.02 | 0.40 ± 0.02 | 0.0004 |
| $Y_{P/X}$ (g g $cells^{-1}$) | 0.44 ± 0.04 | 0.87 ± 0.04 | 0.0002 |

Each value is a mean of three replicates ± standard deviation. All higher values differ significantly according to Tukey-Kramer test.

**Table 5.** Effect of different pH values of the medium containing 50 g sugars from hydrol $L^{-1}$ and corn steep liquor (45 g $L^{-1}$) on *Bt* toxin formation parameters of *B. thuringiensis* (UVM7) in 10 L fermentation medium at 40°C.

| pH | Toxin productivity (mg $L^{-1}$ $h^{-1}$) | Spores ($10^{11}$) $mL^{-1}$ | Yield of toxin (mg/10-$^{11}$spores) |
|---|---|---|---|
| 5.0 | 50 ± $6^b$ | $4.5^b$ | $442^b$ |
| 6.0 | 95 ± $8^a$ | $6.5^a$ | $512^b$ |
| 7.0 | 110 ± $10^a$ | $6.7^a$ | $570^a$ |
| 8.0 | 65 ± $7^b$ | $5.5^a$ | $419^b$ |
| 9.0 | 45 ± $5^b$ | $4.1^b$ | $411^b$ |
| 10 | 30 ± $10^c$ | $3.5^c$ | $366^c$ |
| *P* | 0.0001 | 0.0001 | 0.0005 |

Each value is a mean of three replicates±standard deviation. Values followed by different letters in each column differ significantly at $P \leq 0.05$.

productivity of intracellular toxin when the temperature was increased from 25 to 40°C. Maximum toxins specific productivity was supported by both wild and mutant organisms at 40°C. *Bt* strains can grow up to 45°C but maximum toxin production takes place at 30 to 40°C (Ghribi et al., 2004). This temperature is a little higher than the normal range reported for other *Bt* strains and was a result of mutation in the organism. Conversely, specific productivity progressively decreased over 45°C, though maximal growth occurred at 37 to 45°C. Reduced toxin was probably due to the well-known thermal inactivation of metabolic network at temperatures higher than the optimum in both organisms.

## Determination of activation energy for toxin production

The effect of temperature on the specific rate of toxin formation by strains of *Bt* was also evaluated in terms of activation energy using the Arrhenius equation, which has been used for thermodynamic studies in bioprocesses (Ali et al., 2005). This equation describes the dependence of specific rate of product formation on temperature (Bokhari et al., 2008). The activation energy ($E_a$) for *Bt* strains (both parental and *Bt* UVM7) under study was calculated in the temperature range of 25 to 55°C at pH 7 and was 50.7 and 47.4 kJ $mol^{-1}$ for parental and mutant strain respectively (calculated from Figure 2 b) and was lower than 65.52 kJ $mol^{-1}$ for *Saccharomyces cerevisiae* ITV strain (Ortiz-Muniz et al., 2010), *Schizosaccharomyces pombe* which has a higher activation energy (110 kJ $mol^{-1}$) (Ortiz-Muniz et al., 2010) but was comparable with *P. tannophilus* (S´anchez et al., 2004) which needed 57.1 kJ $mol^{-1}$ for growth and ethanol formation, double mutant of *Aspergillus oryzea* for 3,4-dihydroxyphenyl-L-alanine (Ali et al., 2005) but superior to thermophilic mutant derivative of *Humicola lanuginosa* (Bokhari et al., 2008) which needed 143.59 kJ $mol^{-1}$for ß-glucosidase production.

It has been suggested that the activation energy value reflects if the process is within a biological or diffusional regimen (Brandam et al., 2008). A biological regimen implies that temperature directly affects kinetic growth parameters and a diffusional regimen indicates that physical phenomena, like oxygen transfer, restrict the reaction. It has been reported that when the activation energy is higher than 50.4 kJ $mol^{-1}$, the process is in a biological regimen (Ortiz-Muniz et al., 2010). Our results indicate that bioinsecticide fermentation with *Bt* strains

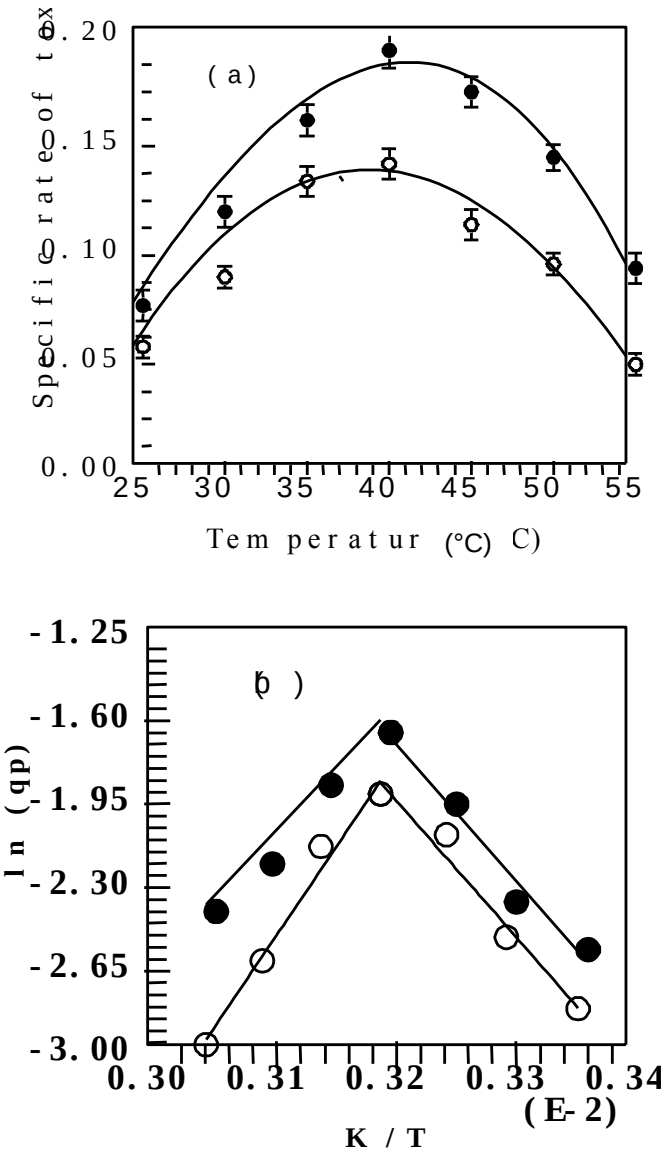


**Figure 2.** Effect of temperature on specific rate of toxin production (a) by parental (○) and mutant (●) strain respectively. Error bars show standard deviation among n = 3 replicates (a) and determination of activation energy demand by the parental (○) and mutant organism (●) respectively in a fully controlled fermenter in optimized medium as described in material and methods.

followed a biological regimen and was a thermostable process better than mutant derivative of *H. lanuginosa* M7 (Bokhari et al., 2008).

## Conclusion

A DG$^r$ mutant derivative (*Bt* UVM7) developed through UV mutagenesis was promising and supported high titres of toxin. Addition of ammonium sulphate in CSL supplements further enhanced enzyme formation rate.

Inoculum 10%, initial pH = 7.0, and fermentation temperature of 40°C for 72 h were optimized in fermenter studies. Aeration rate in fermenter enhanced product formation, cell mass formation and substrate consumption rates. Additional additives like casein may further enhance toxin production in fermenter and optimized media may be used for industrial production of toxin for application in the country.

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## REFERENCES

Aiba S, Humphrey AE, Millis NF (1973). Biochemical Engineering. Academic Press, New York, USA. pp. 92-127.

Ali S, Haq I, Qadeer MA, Rajoka MI (2005). Double mutant of *Aspergillus oryzae* for improved production of 3, 4-dihydroxy phenyl-L-alanine (L-dopa) from L-tyrosine. Biotechnol. Appl. Biochem. 42:143-149.

Amin G, Alotaibi S, Youssef NA, Saleh WD (2008). Optimization of fermentation process for bioinsecticide production by *Bacillus thuringiensis.* World. J. Microbiol. Biotechnol. 24:2465-2471.

Awan MS, Jalal F, Ayub N, Akhtar MW, Rajoka MI (2009) Production and characterization of α-galactosidase by a multiple mutant of *Aspergillus niger* in solid-state fermentation. Food Technol. Biotechnol. 47:370-380.

Bokhari SAI, Latif F, Rajoka MI (2008). Kinetics of high-level of ß-glucosidase production by a 2-deoxyglucose-resistant mutant of *Humicola lanuginosa* in submerged fermentation. Braz. J. Microbiol. 39:724-733.

Bradford MM (1976). A rapid method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72:248-234.

Brandam C, Castro-Mart´ınez C, Delia ML, Ram´ on-Portugal F, Strehaiano P (2008). Effect of temperature on *Brettanomyces bruxellensis*: metabolic and kinetic aspects. Can. J. Microbiol. 54:11-18.

Brar SK, Verma M, Tyagi RD, Surampalli RY, Barnabe S, Valero JR (2007). *Bacillus thuringiensis* porteases: Production and role in growth, sporulation and synergism. Process Biochem. 42:773-790.

Chang L, Grant R, Aronson A (2001). Regulation of the packaging of *Bacillus thuringiensis* δ-Endotoxins into inclusions. Appl. Environ. Microbiol. 67:5032-5036.

Duan YX, Wu G (2002). Control of Plant Nematode Disease. Beijing: China Agriculture Press.

Ghribi D, Zouari N, Jaoua S (2004). Improvement of bioinsecticide production through mutagenesis of *Bacillus thuringiensis* by U.V. and nitrous acid affecting metabolic pathways and/or delta-endotoxin synthesis. J. Appl. Microbiol. 97:338-346.

Içgen Y, Içgen B, Özcengiz G (2002). Regulation of crystal protein biosynthesis by *Bacillus thuringiensis*: II. Effects of carbon and nitrogen sources. Res. Microbiol. 153:605-609.

Miller GL (1959). Use of dinitrosalisylic acid (DNS) for determination of reducing sugars. Anal. Chem. 31:426-428.

Molva C, Sudagidan M, Okulu B (2009). Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey. Food Cont. 20:829-834.

Nonklang S (2003). Growth, sporulation, crystal formation and toxicity of *Bacillus thuringiensis* subspecies *Krustaki* HD-73 grown on various complex nutrient sources, PhD Thesis. Mahidol University, Bangkok, Thailand.

Ortiz-Muniz B, Carvajal-Zarrabal O, Torrestiana-Sanchez B, Aguilar-Uscanga MG (2010) Kinetic study on ethanol production using *Saccharomyces cerevisiae* ITV-01 yeast isolated from sugar cane molasses. J. Chem. Technol. Biotechnol. 85:1361-1367.

Papalazaridou A, Charitidou L, Sivropoulou A (2003). Beta-Glucosidase enzymatic activity of crystal polypeptide of the *Bacillus thuringiensis* strain 1.1. J. Endotox. Res. 9:215-224.

Rajoka MI (2005). Double mutants of C*ellulomonas biazotea* for production of cellulases and hemicellulases following growth on straw of a perennial grass. World J. Microbiol. Biotechnol. 21:1063-1066.

S´anchez S, Bravo V, Moya AJ, Castro E, Camacho F (2004). Influence of temperature on the fermentation of D-xylose by *Pachysolen tannophilus* to produce ethanol and xylitol. Process Biochem. 39:673-679.

Saxena D, Ben-Dov E, Manasherob R, Barak Z, Bousiba S, Zaritsky A (2002). A UV tolerant mutant of *Bacillus thuringiensis* subsp. *Kurstaki* producing melanin. Curr. Microbiol. 44:25-30.

Thamthiankul S, Suan-Ngay S, Tantimavnich S, Panbangred W (2001). Chitinasee from *Bacillus thuringiensis* subsp. *Pakistani* Appl Microbiol. Biotechnol. 56:395-401.

Vu KD, Yan S, Tyagi RD, Valero JR, Surampalli RY (2009). Induced production of chitinase to enhance entomotoxicity of *Bacillus thuringiensis* employing starch industry wastewater as a substrate. Bioresour. Technol. 100:5260-5269.

Yezza A, Tyagi D, Valero J, Surampali Y (2004). Production of *Bacillus thuringiensis* biopesticides in batch and fed-batch cultures using wastewater sludge as a raw material. J. Chem. Technol. Biotechnol. 80:502-510.

Yezza A, Tyagi D, Valero J, Surampali. Y (2006). Bioconversion of industrial wastewater and wastewater sludge into *Bacillus thuringiensis* based biopesticides in pilot fermentor. Bioresour. Technol. 97:1850-1857.

Zi-Quan Y, Qian-Lan W, Bin L, Xue Z, Zi-Niu Y, Ming S (2007). *Bacillus thuringiensis* crystal protein toxicity against plant-parasitic nematodes. C. J. Agric. Biotechnol. 5:13-17.