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Regulation of major cultural components for designing a cost effective medium to increase δ-endotoxin synthesis by *Bacillus thuringiensis*

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The present study was aimed at designing a cost effective medium for increasing the δ-endotoxin (Cry protein) synthesis by *Bacillus thuringiensis (Bt) kurstaki* HD-73 and indigenous *Bt* JSc1 harboring potential *cry* genes active against Lepidoptera insect orders. In this regard, locally available cheap molasses as carbon source, soybean extract as nitrogen source, sea water as trace elements source, cystine as sporulation/growth factor were considered to design a cost effective medium. Molasses and soybean extract in place of glucose and peptone in glucose-peptone (GP) medium supported 78.85% increase in δ-endotoxin synthesis in shake flask culture. The effect of cystine on endotoxin synthesis was highly pronounced in two media with a range of 80.32 to 110% higher δ-endotoxin under comparable fermentation conditions. While, substituting basal salts with sea water, *Btk* HD-73 yielded satisfactory and comparable endotoxin (74.3% of yield with basal salts). It was detected that the rapid decrease of endotoxin synthesis in the culture after 24 h was due to the degradation by the endogenous protease, synthesized with the progress of fermentation. This degradation of the endotoxin was much better protected (1.23 mg/ml endotoxin versus 0.312 mg/ml) by adding 4% ammonium sulfate in the optimized medium. The medium thus formulated with molasses, soybean extract, ammonium sulfate, cystine and sea water was then used in 3.0 L bioreactor cultivation for endotoxin synthesis by both *Btk* HD-73 and *Bt* JSc1 under 30% saturation of dO_2 through cascade control of agitation and aeration producing a higher yield of δ-endotoxin (2.1 and 2.63 mg/ml, respectively). The present results may successfully be used for large scale production of *Bt* biopesticide in Bangladesh.

Key words: *Bacillus thuringiensis* biopesticide, cheap substrates, cystine, protease activity, ammonium sulfate.

INTRODUCTION

The control of pest populations by using biological pesticides has been an attractive alternative to the application of chemical pesticides (Glazer and Nikaido, 1995; Ciche and Ensign, 2003). As hazardous and recalcitrant chemical agents damage the environment by causing soil, water and air pollutions and also triggers development of insect resistance, biological pest management is more preferable for the specific toxicity of

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its component (Massaoud et al., 2010). An entomopathogenic bacterium, *Bacillus thuringiensis (Bt)*, has been used for more than half a century as important biopesticide of which during sporulation produces crystal (Cry) proteins, toxic against various pests (Özkan et al., 2003). Although, *Bt* based microbial insecticides are available in world market, the high cost makes its application impracticable in large-scale in developing countries. The use of *Bt* as commercial insecticides would remain prohibitively and relatively expensive if it is not produced with high titer of insecticidal proteins by large-scale fermentation. More attention has been given to the regulation mechanisms that ensure the efficient production of the insecticidal proteins which could be achieved by application of an adequate fermentation technology (Zouari et al., 2002), essentially with use of appropriate media (Zouari and Jaoua, 1999), overcoming metabolic limitations (Zouari et al., 2002) etc. It was reported that the commercial application depends on the cost of raw materials, strain efficiency, fermentation cycle, maintenance of process parameters, bioprocessing of fermentation fluid, and formulation of the final product. The cost of raw materials is one of the principal costs involved in overall *Bt* production. In the conventional *Bt* production process, the cost of raw materials varied between 30 and 40% of the total cost depending on the plant production capacity (Ejiofore, 1991). Therefore, local production of this insecticide in developing countries should depend on the use of production media made of cheap, locally available sources including agro-industrial by-products (Ampofo, 1995).

For large scale production of *Bt* biopesticide, different approaches were investigated to develop suitable media that could support good production of spores and toxins at reasonable costs. Various agricultural and industrial by-products used as raw material in *Bt* production were citrus peels, wheat bran, corn meal, seeds of dates, beef blood, silkworm pupal skin, ground nut cake, cane molasses, fish meal, cotton seed meal, soybean meal, residues from chicken slaughter house, fodder yeast, cheese whey and corn steep liquor etc (El-Bendary, 2006). Agro-industrial residues and by-products available in southeastern Brazil were used as ingredients for low-cost culture media for liquid fermentation of *B. thuringiensis* var. *kurstaki*. Highest spore yield was obtained with a medium containing cheese whey, soya bean milk and molasses (Alves et al., 1997). Other wastes, such as, sludge and broiler poultry litters were also utilized for biopesticides production (Adams et al., 2002; Vidyarthi et al., 2002). In general, two methods of fermentations are used for production of microbial products, submerged fermentation and solid state fermentation. *Bt* biopesticides are usually composed of spores and crystals protein mixtures, harvested from the production media, readily produced by aerated liquid fermentation. They are easily harvested and have a long shelf life when formulated properly (Ghribi et al., 2007).

Optimizing different culture conditions and regulating some critical factors, it is possible to obtain higher yield in terms of cell mass, Cry protein concentration and toxicity to develop efficient *Bt* formulations (Dulmage et al., 1990). Critical factors e.g. sugars have significant impact on cell growth but when used at high concentrations, they can cause adverse effects on sporulation due to the acids produced by *Bt* δ-endotoxin from carbohydrates (Dulmage et al., 1990) and moreover, the balance of the ratio between carbon and nitrogen, itself is directly important for the crystal protein production (Farrera et al., 1998).

Other important components for the production of crystal proteins are the trace minerals (Rose, 1979). Again, amino acids are important in the formation of spores and crystal proteins (Sachidanandham et al., 1997). Moreover, it was reported that decreasing the proteolytic activity in the fermentation medium increased the accumulation of δ-endotoxin in the insecticidal crystal proteins (Ennouri et al., 2013). Indiscriminate use of chemical pesticide is a common practice in Bangladesh, an agriculture dependant developing country, where agro-industrial wastes like defatted soybean meal, defatted mustard seed meal, molasses, rice husk, rice bran, citrus peels etc are generated in huge amount every year. The present study was, therefore, carried out with a view to design a cost effective medium comparing the effects of carbon and nitrogen sources, amino acid such as cystine and basal salts on growth, sporulation and δ- endotoxin synthesis for large scale production of *Bt* biopesticide with locally available cheap raw materials such as defatted soybean extract and molasses as nitrogen and carbon source respectively and sea water as the substituent of basal salts. The present study also reports the regulation of certain critical factors that affect the growth, sporulation and δ-endotoxin synthesis by reference *Btk* HD-73 and indigenous *Bt* strain JSc1.

MATERIALS AND METHODS

B. thuringiensis strains and inoculum

Reference strain *B. thuringiensis kurstaki (Btk)* HD-73 and the indigenous *Bt* strain JSc1 were used. Reference strain *B. thuringiensis kurstaki (Btk)* HD-73 was kindly provided by Okayama University, Japan from their *Bt* stock collection and the strain *Bt* JSc1 was isolated from Bangladesh (Shishir et al., 2014). Inoculum was prepared by inoculating a single *Bt* colony into a 250 ml Erlenmeyer flask containing 50 ml of Luria Bertani (LB) broth (per litre: tryptone 10.0 g, yeast extract 5.0 g, NaCl 10.0 g) and incubated 12 h at 30°C and 180 rpm. Each time, inoculum was added into the medium aseptically with sterile micropipette in a manner so that the process starts with an OD_{600nm} = 0.1.

Culture medium for bio-insecticide production

Glucose-peptone medium (GP) [10% (w/v) glucose and 5% (w/v) peptone] (Vora and Shethna, 1999) was used either as the control or for substituting the carbon and nitrogen sources, respectively, by

0.5% (w/v) molasses and 10% (w/v) soybean extract (Soybean extract molasses- SeM medium). Optimum cystine concentrations was determined by supplementing it in GP medium. Cystine supplement (10% cystine stock was prepared by suspending 6.0 g cystine in 60.0 ml phosphate buffer) was maintained from 200 to 600 mg/l. The minerals of the soybean extract-molasses (SeM) medium: [(g/l): KH_2PO_4 - 1.0, $MgSO_4.7H_2O$- 0.5, $MnSO_4.H_2O$- 0.1, $FeSO_4.7H_2O$- 0.02, $ZnSO_4.H_2O$- 0.02, $CaCl_2$- 0.01] was substituted with 20% (v/v) sea water (Filtered with Whattman filter paper 1) to formulate Soybean extract molasses sea water medium (SSeM). Ammonium sulfate [4% (w/v)] was added into the medium to inhibit the protease synthesis by *Bt* strains (Liu and Hsieh, 1969). The pH of the medium was adjusted to 6.8 by adding 50 mM phosphate buffer before autoclaving at 121°C for 20 min. Thus, considering different factors and comparing them statistically by paired sample t- test analysis, the final formulation of the medium was 10% soybean extract, 0.5% molasses, cystine 300 mg/l and 20 ml sea water supplemented with ammonium sulphate at a concentration of 4%.

Bioinsectide production

Production in shake flasks

The fermentation method performed in this study was submerged and Batch type. Each flask containing 100 ml of different media were inoculated aseptically with the bacterial inocula of *Btk* HD-73 and *Bt* JSc1 as described in section 2.1 and incubated in an orbital shaker at 30°C with 180 rpm for 72 h. The experiments were performed in triplicate and at least three samples were collected in each case at 24 h interval both from shake flasks and bioreactor for spore count, estimation of δ-endotoxin concentration.

Production in 3.0 L bioreactor

Production experiments were carried out at 30°C in a 3.0 L fully controlled bioreactor (New Brunswick Scientific, USA) containing 2.0 L of finally optimized medium (10% soybean extract, 0.5% molasses, 20 ml sea water, 300 mg/l cystine and 4% ammonium sulfate) as in shake flask. Dissolved oxygen (dO_2) level in the medium was automatically controlled and maintained at 30% by providing aeration, set at 1.0 standard liter per minute (SLPM) and agitation at 250 rpm. Dissolved oxygen was continuously monitored by an oxygen sensor but the pH was not controlled.

Estimation of spore count

The spore counting was performed each time in triplicate with 1.0 ml of sample collected during the culture both from shake flask and bioreactor. It was then heat treated at 80°C for 15 min, serially diluted in sterile distilled water and inoculated 0.1 ml sample using glass rod spreader on the LB agar medium by spread plate method. The plates were then incubated at 30°C for 24 h. The colonies were then counted and multiplied by the dilution factor to estimate their number.

Partial purification and determination of δ-endotoxin concentration

The purification of crystal protein was done by the modified method of Liu et al. (1994) and Öztürk et al. (2009) where 1 ml culture was washed twice with sterile distilled water by centrifugation at 10000 rpm for 10 min. The pellet was treated with 1.0 M NaCl and 5.0 mM EDTA and later with 5.0 mM EDTA alone. Finally, the pellet was re-suspended in 1.0 ml 0.1 N NaOH solution for 1 h at room temperature. Thus, the partially purified crystal protein concentration in the supernatant was estimated by Bradford method (Bradford, 1976).

Proteolytic activity assay

Protease activity was determined by a modified method described by Kreger and Lockwood (1981) using azo-casein (Sigma, USA) as the substrate. In this enzyme assay, 400 µl of the culture supernatant and 400 µl of 1% azo-casein solution (suspended in 0.05 M Tris- HCl; pH 8.5) was taken in microfuge tube. The mixture was then incubated in a water bath at 37°C for 60 minutes. The reaction was stopped by adding 135 µl of 35% TCA and kept on ice for 15 min. The solution was then centrifuged at 13000 rpm for 10 min and 750 µl of the supernatant was collected in which equal volume of freshly prepared 1.0 N NaOH was added by gentle mixing. Absorbance (OD_{440nm}) of the solution was then measured using the solution from a parallel reaction as blank where TCA was added before the enzyme. Enzyme activity was then estimated from the absorbance [OD_{440nm} = 1.0 is equivalent to 100 U Enzyme activity].

SDS-PAGE analysis of delta- endotoxin

SDS-PAGE analysis was performed with the partially purified δ-endotoxin in a 10% separating gel recovered from $(NH_4)_2SO_4$ supplemented and non-supplemented medium to see its influence on inhibiting protease activity (Sambrook et al., 1989).

RESULTS

Efficiency of molasses and soybean extract as C and N source

The δ-endotoxin production was found to be higher in soybean extract-molasses (SeM) medium in comparison to the glucose-peptone (GP) medium by *Btk* HD-73 (Table 1) which can be correlated to the replacement of glucose and peptone with 0.5% molasses and 10% soybean extract as these two formulations differed in carbon and nitrogen sources only. At 24 h, the δ-endotoxin concentration was found to be 0.2 mg/ml in SeM medium which was 78.85% higher than that (0.042 mg/ml) in GP medium. In paired sample t- test analysis, significant difference in endotoxin yield between GP (M = 0.0445, SD = 0.0139) and SeM (M = 0.20933, SD = 0.0139) conditions; t (4) = 14.48 and p = 0.05; was found.

Role of cystine on growth, sporulation and δ-endotoxin synthesis

The maximum sporulation (11.31 Log_{10}CFU/ml) and δ-endotoxin yield (0.215 mg/ml) were obtained at 300 mg/l of cystine at 24 h (Figure 1). The role of cystine (300 mg/l) was also tested in SeM medium and an increase of 1 log in spore concentration and 2 fold in δ- endotoxin yield were observed (Figure 2). In paired sample t-test analysis, there was significant difference in the endotoxin

Table 1. Effect of carbon and nitrogen sources on sporulation and δ-endotoxin synthesis by *Bacillus thuringiensis kurstaki (Btk)* HD-73.

Media	Time (h)	Spore count (Log10 CFU/ml)	δ- endotoxin concentration (mg/ml)
GP medium	24	7.32±0.1	0.0423±0.0478
	48	8.13±0.02	0.13±0.034
SeM medium	24	6.024±0.149	0.2±0.076
	48	6.854±0.24	0.187±0.028

GP = Glucose peptone medium; SeM = Soybean extract molasses medium.

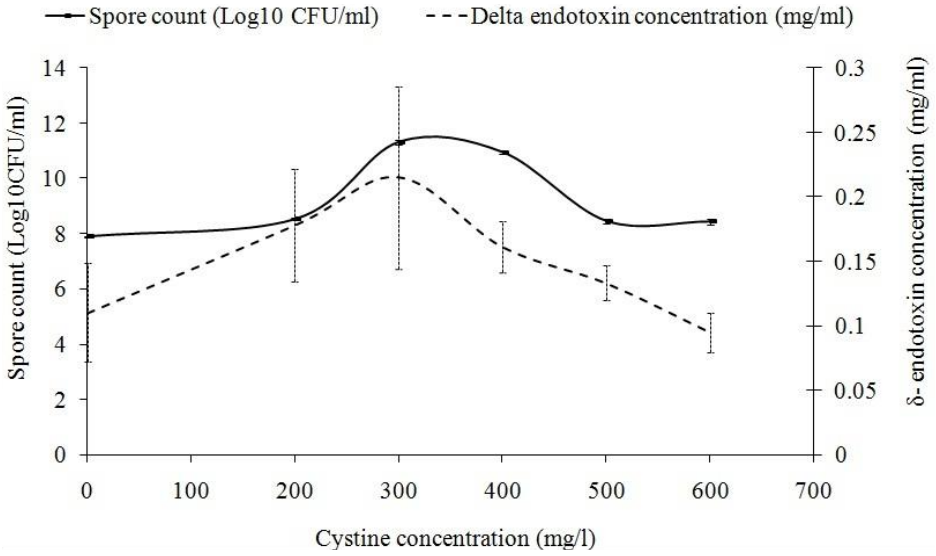

Figure 1. Determination of optimum concentration of cystine (300 mg/l) from the curves of spore concentration (11.31 Log10 CFU/ml) and δ-endotoxin concentration (0.215 mg/ml) by *Btk* HD-73 in Glucose-Peptone (GP) medium. (Culture Period: 36 h).

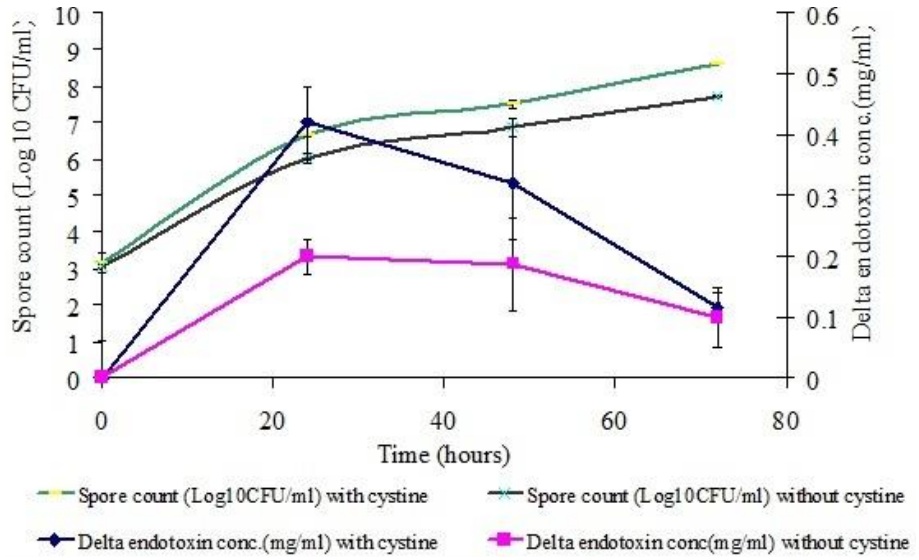

Figure 2. Cystine enhanced both endotoxin yield and sporulation by *Btk* HD-73 similarly in Soybean extract Molasses (SeM) medium.

Table 2. Efficacy comparison between Sea water and basal salts based on sporulation and δ- endotoxin synthesis.

Time (h)	Mineral source	Spore count (Log_{10}CFU/ml)	δ- endotoxin concentration (mg/ml)
0	Basal salts	3.162±0.271	0.001±0.008
	Sea water	3.073±0.0466	0.0014±0.03
24	Basal salts	6.352±0.032	0.420±0.06
	Sea water	6.428±0.072	0.311667±0.0185
48	Basal salts	7.512±0.12	0.320±0.092
	Sea water	6.88±0.047	0.1023 ±0.025
72	Basal salts	8.596±0.0064	0.116±0.024
	Sea water	7.812±0.068	0.075±0.0126

yield at 24 h with cystine (M = 0.42666, SD = 0.061) and without cystine (M = 0.212, SD = 0.061) condition; t (4) = 4.29, p = 0.05. Thus, significant increase in δ- endotoxin yield occurred in the presence of cystine and 80.32 and 110% increase in δ- endotoxin yields were resulted in both GP and SeM media, respectively by *Btk* HD-73 under comparable conditions.

Efficacy of sea water as basal salts substituent

The efficacy of sea water (20%) in sporulation and δ-endotoxin synthesis was comparable with that of basal salts. With both ingredients, maximum sporulation and δ-endotoxin synthesis were obtained at 72 and 24 h, respectively (Table 2). No significant difference was found between endotoxin yield with basal salt (M = 0.4166, SD = 0.384) and sea water (M = 0.313, SD = 0.384) condition; t (4) = 0.33, p = 0.05; and sporulation with basal salt (M = 6.344, SD = 0.058) and sea water (M = 6.439, SD = 0.058) condition; t (4) = 2.00, p = 0.05 at 24 h by paired sample t-test analysis. Maximum yields of sea water in spore count and δ-endotoxin concentration were respectively 90.88 and 74.29% of Basal salt.

Protection of δ-endotoxin degradation by endogenous protease

Sharp decrease in δ- endotoxin concentration was observed to be simultaneous with the rise of endogenous protease activity after 24 h determined by protease assay (Figure 3). When the protease activity reached its maximum, that is, 105.9 U/ml gradually, the δ-endotoxin concentration was also reduced to its minimum (0.075 mg/ml) from the peak (0.312 mg/ml). Protease inhibitor PMSF was added into the culture medium at 0.1 and 0.3 mM concentration to control the proteolytic degradation of δ-endotoxin which inhibited the protease activity partially (Data not shown). As an alternative of PMSF, ammonium sulfate was used and the protease activity was monitored up to 72 h at 24 h interval. Ammonium sulfate (4%) resulted in maximum endotoxin yield (1.2 mg/ml) as well as restricted the protease activity within 10.1 U/ml (Table 3). This result corresponded to a 295% increase in δ-endotoxin productivity (51.43×10^{-3} g/L/h) in presence of 4% ammonium sulfate. Significant differences in δ-endotoxin yield and enzyme activity in the presence and absence of ammonium sulfate were observed while performing the statistical paired sample t-test analysis. The t (4) =62.08, p=0.05 was obtained under condition with endotoxin yield without ammonium sulfate (M=0.32, SD= 0.018) and with ammonium sulfate (M=1.2276, SD= 0.018) at 24 hours. Similarly, the enzyme activity [At 24 hours, enzyme activity without ammonium sulfate (M=44.24, SD= 1.863) and with ammonium sulfate (M= 4.30, SD=1.863) condition; t(4) = 26.2661, p=0.05)]was found to decrease significantly in the presence of ammonium sulfate in paired sample t-test analysis.

Production of reference and indigenous strains in 3.0 L bioreactor

Finally, optimized medium containing soybean extract, molasses, sea water, cystine and 4% ammonium sulfate was used for the production of *Btk* HD-73 and the indigenous *Bt* JSc1 under controlled conditions in 3.0 L bioreactor with an working volume of 2.0 L. Maximum δ-endotoxin yields were 2100 and 2630 mg/l by *Btk* HD-73 and *Bt* JSc1, respectively, at 24 h (Figure 4). Protease activity was negligible for both strains. The indigenous *Bt* JSc1 resulted in 25% higher δ-endotoxin yield than the reference strain.

Qualitative analysis of effect of ammonium sulfate by SDS-PAGE

Prevention of δ- endotoxin degradation due to

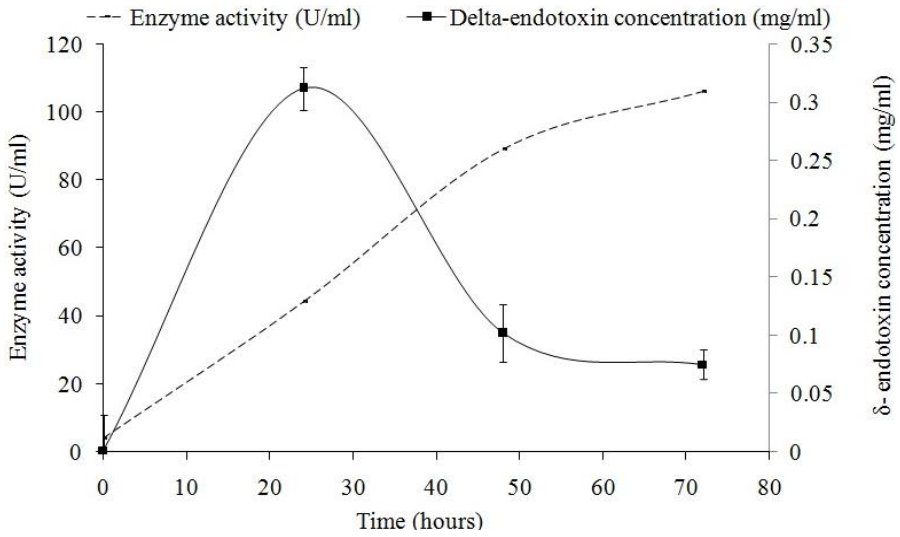

Figure 3. Comparison between increase protease activity and δ-endotoxin degradation with time occurred with *Btk* HD-73 in cystine supplemented SeM sea water medium.

Table 3. Effect of Ammonium sulfate on protease synthesis and δ- endotoxin breakdown in cystine supplemented SeM sea water medium.

Time (h)	δ- endotoxin concentration (mg/ml)		Enzyme activity (U/ml)	
	Without as	With as	Without as	With as
0	0.0016±0.03	0.0011±0.034	4.21±0.020	3.93±0.132
24	0.312±0.0185	1.2343±0.0153	44.2±0.098	4±0.157
48	0.102±0.0025	0.9027±0.0379	89.2±0.056	9.3±0.047
72	0.075±0.0126	0.839±0.0529	105.9±0.038	10.1±0.116

as = Ammonium sulfate.

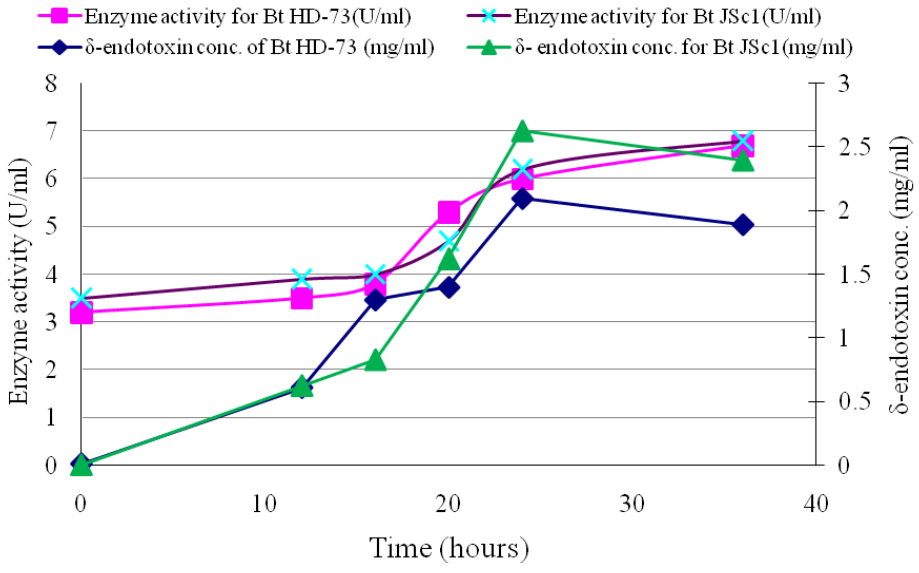

Figure 4. Production kinetics of *Bt* JSc1 and *Btk* HD-73 in cystine and ammonium sulfate supplemented SeM sea water medium in a 3.0 L bioreactor.

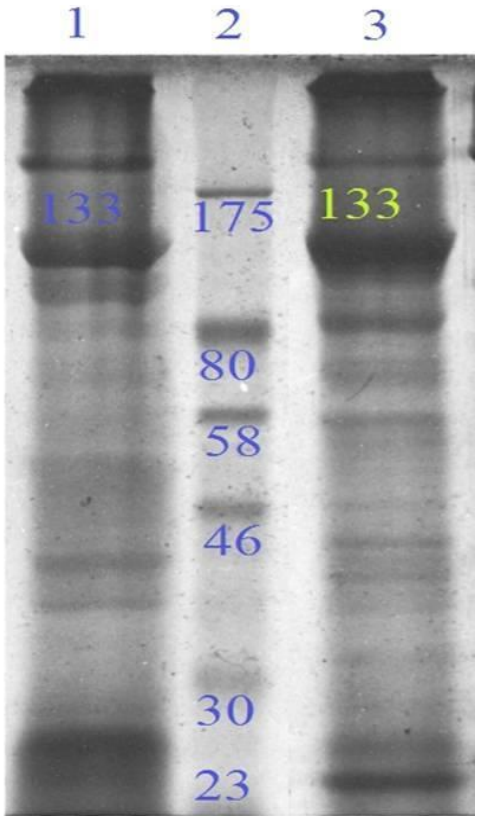

Figure 5. SDS-PAGE analysis of δ-endotoxin partially purified from the cystine supplemented SeM sea water agar medium with and without 4% ammonium sulfate. Lane 1, Without $(NH_4)_2SO_4$; lane 2, marker (ColorPlus Prestained protein marker, Broad range, NEB); lane 3, $(NH_4)_2SO_4$ supplemented.

endogenous protease by ammonium sulfate was visualized by SDS-PAGE analysis (Figure 5). Comparatively thicker band of partially purified 133 kD Cry1Ac protein was observed in the presence of ammonium sulfate than in its absence.

DISCUSSION

One of the main reasons of absence of *Bt* biopesticide in the Integrated Pest Management in Bangladesh agriculture is that it is not yet produced locally. So, industrial production of *Bt* biopesticide can facilitate its application in eco-friendly pest management for which higher yield in spore and δ- endotoxin production with low cost medium is one of the prerequisites to keep the product within farmers' buying capacity. In this connection, present study was performed to formulate a low cost medium regulating different cultural components for higher yield. It was reported that high yield of endotoxin could be achieved by using inexpensive defatted soybean, ground nut seed meal extract for large scale production of *Bt* biopesticide (Vora and Shethna, 1999). In present study, inexpensive substrate such as molasses and soybean was used as carbon and nitrogen sources for biopesticide production instead of relatively expensive glucose and peptone present in commercial media. Soybean extract and Molasses acted as excellent substituent of peptone and glucose respectively as medium containing soybean extract and molasses resulted in 78.85% higher endotoxin yield than that of GP medium. Molasses is cheap, available throughout the year and easy to store. The defatted soybean meal is also a low cost, readily available item and easy to handle. So, a major cost for the production of *Bt* biopesticide might receive a 10-fold reduction. On the other hand, the increase in δ-endotoxin yield may be due to complex carbon and nitrogen source that permits high sporulation and biomass production.

The optimum concentration of cystine was determined to be 300 mg/l which enhanced the sporulation and δ-endotoxin synthesis by *Bt* strains whereas higher concentration inhibited this phenomena. The cystine was also found to keep statistically significant positive impact on yield. This increase could be explained as the fact that cystine might have interfered with some of the macromolecular changes during sporulation and parasporal crystal formation (Rajalakshmi and Shethna, 1980). On the other hand, when basal salts were replaced with sea water containing most of the minerals, comparable effects on δ-endotoxin synthesis and sporulation were observed as ca. 75 and 90% yield respectively of basal salts was obtained and the differences were not significant at 95% confidence level. So, another cost incurring ingredient, that is, minerals could successfully be replaced with sea water without much negative influence on yield. It was reported in a study that 20% sea water (source: Mediterranean sea) improved the yield in δ-endotoxin concentration and spore count by 2 and 4%, respectively (Ghribi et al., 2007) whereas this study reports slightly lower yield. It indicates that the yield might be variable based on the source of sea water too. Endogenous protease activity was responsible for the decrease in δ-endotoxin concentration that occurred after 24 h (Figure 3). So, initially PMSF and later ammonium sulfate were used to control this protease synthesis and to protect δ-endotoxin degradation. PMSF inhibited the protease synthesis partially and it might be due to the fact that *Btk* HD-73 secreted not only serine protease but also some other classes of proteases which could not be inhibited by this serine protease inhibitor (PMSF). But 4% ammonium sulfate inhibited protease synthesis (10.1 U/ml) to great extent and also resulted in higher endotoxin yield (1.23 mg/ml).

Finally, the medium containing 10% soybean extract, 0.5% molasses, 20% sea water, 300 mg/l cystine and 4%

ammonium sulfate thus found suitable for optimum production of both spores and δ-endotoxin in shake flask culture, was selected for bioprocess development in a 3.0 L bioreactor. From this study, it was found that the fermentation broth should be harvested at 24 h for the strains both reference, *Btk* HD-73 and indigenous, *Bt* JSc1 to recover δ-endotoxin at its maximum yield which also reduces the power consumption, hence cost. So, a low cost medium was designed thus which will facilitate large scale industrial production of *Bt* biopesticide in Bangladesh in a cost effective manner.

Conflict of interests

The authors did not declare any conflict of interest.

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