Screening of Different Adjuvants for Wastewater/Wastewater Sludge-Based Bacillus thuringiensis Formulations

SATINDER K. BRAR,1, 2 M. VERMA,1, 2 R. D. TYAGI,1, 3, J. R. VALÉRO,1, 2 AND R. Y. SURAMPALLI4


ABSTRACT Screening of different adjuvants, namely, suspending agents, phagostimulants, stickers, antimicrobial agents, and UV screens to develop aqueous biopesticidal suspensions of Bacillus thuringiensis (Bt) variety kurstaki HD-1 fermented broths, specifically, nonhydrolyzed sludge, hydrolyzed sludge, starch industry wastewater, and soya (commercial medium), were investigated. The selected suspending agents [20% (wt:vol)] included sorbitol, sodium monophosphate, and sodium metabisulfite with corresponding suspendibility of 74–92, 69–85, and 71–82%, respectively. Molasses [0.2% (wt:vol)] increased adherence by 84–90% for all fermented broths. The optimal phagostimulants [0.5% (wt:vol)], namely, soya and molasses, caused entomotoxicity increase of 3–13 and 7–13%, respectively. Sorbic and propionic acids showed high antimicrobial action [0.5% (wt:vol)], irrespective of fermentation medium. Sodium lignosulfonate, molasses, and Congo red, when used as UV screens [0.2% (wt:vol)], showed percent corresponding entomotoxicity losses of 3–5, 0.5–5 and 2–16, respectively. The Bt formulations, when exposed to UV radiation, showed higher half-lives (with and without UV screens) than the fermented broths or semisynthetic soya medium and commercial Bt formulation. UV screen-amended nonhydrolyzed, hydrolyzed, and starch industry wastewater formulations showed 1.3–1.5-fold higher half-lives than commercial Bt formulation. Thus, the recommended formulation comprises sorbitol, sodium monophosphate, sodium metabisulfite (suspending agents); molasses, soya flour (phagostimulants); molasses and skimmed milk powder (rainfasteners); sorbic and propionic acids (antimicrobial agents) and sodium lignosulfate; and molasses and Congo red (UV screens). These waste-based Bt formulations offer better UV resistance in comparison with commercial formulation.

KEY WORDS adjuvants, Bacillus thuringiensis, formulations, wastewater, sludge
Phagostimulant (gustatory stimulation) studies began with sugar-based derivatives, namely, lactose and sucrose (Bartelt et al. 1990). With the progress in understanding of the actual and specific action of phagostimulants, newer options of amino acids, starch, ascorbic acid, and nature-derived cucurbitacins and garbanzo beans were explored (Bartlet et al. 1994, Gillespie et al. 1994, Lopez et al. 1994). As the phagostimulant interest soared, some commercial products such as Coax, Entice, Gusto Konsume, and Mo-Bait were introduced to increase feeding responses of pests in the field (Lopez and Lingren 1994, Farrar and Ridgway 1995).

Fermented biopesticidal broths are highly susceptible to foreign microorganisms because of their biodegradable characteristics (live microbial cells, toxin crystals, and spores). This necessitates lowering of the pH and adding antimicrobial agents to inhibit the growth of various microorganisms (Burges 1998). Occasionally, microflora such as yeast and mold and fecal cocci/enterococci colloforms such as Escherichia coli, Staphylococcus aureus, and Salmonella typhae are likely to infest these broths (Lisansky et al. 1993, Burges 1998). Therefore, International Union of Pure and Applied Chemistry (IUPAC) has established maximal allowable limits of these probable contaminants in formulations (Quinlan 1990).

Another problem with Bt formulations is the loss of residual entomotoxicity (Tx) on exposure to UV under field conditions (Cohen et al. 1991). Several approaches on UV protection of Bt formulations included the use of oil-soluble sunscreens with oil-carriers, oil–water emulsions (Burges 1998), water-soluble or suspendable absorbers or blockers with water carriers (Shasha et al. 1998), or encapsulation (e.g., insoluble starch) with a water carrier (McGuire et al. 1996, Behle et al. 1997a,b, Tamez-Guerra et al. 2000). Other studies included incorporation of various UV screens such as Congo red, folic acid, molasses, lignin, alginate, cellulose, shellac yeast, and p-aminobenzoic acid with mixed results (Dunkle and Shasha 1989; Shapiro 1989; McGuire et al. 1996; Behle et al. 1997a,b; Ragaei 1998; Wirtz et al. 1999). Certain fluorescent brighteners, especially compounds of stilbene type also have been found to enhance biological activity up to 1000-fold and protect Bt from UV exposure (Shapiro et al. 1992). However, external addition of UV screens and modification of formulation matrix are expensive methods. This cost could be reduced if the fermentation medium possessed inherent characteristics that provided UV resistance compared with commercial formulations.

This study focuses on screening of different formulation adjuvants (suspending agents, phagostimulants, stickers, antimicrobial agents, and UV screens) for wastewater/wastewater sludge-based Bt liquid suspensions to assist in development of stable formulations for field application. Furthermore, the study investigates the effect of UV on Tx of Bt-fermented broths and formulations (with and without UV screens) of starch industry wastewater (SIW), wastewater sludge (raw and hydrolyzed [H]), and soya (semisynthetic commercial medium).

### Materials and Methods

**Bt Production Media.** Three media were used for Bt growth: 1) conventional semisynthetic soybean medium (control) (Lisansky et al. 1993) that was made up of (in 1 liter of water) 15 g of soybean meal, 5 g of glucose, 5 g of starch, 1 g of KHP04, 0.3 g of MgSO4(0.7H2O), 0.02 g of FeSO4(0.7H2O), 0.02 g of ZnSO4(0.7H2O), and 1g of CaCO3; 2) SIW (complete medium) from ADM-Urbaine du Quebec (Quebec, Canada) wastewater treatment plant (complete medium without addition of external ingredients); and 3) SIW (complete medium) from ADM-Oglvie (Candiac, Quebec, Canada). The wastewater sludge and SIW were used with a minimum delay (within 1 wk of sampling) for fermentation as long-term storage at even 4°C would lead to deterioration (slow endogenous respiration of microbes). The sludge sampled at different times of the year (incorporates seasonal, day and night, and other possible variations) did not vary much in terms of Tx at optimal sludge solids concentration (Yezza et al. 2005).

**Characterization of Wastewater Sludge/SIW.** Different characteristics of raw wastewater sludge/SIW as presented in Table 1 were determined according to standard methods (AWWA 1998).

**Solids Amendment and Pretreatment Procedure.** The sludge was concentrated from ≈1.7 to 5% (wt/vol)
on 09 October 2017

Sixty vials containing 1 ml of artificial diet (C1) were used as a control, and another control contained sterilized NH sludge/H sludge/SIW/soya medium (C2). One L3-L4 larva was placed into each vial and allowed to feed ad libitum for 7 d at 25 ± 1°C. Mortality was monitored after 7 d. If mortality in control vials was higher than 10%, the sample was repeated. 

For bioassay, 76B contained spores and crystals of Bt variety kurstaki at a potency of 20.1 × 10⁹ IU/liter measured against cabbage looper, Trichoplusia ni (Hübner). On comparison of Tx of Bt-fermented sludge samples, we found that the SBU was 20–25% higher than IU. Data were analyzed with one-way analysis of variance (ANOVA) for significant test (Chatfield 1983). The standard deviation for Tx measurement was 8–10%.

Harvesting Techniques. Preformulation Step. Temperature of fermented broths (NH sludge, H sludge, SIW, and soya) was gradually lowered from 30 ± 1 to 25 ± 1°C. Individually sterilized 4 M H₂SO₄ and 4 M NaH₂PO₄ were mixed in 1:1 volume ratio, and the mixture was added to fermented broths via automated peristaltic pumps integrated into the fermentation setup to lower the pH from 7 ± 0.1 to 4.5 ± 0.1. If 4 M NaH₂PO₄ was used singly for pH adjustment, the volume required was 10 times more than the mixture mentioned previously, and there was a loss of approximately 45% Tx (initial Tx of fermented broth at 12,000 SBU/μL gave Tx of 7,000 SBU/μL after pH adjustment) during measurement because of dilution. Later, the broth at pH 4.5 ± 0.1 was collected aseptically in sterile high-density polyethylene (HDPE) containers (12-liter capacity, VWR Canlab), sealed with Parafilm, and stored in a freezing chamber (DuPont, Wilmington, DE) at −20°C until used for formulation studies.

Preformulation Step. The frozen acidified broth (NH sludge, H sludge, SIW, and soya from postfermentation step) was freeze thawed by holding the container in water at 30 ± 1°C for 1 d. To compare the results of different acidified fermented broth formulations at 19.5 × 10⁹ SBU/liter, the fermented broths (after thawing) with initial Tx values of 9.54, 12.7, 16.5, and 18.9 × 10⁹ SBU/liter for soya, NH sludge, SIW, and H sludge, respectively, needed to be concentrated to higher Tx values. Thus, the acidified fermented broths were centrifuged in sterilized 500-ml HDPE bottles.
The concentrated broths of H sludge and NH sludge at 9,682 × g at 20 ± 1°C. The concentrated broths of H sludge and SIW at ≈70 g/liter solids concentration yielded an approximate Tx of 25.4 × 10^9 SBU/liter and NH sludge and soya at 120 g/liter solids gave a Tx of 24.6 and 18.8 times 10^9 SBU/liter, respectively. The estimation of Tx at this stage was essential for further studies on adjuvant screening.

**Screening/Selection Tests.** Specific adjuvants with known characteristics/property and recommended concentrations were selected from literature (Lisansky et al. 1993, Burges 1998, Bishop 2002). The screening/selection criteria of each adjuvant was based on specific characteristics/properties (e.g., suspendibility—suspension; phagostimulant—feeding action (increase in Tx); stickers/adherents—rainfastness; antimicrobial agents—percentage of contamination; and UV screens—percentage of viable spore [VS] and Tx losses) as listed in protocol (Table 2).

**Preparation of Samples (Suspending Agents/Phagostimulants/Stickers).** Each selected adjuvant was tested individually (one at a time basis) per the concentrations given in Table 2 for specific properties by amending the concentrated fermented broth (from preformulation step described above) in 125-ml HDPE bottles (100-ml formulation). The adjuvant amended concentrated broth was further fortified with 0.5% (wt:vol) propionic acid to eliminate contamination followed by addition of 0.5% (wt:vol) sodium monophosphate as a buffer to maintain pH (because addition of propionic acid and individual adjuvants may alter the pH). Furthermore, the volume of individual samples thus obtained after amendment with respective suspending/phagostimulating/sticking agents was made up with supernatant of centrifuged broth to attain final Tx of 19.5 × 10^9 SBU/liter approximately equivalent to industry standard Foray 76B (Abbott Laboratories, Chicago, IL). The adjuvant/additives composition (mentioned above and in Table 2) was the final concentration found in each adjuvant-amended formulation (19.5 × 10^9 SBU/liter) of respective broths. The screening agents comprised five replicates of each adjuvant in each category (class of adjuvant, namely, suspending agents, rainfasteners, and phagostimulants). Thirty (six adjuvants × five replicates) adjuvants were used for each treatment. The value of five replicates was treated as the experimental unit. Data were subjected to one-way ANOVA (Chatfield 1983). Therefore, five (replicates of each adjuvant) × six (adjuvants) = 30 samples were tested for each adjuvant category resulting in 30 by three (categories) = 90 total samples. Each screened preparation also included a control (fermented centrifuged broth) without adjuvants at pH 4.5 ± 0.1. The bottles were stored at 20 ± 1°C for 24 h.

**Assessment of Screening/Selection Criteria.***Suspendibility Tests.** The tests were used to adjudge the suspension properties of formulations during shelf storage. Each suspending agent formulation (50 ml) was suspended in 100 ml of deionized water (turbidity-free Milli-Q water). The sample was compared with a well mixed suspension of industry standard

| Table 2. Screening and selection of different adjuvants/additives for Bt formulations |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Suspending agentsa, b, c, d | Sticking agentsa,b,c,d | Phagostimulantsa,b,c,d | Anti-microbial agentsa,b,c,d |
| **Susceptibility** | **50% increase in** | **VS/contamination** | **UV screens** |
| Sodium metabisulphite (D3)a,b,c,d | Salicylic acid (P1)a,b,c,d | Xanthan gum (R3)a,b,c,d | PABA (UV5)c |
| Veegum (regular grade) (D6) | Lactic acid (P4)a,b,c,d | Citric acid (AM-5) | Propionic acid (AM-6)a,b,c,d |
| Sorbitol (D1)a,b,c,d | Molasses (P3)a,b | Sodium metabisulphite (AM-1)a,b,c,d | Congo Red (UV3)a,b,c,d |
| Glucose (P1) | Molasses (R1)a,b,c,d | Sodium lignosulphate (UV1)a,b,c,d | Sodium monophosphate (D4)a,b,c,d |
| Molasses (P2)a,b,c,d | Ghatti gum (R2) | Methyl para benzoate (AM-2) | Lactic acid (AM-4) |
| Soya flour (P5)a,b,c,d | Sodium silicate (D5) | Skimmed milk powder (R4)a,b,c,d | Bismaleimide sulfonic acid (BSA) (UV6) |
| Sucrose (D2) | Cornmeal (P6) | Oatmeal (P4)d | Polyvinylpyrrolidone (R6) |
| Sodium metabisulphite (D4)a,b,c,d | Soya flour (P5)a,b,c,d | Xanthan gum (R3)a,b,c,d | PABA (UV5)c |

Superscript letters: a, NH formulations; b, H formulations; c, SIW formulations; d, soya formulations. The adjuvants with superscript a, b, c, d represent >50% suspendibility, >50% VS and Tx losses, <50% microbial contaminant, and <50% suspending properties of formulations during shelf storage. Each adjuvant was tested independently (one-at-a-time-basis).
Foray 76B (Abbott Laboratories) as control. The suspended sample was well dispersed by shaking in a separating funnel several times and then letting it stand undisturbed for 30 min. Afterward, 20 ml of the sample was withdrawn from the bottom to determine the amount of settled solids (dry weight basis, concentration in grams per liter). The supernatant from the top was used to determine the apparent turbidity (nephelometric turbidity units, NTU) as optical density using UV-visible spectrophotometer (Varian Cary 100 Bio, Mississauga, Ontario, Canada) at λmax of 430 nm (AWWA 1998). The suspendibility of formulations was defined as per equation 2:

\[
\text{Suspendibility} = \frac{\text{Turbidity (NTU)}}{\text{Total solids (g/L)} - \text{Settled solids (g/L)}} \quad [2]
\]

The percentage relative suspendibility (equation 3 was calculated, based on industry standard Foray 76B (control).

\[
\text{Percentage Relative Suspendibility} = \left(\frac{\text{Turbidity}}{\text{Total solids} - \text{Settled solids}}\right)_{\text{sample}} \times 100 \quad [3]
\]

\[
\text{Percentage standard deviation for suspendibility measurement was 2–5%}.
\]

**Rainfastness Tests.** A simple bench top modified glass slide (precleaned ground edge blood smear slides 25.4 by 76.2 by 645.16 mm, Surgipath, Franklin, Germany) assay method was developed instead of the conventional simulated rainfall in a greenhouse. Precleaned and preweighed glass microscopic slides were used for the purpose.

One milliliter of sticker formulation sample was uniformly spread on these slides, and it was allowed to air dry (for quick drying, kept inside a laminar flow chamber). The slides were held approximately at 2 cm under a stream of distilled water released from a burette (high precision 100-ml burette, VWR Canlab). Approximately 40 ml of distilled water was poured from the burette at a rate of 20 ml/min over the slide, which was continually moved back and forth using a jack-mounted slide. Slides were air-dried, and the procedure was repeated for three wash-dry cycles. Slides were held approximately at 2 cm under a stream of distilled water released from a burette (high precision 100-ml burette, VWR Canlab). The slides were air-dried, and the procedure was repeated for three wash-dry cycles. Slides were held approximately at 2 cm under a stream of distilled water released from a burette (high precision 100-ml burette, VWR Canlab). The percentage of sticking was 10–12%.

**Phagostimulant Tests.** The phagostimulant action was estimated by increase in Tx of each phagostimulant-amended formulation in comparison with unamended sample determined by bioassay tests (discussed above).

**Preparation of Samples for Antimicrobial/UV Tests.** The concentrated broths [≈7 or 12% (wt:vol) solids; obtained from preformulation step] with respective Tx (mentioned above) were amended with basic adjuvants (in % wt:vol); glycerol (2%, humectant), Tween 80 (0.2%, surfactant), and Triton X-100 (0.1%, surfactant) as reported by Lisansky et al. (1993) and sorbitol (21%, prescreened suspending agent). This suspension was further used to screen antimicrobial agents and UV blockers and investigate half-life. The final antimicrobial agent/UV amended broth was stoichiometrically made up with supernatant to attain Tx of 19.5 × 10⁹ SBU/liter as described above. The antimicrobial agent/UV formulations comprised five replicates in each category of adjuvant. Thirty (six adjuvant × five replicates) adjuvants were used for each treatment. The value of five replicates was treated as the experimental unit. Data were again subjected to one-way ANOVA (Chatfield 1983). Therefore, five (replicates of each adjuvant) × six (adjuvants) = 30 samples were performed for each screening agent resulting in 30 by 2 (categories) = 60 total samples. Each screened preparation consisted of a control-I at pH 4.5 ± 0.1 for each formulation (raw, fermented centrifuged broth) without screened agents. Likewise, an additional control-II for molluses at 0.2% (wt:vol), unexposed to UV radiation was set up to account for the phagostimulant effect, if any.

**Microbiological Purity Testing.** The above-stated suspension was amended with five (replicates of each adjuvant) × six (antimicrobial agents) as stated in Table 2 and was stored in HDPE bottles at room temperature (20 ± 1°C). Samples were drawn at a period of 1, 7, 15, 30, and 60 d for microbial contamination. Each sample was appropriately diluted in sterile saline solution (0.85% NaCl) to detect microbial contamination on selective agars by using protocol given in Table 3. Various nutrient media were purchased from EM Science (Merck, Darmstadt, Germany).

**Sunlight Exposure/UV Inactivation Tests.** The suspensions (preparation method described above) with UV blockers were transferred in cylindrical HDPE sample vials (1.5 cm in diameter, 10-ml capacity, VWR Canlab). The sample vials (each containing 1 ml of sample) were placed at a 20-cm distance from the UV radiation source as seen in the laboratory setup (Fig. 1). A 15-W UV-A tube (emission maxima at 366 nm) and a 15-W UV-B tube (emission maxima at 312 nm) (Transilluminator, UVP, San Gabriel, CA) provided specific UV radiation. These tubes were mounted parallel to each other in a customized setup with aluminum sheet covering the entire interior of the compartment so as to reflect the stray radiation (Fig. 1).
The combination of UV-B/UV-A tubes produced 4,788 W/cm², which emitted 1,403 W/cm² UV-B radiation (280–320 nm), 2,952.5 W/cm² UV-A radiation (320–400 nm), and 421.9 W/cm² visible light (400–800 nm) as per details provided by VWR Canlab. UV agents were screened by exposing samples for 8 h in the laboratory setup. The absorbance spectra of different UV screens and NH and H fermented sludge were scanned by using UV-visible spectrophotometer (Varian Cary 100 Bio, Mississauga, Ontario, Canada).

For half-life studies, Bt suspensions or formulations (duplicate samples) were set up as follows: 1) fermented broths were exposed to light for different time intervals of 0, 1, 6, 10, 16, 28, 32 and 52 h; and 2) formulations (obtained from different fermented media) were exposed to 0, 5, 10, 20, 30, 45, 90, 300, and 1,000 hours. Half-life (T_{0.5}, time required for Bt formulation to lose half of its Tx by UV action) was calculated from residual Tx versus exposure time plots. Eight hours in the laboratory under these UV conditions was considered equivalent to 1-d field exposure to the complete UV spectrum. This assumption was in concordance with McGuire et al. 1996 who used Sun Test apparatus to test solar stability of Bt formulations against Plutella xylostella (L.) The control samples (without UV protection agents) were wrapped with thick aluminum sheet and stored at ambient temperature (20 ± 1°C) for same period of exposure. Samples were taken at regular intervals for VS and Tx measurement and a standard deviation of 8–12% for Tx and VS measurement was observed.

### Table 3. Microbiological purity test schedule

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Medium</th>
<th>Environmental conditions</th>
<th>Incubation time</th>
<th>Inference test</th>
<th>Upper limits of concn (CFU/ml)</th>
<th>Data from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast and mold</td>
<td>Malt extract agar</td>
<td>pH 1.5, 5, 30 ± 1°C</td>
<td>3-5 d</td>
<td>Colonial morphology (dumb bell shaped under 100× magnification)</td>
<td>&lt;100</td>
<td>Quinlan 1990, Lisansky et al. 1993, Burges 1998, and Bishop 2000.</td>
</tr>
<tr>
<td>Fecal streptococci</td>
<td>Slanetz and Bartley agar</td>
<td>37 ± 1°C</td>
<td>48 h</td>
<td>Pink or blue colonies (chain-like, under the microscope)</td>
<td>&lt;3 x 10⁴</td>
<td>According to IUPAC recommendation, upper limit of concentration for microbial contaminants should be expressed in colony-forming units (CFU) per gram and CFU per milliliter for solid and liquid formulations, respectively.</td>
</tr>
<tr>
<td>California Fecal coliforms</td>
<td>Luria’s broth, MFC agar/m-endo agar</td>
<td>37 ± 1°C</td>
<td>48 h (3–24 h)</td>
<td>Pink or blue colonies (chain-like, under the microscope)</td>
<td>&lt;10⁴</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>TSB; plating on Baird Parker agar</td>
<td>35±1°C</td>
<td>24 h</td>
<td>Gram stain positive colonies (black and glossy); resemble &quot;bunch of grapes&quot; under the microscope</td>
<td>10⁴</td>
<td></td>
</tr>
<tr>
<td>Salmonella and Shigella</td>
<td>Lactose broth; selenite (10 ml) and tetrathionate broth (10 ml) and testing growth on Brilliant green phenol red lactose sucrose agar and bismuth sulfit agar</td>
<td>20–25°C</td>
<td>18–24 h</td>
<td>Blackening with sheen (rod shaped without spots or crystal protein and characteristic flagella under the microscope); green, moist, flat, and transparent colonies represent Shigella.</td>
<td>10²</td>
<td></td>
</tr>
</tbody>
</table>

The combination of UV-B/UV-A tubes produced 4,788 x 10⁻⁸ W/cm², which emitted 1,403 x 10⁻⁸ W/cm² UV-B radiation (=280-320 nm), 2,952.5 x 10⁻⁸ W/cm² UV-A radiation (=320-400 nm), and 421.9 x 10⁻⁸ W/cm² visible light (=400-500 nm) as per details provided by VWR Canlab. UV agents were screened by exposing samples for 8 h in the laboratory setup. The absorbance spectra of different UV screens and NH and H fermented sludge were scanned by using UV-visible spectrophotometer (Varian Cary 100 Bio, Mississauga, Ontario, Canada).
Results and Discussion

Suspending Agents. The percentage of suspending ability (suspendingibility response) for different screened suspending agents is shown in Fig. 2a. Sorbitol (D1), sodium monophosphate (D3), and sodium metabisulfite (D4) showed better suspending ability for all formulations (Fig. 2a). The suspending ability of the three suspending agents ranged between 68 and 92% with significant difference ($F = 3.29; df = 5, 24; P < 0.05$). These results agreed with the work of other researchers who have pointed out the role of sorbitol in better suspensions in food industry and biopesticidal sprays (Smirnoff and Juneau 1982; Petersen et al. 2004). Addition of sorbitol produced well dispersed suspensions as it would carry out hydrophilic stabilization of proteins and provide cryoprotection of the fermented broths (all broths in this study are highly proteinaceous in nature). The cryoprotection property of sorbitol (Petersen et al. 2004) would aid further in better stability of Bt formulations during storage at lower temperatures, which are commonly encountered in many parts of Canada and United States. The low temperatures refer to the storage temperatures as when the biopesticides will be stored in warehouse facilities, there is a possibility of encountering low temperatures, which may affect the properties of formulation. Furthermore, the unused/partially used biopesticide barrels are often left in the open sites, which may affect biopesticide stability.

In addition, sodium monophosphate finds widespread use as a dispersing agent in pesticidal sprays and acts as buffering agent (WHO 1999). This will also aid in maintenance of pH during shelf storage and further retain the physical consistency of formulations. Although sodium metabisulfite has been reported as a preservative (WHO 1999), it also can stabilize suspensions as established in this study. So, sodium metabisulfite could be used for its properties of suspension as well as preservative (antimicrobial agent). However, there will be a difference in concentrations at which it can serve as a suspending agent [20% (wt:vol)] or preservative [0.2–0.5% (wt:vol)], but all the same, suspending agent concentrations also can provide antimicrobial properties (saving on formulation economy). Similarly, sodium monophosphate offered dual-advantage of buffering and structuring of suspensions.

Phagostimulants. The phagostimulants were screened depending on the feeding response of spruce budworm larvae on formulations (determined by relative Tx increase compared with control) as presented in Fig. 2b. The soya flour (P5) showed increase in Tx by 13–14% ($F = 2.84; df = 5, 24; P < 0.05$) for all formulations, except fermented H sludge compared with control (no phagostimulants). However, molasses (P3) showed 9% increase in Tx for H sludge formulations as well as 13% increase for NH sludge formulations ($F = 2.97; df = 5, 24; P < 0.05$). The maximum phagostimulant action was based on increase in Tx obtained for each phagostimulant treatment. Soya flour showed higher Tx increase for most of the formulations, probably because of the feeding preference of larvae because of granular consistency of soya, known to possess high phagostimulant response (Castillejos et al. 2002). It is possible that the spruce budworm larvae have selective taste for soya formulations. Furthermore, molasses provided phagostimulant response in NH and H sludge formulations selectively because of the enhancement of odor and sugar content of these formulations.

Stickers/Rainfasteners. The screening of various stickers based on percentage of sticking property is presented in Fig. 2c. It was observed that molasses (R1) increased adherence/rainfastness by 84–90% of all media-based Bt formulations, except soya formulations. Indeed, molasses has been shown to have good sticking property and is widely used in various biopesticidal formulations (Burges 1998). Similarly, skimmed milk powder (R4) increased sticking by 93 and 64% for fermented NH sludge and soya formulations, respectively, in comparison with formulations not amended with stickers and the values were significantly different ($F = 4.63; df = 5, 24; P < 0.05$) between each sticker treatment. The stickiness (resistance to washoff) of the molasses was a result of sugar content as any sugar on drying (here, glass slide simulated as leaf of the tree) will lend the characteristic sticking property as it forms a cohesive bond with the glass slide that aids in adherence and probable stickiness during rainfall. Furthermore, polymeric chain of amino acids in molasses cross-links to form a contiguous, three-dimensional molecular network in solution. This network traps the water within to form a firm, flow-resistant structure (Xu et al. 2005) that may be difficult to wash off by rain. Indeed, gelling of molasses has been found to cause pumping problems from storage tanks for use in animal feed. Likewise, skimmed milk powder-based formulation when air-dried during field application would lend adherence to the formulation (probably because of presence of casein protein molecules-polypeptide chains) preventing washout.

The screening of different formulation adjuvants would aid in further quantification for use in development of stable formulations. Additionally, it will be more cost-effective to use multifunctional adjuvants, for example, molasses, which possess sticking, phagostimulation, and UV resistance properties (discussed below).

Antimicrobial Agents. Screening results of different antimicrobial agents for NH sludge, H sludge, SIW, and soya formulations are presented in Table 4, and the corresponding profiles after a period of 2 mo are given in Fig. 3. There was no contamination for first
7 d; however, after a period of 7 d, a contamination was detected in some of the antimicrobial agent-treated samples as illustrated in Table 4. This contamination could be because of the adjuvants added and the ambient air in the amendment room, which might have become contaminated over the 7-d period. Meanwhile, the room where manipulations were made was free of contamination as tested through exposure of agar plates from 0 to 5 d. Thus, the contamination would have set on seventh day. Furthermore, other possible sources of contamination—fermented broth (not contaminated on day 1; Table 4) and sampling (use of sterile equipment)—were ruled out. It was evident that soya suspensions showed no contamination with any of the agents except sodium metabisulphite (AM-3), citric acid (AM-4), and lactic (AM-5) acids, and the results were significantly different (F = 3.79; df = 5, 24; P < 0.05) for each anti-

![Fig. 2. Screening profile of different adjuvants for different media, namely, NH, H, SIW, and soya: a) suspending agents, b) phagostimulants, c) stickers, and d) e), UV screens (UV1 to UV5). Error bars represent standard deviations. Control refers to sample without adjuvants. All symbols D_x, P_x, R_x, UV_x (x = 1, 2, 3, 4, 5, or 6) are defined in Table 2. Each adjuvant was tested independently (one at a time-basis).](https://academic.oup.com/jee/article-abstract/99/4/1065/2218516/Screening-of-Different-Adjuvants-for-Wastewater/1072)
Table 4. Screening results of different antimicrobial agents

<table>
<thead>
<tr>
<th>Yeast and mold</th>
<th>E. coli</th>
<th>S. typhae</th>
<th>Staphylococcus sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NH</td>
<td>H</td>
<td>SIW</td>
</tr>
<tr>
<td><strong>7 d</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM-1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>AM-2</td>
<td>7</td>
<td>8</td>
<td>37</td>
</tr>
<tr>
<td>AM-3</td>
<td>55</td>
<td>34</td>
<td>31</td>
</tr>
<tr>
<td>AM-4</td>
<td>108</td>
<td>31</td>
<td>26</td>
</tr>
<tr>
<td>AM-5</td>
<td>132</td>
<td>34</td>
<td>61</td>
</tr>
<tr>
<td>AM-6</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>143</td>
<td>115</td>
<td>213</td>
</tr>
<tr>
<td><strong>15 d</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM-1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>AM-2</td>
<td>10</td>
<td>21</td>
<td>69</td>
</tr>
<tr>
<td>AM-3</td>
<td>54</td>
<td>95</td>
<td>59</td>
</tr>
<tr>
<td>AM-4</td>
<td>9.1</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td>AM-5</td>
<td>9.2</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>AM-6</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>21</td>
<td>2.9</td>
<td>6.8</td>
</tr>
<tr>
<td><strong>30 d</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM-1</td>
<td>2*</td>
<td>1*</td>
<td>2.3</td>
</tr>
<tr>
<td>AM-2</td>
<td>1.59</td>
<td>5*</td>
<td>3.4</td>
</tr>
<tr>
<td>AM-3</td>
<td>1.2*</td>
<td>3*</td>
<td>2.7</td>
</tr>
<tr>
<td>AM-4</td>
<td>3.1*</td>
<td>4*</td>
<td>2.4</td>
</tr>
<tr>
<td>AM-5</td>
<td>6.6</td>
<td>0.68</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>6.5</td>
<td>0.68</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Microbial concentrations are reported in colony-forming units (CFU) per milliliter and are average of five replicates ($P < 0.05$). At 1 d, the concentration was nondetectable (nd) for all antimicrobial agents for all fermented broths. The control (without antimicrobial agents) was not considered further on the basis of range of contamination observed.

*Actual value $= \times 10^2$ CFU/ml.

bActual value $= \times 10^3$ CFU/ml in the row.
microbial treatment. Moreover, the best antimicrobial agents for all liquid suspensions were sorbic (AM-1) and propionic acid (AM-6), which controlled the contamination by $10^2$ to $10^6$ times in comparison with other antimicrobial agents. This could be because the $pK_a$ (negative logarithm of dissociation constant) of sorbic acid (4.8) and propionic acid (4.87) are in the working pH range of 4.5 ± 0.1, leading to more effective form (dissociated) with an ability to permeate into microbial cells causing maximum antimicrobial activity in comparison with sodium metabisulfite ($pK_a = 1.89$). Indeed, Tyagi et al. (1998) have reported that
organic acids in the dissociated form were more toxic in inhibiting bacterial growth. Likewise, metals in ionic form have been found to be toxic to Bt metabolism (Entwistle et al. 1993). Sorbic acid has been shown to inhibit gram-positive and -negative, catalase-positive and -negative aerobes and anaerobes and thermophilic, mesophilic, and psychrotrophic bacteria (Bracey et al. 1998). This also could have resulted in inhibition of Bt cells, but periodic Tx measurements yielded stable results (data not reported), as deduced from Brar et al. (2004b). Thus, development of formulated product of different fermentation broths without addition of antimicrobial agents is impractical and may lead to serious contamination problems degrading the product, reducing its effectiveness and integrity and hence shortening the overall shelf-life. Propionic (AM-6) and sorbic (AM-1) acid salts (weak organic acids) gave better performance than other antimicrobial agents owing to their well defined and well established mechanism of growth inhibition and excellent safety records (Bracey et al. 1998). The growth inhibition could be because of inhibition of essential metabolic reactions and accumulation of toxic anions (Piper et al. 2001).

Thus, the best antimicrobial agents were propionic and sorbic acids with ability to act on a wide spectrum of contaminants. More studies need to be carried out on quantitative optimization of these antimicrobial agents.

**UV Screens.** The UV blockers screening results (8-h exposure) are shown in Fig. 2d and e and corresponding UV absorption spectra scans of screened agents as well as those of media used for Bt formulation are presented in Fig. 4. Semisynthetic soya medium and SIW did not show any absorbance, whereas fermented NH and H sludge showed average absorbance of 10 and 2, respectively. It was seen that the package formulations containing each of sodium lignosulfonate (UV1), molasses (UV2), and Congo red (UV3) served as universally good UV screens with the exception of p-amino benzoic acid (PABA, UV5), which was good for SIW formulations based on significant difference between all UV treatments \((F = 4.48; \text{df} = 5, 24; P < 0.05)\). Moreover, Tx losses were reported as: control-I (without UV screens, exposed to UV radiation), 70–80% Tx losses (depending on broth); control-II (with 0.2% (wt:vol) molasses, without UV exposure), 2% Tx increase which was negligible; and sample (with UV screens, exposed to UV radiation), 10–15% Tx losses. Furthermore, molasses at 0.2% (wt:vol) is used as UV screen agent that will distinguish it from its action as a phagostimulant at \(\geq 0.5\%\) (wt:vol) (Burges 1998).

Despite all the screening agents possessing their wavelength spectrum in the range of UV-A (320–400 nm) and UV-B (280–390 nm) as seen in Fig. 4, only sodium lignosulfonate (UV1) and molasses (UV2) gave higher average absorbance of 10 and 3.5, respectively, over a broad range of spectrum, especially for sodium lignosulfonate. Congo red gave lower average absorbance of 0.09, but the absorbance was spread over a wide wavelength range from 200 to 400 nm, which provided better UV protection. Moreover, Congo red (synthetic azo dye) can remain inert under field conditions and provide enhanced UV resistance. However, folic acid (UV absorbance 0.7) has a tendency to degrade rapidly in heat, cold, and exposure to light, including sunlight, which would ultimately affect its overall photostabilization properties.

**Half-Life Studies.** The half-lives of Tx potential of different Bt fermented broths and formulations (with and without UV screens) is presented in Fig. 5. The residual Tx (percentage of Tx remaining after UV exposure) data of different fermented broths and package formulations fitted fairly well into first order rate model \((R^2 = 0.93)\) and first order rate constants \([k] = 0.1735, 0.1118, 0.2629,\) and \(0.5835 \text{ d}^{-1}\) for NH sludge, H sludge, SIW, and soya fermented broths and \(k = 0.0622, 0.0729, 0.0768,\) and \(0.2423 \text{ d}^{-1}\) for NH sludge, H sludge, SIW, and soya formulations, respectively with truncation at 6.5 d (fermented broths), 50 d (package formulations with UV screen), and 125 d (package formulations without UV screen). The difference in UV resistance between Bt fermented broths and package formulations could be because of two reasons: 1) stabilization of the broth by adjuvants/additives; and 2) centrifugation of the broth resulting in higher suspended solids concentration, leading to less exposure of spores, \(\delta\)-endotoxin, and other virulence factors such as chitinases, vegetative insecticidal proteins, phospholipases, and cytolytic toxins. The virulence factors, spore and crystal toxin, were probably embedded inside the concentrated solids rather than being dispersed free in the supernatant.

Spore count has been used to calculate \(T_{0.5}\) as a measure of UV resistance by many researchers (Cohen et al. 1991; Burges 1998; Bishop 2002). However, spore count cannot be an accurate representation of Tx as reported in our previous work (Yezza et al. 2005). Therefore, in this study, half-lives were calculated on the basis of residual Tx as it would provide direct indication of UV inactivation and the results for UV treatments were significantly different \((F = 3.67; \text{df} = 5, 24; P < 0.05)\). Herman et al. (2002) also advocated that it was more precise if calculations of \(T_{0.5}\) were based on residual Tx.

**Fermented Broths.** The half-lives (Fig. 5) and \(T_{0.9}\) (time required for Bt formulation to lose 90% of its Tx by action of UV) of different fermented broths, followed the order: H > NH > SIW > soya and were found to be (in d) as 6.2 > 3.99 > 2.63 > 1.19 and 20.59 > 13.27 > 8.76 > 3.95, respectively. \(T_{0.5}\) of H sludge was probably higher because of lower concentration of protease in the medium (protease of 0.5 IU/ml for H sludge vis-à-vis 2.4 IU/ml for NH) which resulted in lower inactivation of crystal protein during storage and hence better residual Tx. Proteases are well known to cause degradation of crystal proteins and act as inhibitors of potency of Bt formulations (Burges 1998). On the contrary, although protease activity of fermented NH sludge was higher than SIW and soya and yet \(T_{0.5}\) of NH sludge was higher than SIW and soya. This was probably because of overriding factor of relatively bigger floc size of NH sludge.
Fig. 4. UV spectra scans of NH and H fermented sludges and different UV screens. Digits in parentheses represent average absorbance (AA).
and H sludge (particle size of Bt fermented broths: NH sludge, 35 μm; H sludge, 25 μm; SIW, 6 μm; and soya, 3 μm), which formed a sheath around spores, protected the crystal protein, and other virulence factors against UV effect. Lower particle size in case of soya and SIW did not provide the advantage of UV resistance (Brar et al. 2004a). Lower particle size in case of soya formulations showed higher residual Tx relative to NH sludge, H sludge, and SIW-based formulations in field conditions usually ranged from 16 h to 2 d (Ragaei 1998). Moreover, when UV spectra of nonsterilized NH sludge and H sludge were taken (Fig. 4), absorbance was 10 (very high) for NH sludge (wavelength range 200–400 nm) and 2 for H sludge (wavelength range 200–300 nm). Furthermore, domestic sludge has been reported to possess components with chromophoric compounds or auxochromes (majority of fulvic, humic, and hydromelanic acids) with absorbance at 350 nm (Manka et al. 1974). These components in the concentrated suspensions (more cations) could have acted as natural UV screens in NH sludge and H sludge based formulations. Meanwhile, the UV resistance could be further enhanced by addition of UV screens.

Formulations (with UV Screens). When the formulated package products (without UV screen) were exposed to UV, half-lives of Tx were higher relative to fermented broths (Fig. 5). This was because the protease activity was not observed in formulations at acidic pH (4.5 ± 0.1) where proteases were not active. T0.5 (in days) was found to be in the order NH sludge > H sludge > SIW > soya as 11.14 > 9.51 > 9.02 > 2.8. The NH sludge, H sludge, and SIW-based formulations showed higher residual Tx relative to soya formulations. Half-lives of conventional soya-based Bt formulations in field conditions usually ranged from 16 h to 2 d (Ragaei 1998). Moreover, when UV spectra of nonsterilized NH sludge and H sludge were taken (Fig. 4), absorbance was 10 (very high) for NH sludge (wavelength range 200–400 nm) and 2 for H sludge (wavelength range 200–300 nm). Furthermore, domestic sludge has been reported to possess components with chromophoric compounds or auxochromes (majority of fulvic, humic, and hydromelanic acids) with absorbance at 350 nm (Manka et al. 1974). These components in the concentrated suspensions (more cations) could have acted as natural UV screens in NH sludge and H sludge based formulations. Meanwhile, the UV resistance could be further enhanced by addition of UV screens.

Acknowledgments

We thank the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, Canada Research Chair) for financial support. We also thank the Natural Sciences and Engineering Research Council of Canada, Canadian Forestry Service, and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing scholarship to S.K.B.

References Cited


Received 2 November 2005; accepted 22 February 2006.