Purification and characterization of amylase from *Bacillus thuringiensis* subsp. *kurstaki*

Robinson Babysarojam Smitha, Sreedharan Sajith, Prakasan Priji, Kizhakepawthial Nair Unni, Trikaryoor Asokan Nidheesh Roy, Sailas Benjamin

Enzyme Technology Laboratory, School of Biosciences, University of Calicut, Kerala – 673635, India

Corresponding author email: benjamin@uoc.ac.in

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Abstract In a biphasic fermentation strategy, *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) was cultivated in potato flour (10% w/v) supplemented Luria-Bertani medium for enhancing the production of δ-endotoxin. After 12 h fermentation, the supernatant was collected for the purification of amylase. The amylase secreted by *Btk* was purified to homogeneity (15 folds purified with 79% yield) through a series of steps including (NH₄)₂SO₄ fractionation and sephadex G-100 gel filtration chromatography. From the profile on SDS-PAGE, the molecular weight of the purified amylase was judged as 51kDa. The maximum amylase activity (8088 U/gds) was obtained with 2.5% soluble starch at 60 °C in the presence of 3µ M Ca²⁺ (pH 6); however, SDS and EDTA retarded the activity. The amylase hydrolyzed starch with a *Km* of 2.9 mg/ml and *Vmax* of 0.053µ mol/ml/min. This is the first report describing the purification and characterization of an amylase from a *Bt* strain; since amylase can be purified as byproduct with no additional cost during the harvest of δ-endotoxin, recovery of valuable amylase from the effluent would be advantageous for the *Bt* bioindustry.

Keywords *Bacillus thuringiensis* subsp. *kurstaki*; amylase; characterization; biphasic fermentation

Background

Amylases (EC 3.2.1.1) constitute a class of industrially significant enzymes, which contribute approximately 30% to the world enzyme market (Benjamin et al., 2013). Amylases have wide range of applications in a wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries (Liu and Xu, 2008, Benjamin et al., 2013). Amylases play a pivotal role in the carbohydrate metabolism of all organisms. Amylases catalyze the formation of a range of important products useful to foods and pharmaceuticals with different physical and chemical properties of (Prakash et al., 2009). Although amylases can be derived from several sources including plants, animals and microorganisms; microbial amylases generally meet industrial demands (Hmidet et al., 2008).

Either submerged fermentation (SmF) or solid state fermentation (SSF) strategies are employed for the production of enzymes including amylase from bacteria and fungi; the former is preferred for bacteria, while the later for fungi. However, a few studies used SSF strategy for the production of enzymes using bacteria (Saxena and Singh 2011; Smitha et al. 2013a; Saha et al. 2014). Species of bacterial genera; *Pseudomonas*, *Achromobacter*, *Streptomycyes*, *Bacillus*, *Streptiococcus* etc. are the producers of hydrolytic enzymes. Of them, *Bacillus* spp. are the major producers of amylases. Amylase produced by *Bacillus* spp. may belong to α, β or γ subtypes; of which α-amylase represent the predominant subtype, which is invariably produced by almost all *Bacillus* spp., especially in the presence of starchy substrates (Benjamin et al. 2013). Various agricultural residues such as molasses, whey, wheat bran, rice bran etc. have been used as substrate for the production of amylase (Kunamneni et al. 2005). Mostly, in such preparations, the commercial media like Luria-Bertani (LB) and nutrient broth (NB) are used as the base, to which agricultural residue is supplemented as extra nutrient (Smitha et al. 2015).

Owing to the characteristic production of insecticidal δ-endotoxin, *Bacillus thuringiensis* (*Bt*) is known as a
natural bioinsecticide; which is known to produce various hydrolytic enzymes such as protease (Akcan and Uyar 2011) and amylase (Demirkan et al. 2011; Smitha et al. 2013a). Of these reports; only the study employing Bt subsp. kurstaki (Btk) Smitha et al. (2013a) proposed the recovery of amylase as a byproduct from the effluent, released during the harvest of δ-endotoxin. In continuation to this report, the present study explores the purification, characterization of amylase produced by Btk, which recovered from the effluent (supernatant) obtained during the biphasic fermentation strategy adopted for the production of δ-endotoxin (Smitha et al. 2013b).

1 Results
As reported previously (Smitha et al., 2013a, Smitha et al., 2013b), potato flour (10%w/v) supplemented LB supported the maximum amylase production by Btk at 12 h fermentation; hence, supernatant obtained from this medium (after 12 h fermentation) was used for the purification of amylase.

1.1 Partial purification of α-amylase by ammonium sulphate fractionation
The supernatant was fractionated with (NH₄)₂SO₄; of this, 40-60% fraction showed the maximum amylase activity (Figure 1), which was used for the characterization studies. It was 3.5 folds purified fraction with 81% yield (Table 1). The SDS-PAGE profile of crude as well as partially purified amylase showed that the apparent MW of the Btk-amylase is 51 kDa, the supposed α-amylase (Figure 2).

1.2 Purification of α-amylase by Sephadex G-100 gel permeation chromatography
Seventy fractions (2 ml/10 min) were collected and the OD at 280 nm showed major peak in between fraction 37 and 43 (Figure 1). These amylase active fractions were pooled and concentrated by lyophilization. Purified amylase was seen as single band on SDS-PAGE image, whose apparent MW was 51 kDa, as that of crude and partially purified (NH₄)₂SO₄ preparations (Figure 2). The purification fold of sepahadex G-100 fraction of α-amylase was 15 with 79% yield (Table 1).

1.3 Enzyme characteristics
1.3.1 Effect of pH on enzyme activity
The maximum amylase activity (5643 Units/gram dry substrate (U/gds)) was noticed at pH 6.0, and the lowest activity (843 U/gds) was at pH 3.0 (Figure 3). The amylase activity was considerably decreased with low (acidic) as well as at high (alkaline) pH (Figure 3).

1.3.2 Effect of temperature on enzyme activity
Results indicate that the temperature optimum for amylase from Btk was 60 °C (5498 U/gds), with comparable activities at 50 °C, 55 °C and 65 °C (Figure 4). In fact, about 17% activity was retained at 100 °C (931 U/gds).
Table 1 Summary of purification of extracellular Btk amylase from the supernatant in the potato flour supplemented LB medium

<table>
<thead>
<tr>
<th>Purification</th>
<th>Total protein (mg)</th>
<th>Total activity (U/gds)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>3.36</td>
<td>2903.27</td>
<td>864.00</td>
<td>100%</td>
<td>1</td>
</tr>
<tr>
<td>40-60% (NH₄)₂SO₄ Fraction</td>
<td>1.8</td>
<td>5370.18</td>
<td>2983.43</td>
<td>81.36</td>
<td>3.45</td>
</tr>
<tr>
<td>Sephadex G-100 column Fraction</td>
<td>0.18</td>
<td>2295.27</td>
<td>127512</td>
<td>79.05</td>
<td>14.75</td>
</tr>
</tbody>
</table>

Figure 3 Effect of pH on partially purified Btk amylase obtained at 12 h fermentation of potato flour (10%, w/v) supplemented LB medium. This activity was with varying pH and fixed temperature (~32 °C) and substrate concentration (1%) and incubation (5 min).

Figure 4 Effect of temperature on Btk amylase, this activity was at varying temperature with fixed pH (6.0) and substrate concentration (1%) and incubation (5 min).

1.3.3 Effect of complex compounds on amylase activity
Of the complex compounds, the chelating agent EDTA (553 U/gds) and detergent SDS (349 U/gds) have tremendously decreased the amylase activity, while β-mercaptoethanol completely abolished it, and the amino acid cysteine partially retarded (by 15%) the activity (4713 U/gds) (Table 2).

Table 2 Effect of metal salts on Btk amylase

<table>
<thead>
<tr>
<th>Metals (µM)</th>
<th>Activity (U/gds)</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn²⁺</td>
<td>3810.91</td>
<td>±1.82</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>5294.52</td>
<td>±4.84</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>5410.91</td>
<td>±1.57</td>
</tr>
<tr>
<td>Thiourea</td>
<td>4218.18</td>
<td>±5.09</td>
</tr>
<tr>
<td>EDTA</td>
<td>552.727</td>
<td>±1.68</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>3723.64</td>
<td>±4.73</td>
</tr>
<tr>
<td>SO₃⁻</td>
<td>3374.55</td>
<td>±4.84</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>5847.27</td>
<td>±5.77</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>5880.00</td>
<td>±6.18</td>
</tr>
<tr>
<td>Na⁺</td>
<td>3723.64</td>
<td>±1.2</td>
</tr>
<tr>
<td>Mo²⁺</td>
<td>5498.18</td>
<td>±3.58</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>5934.55</td>
<td>±1.68</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>5556.36</td>
<td>±4.38</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>5381.82</td>
<td>±9.87</td>
</tr>
<tr>
<td>Cysteine</td>
<td>4712.73</td>
<td>±5.36</td>
</tr>
<tr>
<td>SDS</td>
<td>349.09</td>
<td>±1.83</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

1.3.4 Effect of metal salts on amylase activity
Table 2 shows that the metal ions Ag⁺, Ca²⁺, Cu²⁺, Mg²⁺, and Mo²⁺ generally enhanced the amylase activity. The reaction mixture was incubated with these metals at 0.5, 1.0, 2, 3, 4 or 5 µM concentrations. These results suggest that 3µM Ca²⁺ supported the highest amylase activity (6080 U/gds) (Figure 5). This was about 10% increase over other factors as described above.

Figure 5 Effect of varying metal salts concentration (µM) on the activity of Btk amylase with fixed pH (6.0), temperature (60 °C) and substrate concentration (1%) and incubation (5 min).
1.3.5 Effect of substrate concentration
The activities of amylase purified from Btk as described above were with 1% starch substrate for 5 min incubation. Figure 6 illustrated the effect of varying substrate concentration (1-3%) on amylase activity for 30 min incubation at 60 °C. The maximum activity (8088 U/gds) was obtained at 2.5% starch concentration. Interestingly, amylase activity was almost stable for 1 h at 60 °C (Figure 6).

![Figure 6](image)

Figure 6 Effect of soluble starch concentration (%) on the activity Btk amylase with fixed pH (6.0), temperature (60 °C) and Ca ^{2+} (3μM) concentrations at varying incubation time (5 – 60 min)

Briefly, optimized conditions for Btk amylase activity are: 2.5% starch at 6.0 pH and 60 °C incubation for 30 min in the presence of 3 μM Ca ^{2+}.

1.3.6 Enzyme kinetics
The Km and Vmax values for Btk amylase were calculated using the data obtained with different substrate concentrations. The Km and Vmax values were found to be 2.9 mg/ml and 0.053 μmol/ml/min, respectively. This was calculated using the software, Hyper 32 (Figure 7).

![Figure 7](image)

Figure 7 Km and Vmax values of Btk amylase with soluble starch as substrate

2 Discussion
No report is available in literature documenting the purification and characterization of Btk amylase either by SmF or SSF; however, Smitha et al. (2013a) showed that amylase could be recovered from the effluent (supernatant) discarded during the production of δ-endotoxin. Present study, therefore, is the first ever attempt towards the purification and characterization of a Bt amylase. Supernatant obtained from 10% (w/v) potato flour supplemented LB medium after 12 h fermentation of Btk was used for the purification and characterization of amylase.

The first step was to fractionate the crude supernatant using (NH₄)₂SO₄; 40–60% fraction showed the maximum amylase activity. Hence, this fraction was used for the characterization studies, and also for further purification by gel filtration. Btk was capable of secreting significant amount of amylase, which was purified (15 folds with 79% yield) through (NH₄)₂SO₄ fractionation and sephadex G-100 gel permeation chromatography. No report is available demonstrating the purification of amylase from Btk, but there are reports supporting the present data from other Bacillus spp. Krishnan and Chandra,(1983) purified α-amylase produced by B. licheniformis CUM 305 to 212 fold with 42% yield. Hmidet et al. (2008) reported a thermostable α-amylase produced by B. licheniformis NH 1, which was purified to homogeneity through 40–60% (NH₄)₂SO₄ precipitation, sephadex G-100 gel filtration and sepharose mono-Q anion exchange chromatography. It showed 3 folds increase in specific activity and 16% recovery. Das et al. (2004) isolated B. subtilis strain DM-03 from starter culture, being used for the production of alcohol by local Assam tribes, and the α-amylase purified by ion exchange, gel filtration and HPLC techniques to 80 folds purity. Ezeji and Bahl, (2006) purified α-amylase secreted by Geobacillus thermodenitrificans HRO-10 through a series of steps, which resulted in 14 folds purification with 11.5% recovery. All these findings show varying purification folds and yields. In the present study, about 15 folds purification and 79% yield were obtained, which are within the limit of reported values for different Bacillus spp.

The amylase purified from Btk showed a single protein band on SDS-PAGE with an apparent MW of 51 kDa. This finding corroborates well with previous
reports as highlighted below on α-amylase from various species of Bacillus: 52 kDa from B. amylo liquefaciens (Demirkan et al., 2005); 55 kDa from B. licheniformis (Faber et al., 2007); 51 kDa from Bacillus sp. strain LI1711 (Bernhardsdotter et al., 2005); 58 kDa from B. licheniformis NH 1 (Hmidet et al., 2008); 53 kDa B. subtilis KCC-103 (Nagarajan et al., 2007), and Liu and Xu (2008) purified 53 kDa amylase from B. subtilis WB600 by (NH₄)₂SO₄ fractionation, anion exchange and gel filtration. It indicates that the MW of α-amylase secreted by the species of Bacillus varies from 51-58 kDa.

Purified amylase from Btk showed its pH and temperature optima as 6.0 and 60 °C, respectively. At this condition, the activity was persisted for over 1 h. These optimal pH and temperature are in the ranges reported for α-amylase from some species of Bacillus by several authors. Demirkan et al. (2005) purified α-amylases from B. amylo liquefaciens and a mutant strain, whose degradation abilities with starch granules from various botanical sources were tested. They found that the purified amylase was best active at pH 6.0 and 55°C. Hmidet et al. (2008) purified thermostable α-amylase from B. licheniformis NH 1, which was highly active at pH 6.5 and 90 °C. Kiran and Chandra (2008) found that the pH and temperature optima of α-amylase from Bacillus sp. strain TSCVKK were 7.5 and 55 °C, respectively. Ohdan et al. (2000) produced α-amylase from B. subtilis X 23 with 5.5 pH and 65 °C temperature optima. A malto oligosaccharide forming α-amylase from B. subtilis KCC 103 was shown highly active over a broad range of pH from 5 to 7 and temperature 65-70 °C (Nagarajan et al., 2005). It shows that the amylase purified from Btk has the optimum pH and temperature characteristics, comparable to those α-amylases produced by various species of the genus, Bacillus.

Complex compounds like the chelating agent (EDTA) and detergent (SDS) tremendously decreased the activity of Btk amylase; while cysteine partially retarded the activity and β-mercaptoethanol completely abolished it. The thermostable α-amylase from B. licheniformis NH 1 was almost inactivated by the chelating agent EDTA, retaining only 17% activity, while SDS and β-mercaptoethanol completely abolished the activity (Hmidet et al., 2008). Krishnan and Chandra (1983) observed that α-amylase from B. licheniformis CUM 305 was highly inactivated by EDTA, but EDTA retained 50% activity of α-amylase obtained from B. subtilis KCC-103 (Nagarajan et al., 2006); while the activity of amylase from B. subtilis strain DM-03 was completely abolished in the presence of phenylmethylsulfonyl fluoride, bromophenacyl bromide and SDS (Das et al., 2004). Urea, SDS, Na-sulphide, EDTA, ZnCl₂, NaCl and CaCl₂ inhibited the activity of amylase from Bacillus sp. A3-15 (Arikan 2008). These inhibitors should have irrecoverably complexed with the amylase, so that its conformation would have changed leaving the enzyme with total or partial loss of activity.

Of different metal salts tested to evaluate their effects on Btk amylase activity, 3 µM Ca²⁺ was found as suitable ion for maintaining and enhancing its activity. Presence of Ag⁺, Ca²⁺, Cu²⁺, Mg²⁺ and Mo⁷⁺ in the reaction mixture generally enhanced α-amylase activity, while other metal salts Mn⁴⁺, Fe²⁺, Fe³⁺, Na⁺,Zn²⁺,SO₄²⁻ and SO₃⁻ showed inhibitory action, up to 45%. This data gets support from several published work. Krishnan and Chandra, (1983) purified α-amylase from B. licheniformis CUM 305 and found that the cations Na⁺, Ca²⁺ and Mg²⁺ showed stimulatory effect; whereas, Hg²⁺, Cu²⁺, Ni²⁺, Zn²⁺, Ag⁺, Fe²⁺, Co³⁺, Cd²⁺ Al³⁺ and Mn²⁺ were found inhibitory, and the anions azide, SO³⁻ and SO₄²⁻ showed excitant effect. Amylase from B.licheniformis NH 1 showed enhanced activity in the presence of Hg²⁺ and Zn²⁺ ions (Hmidet et al., 2008). Demirkan et al. (2005) showed that α-amylases from B. amylo liquefaciens was stimulated by the divalent metal ions like Mg²⁺, Ba²⁺ and Cu²⁺; while Hg²⁺, Fe³⁺, Zn²⁺ and Ag⁺ strongly inhibited the activity. Huang et al. (2005) found that Ni²⁺ and Mn²⁺ have stimulatory effects on the α-amylase produced by Bacillus sp. strain TS 23. Briefly, divalent cations are found potent enhancers of α-amylase activity, as explained above. Ca²⁺ indicated as the most crucial ion in all these studies. Crystal studies also showed that Ca²⁺ is the most favored ion by α-amylases for their better stability and activity (Benjamin et al. 2013). Binding of metal ions impart major conformational rearrangement on the enzyme. The metal induced disorder→order transition observed in B. licheniformis α-amylase lead to the formation of the extended substrate binding site,
which explains the calcium dependency of α-amylases at structural level (Machius et al., 1998).

The $K_m$ and $V_{max}$ values of amylase produced by *Btk* were found to be 2.9 mg/ml and 0.05335μmol/ml/min, respectively. $K_m$ approximates the affinity of enzyme for its substrate. A small $K_m$ indicates high affinity, and a substrate with a smaller $K_m$ will approach $V_{max}$ more quickly. Literature shows that the $K_m$ of various α-amylases from *Bacillus* spp. varies from 1.3 to 7.8 mg/ml. The $K_m$ and $V_{max}$ of extracellular thermostable α-amylase from *B. subtilis* were 7.79 mg/ml and 11.176 mg/ml/h, respectively towards potato starch substrate (Konzula and Kyriakides, 2006). Bernhardsdotter et al. (2005) isolated an alkalophilic amylase producing bacterium, *Bacillus* sp. strain L1711, whose $K_m$ and $V_{max}$ values were calculated as 1.92 mg/ml and 0.051μmol/min, respectively. Ezeji and Bahl (2006) purified α-amylase secreted by *G. thermodenitrificans*, whose $K_m$ and $V_{max}$ were 3 mg/ml and 7 U/ml, respectively. $K_m$ and $V_{max}$ of α-amylase from *B. subtilis* KCC-103 were 2.6 mg/ml and 909 U/mg, respectively (Nagarjan et al., 2006). From this, comparatively, it is evident that amylase purified from *Btk* is a fast acting enzyme, which would find better position in industry. The substrate with the lowest $K_m$ upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes. In fact, in the present study, partially purified enzyme was used, and thus the kinetic behavior of pure enzyme from *Btk* would be far excellent than the present values if pure *Btk* amylase was used.

3 Materials and methods

3.1 Enzyme purification and characterization

Amylase was purified by the method of Ezeji and Bahl, (2006). The strategy included fractionation by (NH$_4$)$_2$SO$_4$, followed by dialysis and sephadex G-100 gel permeation chromatography. The purity was checked on gel by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970).

3.2 Ammonium sulphate fractionation

Finely powdered (NH$_4$)$_2$SO$_4$ was added slowly into the crude enzyme preparation until it reached 80% saturation (0-20%, 20-40%, 40-60% or 60-80%). A magnetic stirrer was used for the continuous stirring, and the procedure was carried out at 4 °C in an ice bath. The precipitated protein was removed by centrifugation (2400 × g for 10 min at 4 °C). The pellet was re-suspended in a minimum volume of 0.02 M sodium phosphate buffer (pH 6.9).

The precipitate obtained after (NH$_4$)$_2$SO$_4$ fractionation was dialyzed against sterile ddH$_2$O for 24 h at 4 °C with continuous stirring in a cold room. Cellulose membrane dialysis tubes were used for dialysis. The amylase activity and protein content of the dialysate were determined by DNS and Lowry’s methods, respectively (Smitha, 2010).

3.3 Gel permeation chromatography

The dialysate obtained by 40-60% (NH$_4$)$_2$SO$_4$ fraction was used for gel permeation chromatography, for which sephadex-G100 (Sigma Aldrich, USA). The gel permeation chromatography (BioRad Biologic LP, Italy, 50 × 1.5 cm) was also done in National Institute for Interdisciplinary Science and Technology, Papanamcode, Thiruvananthapuram.

3.4 Electrophoresis

The purified enzyme was subjected to SDS-PAGE (10%) to confirm the purity and to determine the approximate molecular weight (MW) of the purified protein. SDS-PAGE was conducted using a vertical mini gel (8×7cm) slab with notched glass plate system (BioTech, India); gels of 1.5 mm thickness were prepared for the whole study.

3.5 Characterization of amylase

The purified enzyme was characterized and its various properties were studied. Effects of pH, temperature, substrate concentration, chelating agents and different metal ions on enzyme activity were the factors studied (Smitha, 2010).

3.5.1 Effect of pH

The optimum pH on the amylase activity was studied by performing the assay at different pH using Tris acetate (pH 4.0-5.0) and Sodium phosphate (pH 6.0-8.2) buffers.

3.5.2 Effect of temperature

The optimum temperature required for amylase activity was estimated by incubating the reaction mixture for 5 min at different temperatures (30-100°C) at pH 6.0.
3.5.3 Effect of different metal salts
The effect of different metal ions on amylase activity was determined by incubating the reaction mixture with different metal salts, i.e., Ag⁺, Ca²⁺, Cu²⁺, Fe²⁺, Mg²⁺, Mn²⁺, Mo⁶⁺, Na⁺, SO₄²⁻, SO₃⁻ and Zn²⁺ to a final concentration (μM) of 0.5, 1.0, 2.0, 3.0, 4.0 or 5 at pH 6.0 and 60 °C.

3.5.4 Effect of complex compounds
The role of complex compounds like ethylene diamine tetra acetic acid (EDTA), β- mercaptoethanol, SDS and thiourea were tested for their influence on amylase activity, in addition to cysteine.

3.5.5 Effect of substrate concentration
The enzyme was treated with soluble starch at a concentration (%) of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0. The reaction mixture was incubated with 5 min time intervals ranging from 5 to 60 min at pH 6.0, 60 °C and Ca²⁺.

3.6 Calculation of Km and Vmax
The Km and Vmax values were calculated using the software, Hyper 32.

4 Conclusions
This is the first study demonstrating purification and characterization of an amylase from Bt. In comparison, the amylase reported herein shows the characteristics of various α-amylases produced by different species of Bacillus, the prominent amylase producers for industry. It seems that the Btk amylase is highly efficient than most of the amylase reported from bacteria. Thus, the Bt industry should utilize the added profit of amylase, to be recovered as a byproduct from the discarded effluent during the process of δ-endotoxin production.

Author’s Contributions
SB designed and prepared the manuscript, RBS did the experiments, PP and SS set the reference and figures, KNU and TANR collected the literature.

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