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Downstream processing of mosquitocidal toxins from solid state fermentation of *Lysinibacillus sphaericus* and *Bacillus thuringiensis israelensis*

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ABSTRACT

Aims: The aim of this study was downstream processing of moquitocidal toxins produced by *Lysinibacillus sphaericus* (*L. sphaericus*) and *Bacillus thuringiensis israelensis* (*Bti*) under solid state fermentation.
Methodology and results: Two mosquitocidal strains (*L. sphaericus* and *Bti*) were grown separately in trays under solid state fermentation for toxin production. The best conditions for extraction of crude toxins from fermented solids of both cultures were tap water at 5-50 °C, for 10 min under static conditions. Also, concentrated mosquitocidal toxins were efficiently extracted from fermented solids by 4 constitutive additions of 500 mL tap water to 1 kg of fermented culture at room temperature (25 °C) for 5 min each under static conditions. Both extracted toxins were formulated with talcum powder and they were stable for 8 months at room temperature.
Conclusion, significance and impact of study: It is very important to study the operating conditions for mosquitocidal toxins extraction from solid state fermentation (SSF) and its formulation in cost effective manner.

Keywords: Mosquitocidal toxins, solid state fermentation, *Lysinibacillus sphaericus*, *Bacillus thuringiensis israelensis*, downstream processing

INTRODUCTION

Lysinibacillus sphaericus (*L. sphaericus*) and *Bacillus thuringiensis* (*Bt*) are the most widely used entomopathogenic microorganisms. Some strains of these bacteria are important agents used in insect control programs to reduce vector species that transmit diseases such as malaria, yellow fever and dengue. These bacteria produce toxin proteins that are toxic to susceptible insect larvae upon ingestion. They are spore-forming bacteria, resist the environmental conditions and persist in the field. Therefore, they are suitable for industrial production and field application (Luna-Finkler and Finkler, 2012). A biotechnological process for the production of microbial proteins can be divided into two steps: the fermentation and downstream processing steps as reported by Wenzig *et al.* (1993).

Several practical and economic advantages have been reported to solid state fermentation (SSF) compared with submerged fermentation such as low production cost, saving water and energy, low capital investments, low waste effluent, more concentrated product, stability of the product and minimizing operational downstream processing (Singh *et al.*, 1999; Holker and Lenz, 2005). In SSF, microbial products are formed at the surfaces of the solid materials with low moisture content (Selvakumar and Pandey, 1999). The advantages of SSF technology should be maintained during the extraction step for the recovery of the microbial products. Sometimes, the natural solid substrates in SSF can hinder downstream process and a dilute extract can defeat economically important SSF advantages (Castilho *et al*., 2000; Singhania *et al.*, 2009). So, it is necessary to select the most appropriate solvent for leaching out the product from the fermented solids depending upon its application, process economics and further downstream processing. Mussatto *et al.* (2012) reported that solvent type, solvent concentration, solvent to the solid ratio, contact time, extraction temperature and pH are important factors affecting the efficiency of microbial product extraction.

Although extensive research has led to production of mosquitocidal toxins using cost effective media, the major factors limited their commercial success are scaling up and formulation. Formulation of biocontrol agents encountered number of challenges such as ease of production, application and storage and shelf life during transportation (Boyetchko *et al.*, 1999). Also a successful formulation should be compatible with end user equipment and its activity must be stable under various environmental conditions (Brar *et al.*, 2006).

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Although, there are many literatures dealing with production of mosquitocidal toxins by *L. sphaericus* and *Bacillus thuringiensis israelensis* (*Bti*) under SSF, none considering the leaching of these toxins from fermented solids. Thus, the aim of the present work was to study the extraction conditions of crude mosquotocidal toxins produced by *L. sphaericus* and *Bti* under SSF in trays. Also, concentrated toxins extraction was studied for more downstream processing or for direct formulation.

MATERIALS AND METHODS

Microorganisms

Egyptian strain, *L. sphaericus* 14N1, (El-Bendary *et al.*, 2003) and the standard strain of *Bti* are used in this study. *Bacillus thuringiensis israelensis* was obtained from Prof. Dr. Fergus G. Priest, Heriot-Watt University, United Kingdom.

Solid state fermentation

SSF was performed as previously reported by El-Bendary *et al.* (2016). Initially, fine sand used as a carrier material (less than 0.35 mm in diameter). Wheat germ meal, linen meal (4.5% of each) and 0.2% yeast extract were used for production of *L. sphaericus* 14N1 while, sugar beet pulp, sesame meal (4.5% of each) and 0.2% beef extract were used for production of *Bti*. Carrier and substrates were taken in aluminum trays (35×25×10 cm^3) then moistened with tap water and autoclaved. These trays were inoculated with tested organisms and incubated at 30 °C for 5 and 7 days for growth of *L. sphaericus* 14N1 and *Bti*, respectively with daily manual agitation.

Crude toxin extraction conditions

Solvent type

One gram of final fermented culture was put in Erlenmeyer flasks containing 100 mL solvent and then the crude toxins extracted under static and shaking conditions for 1 h. The solvent containing crude toxins was separated from sand by decantation. Other solid materials were removed from solvent by filtering through nylon cloth. The tested solvents were listed in Table 1.

Table 1: Effect of different solvents on extraction of toxin from fermented cultures of *L. sphaericus* 14N1 and *Bti* grown under SSF.

Sovent /conc	Mortality % of *C. pipiens* larvae at 2×10^{-5} dilution after 48 h			
	L. sphaericus 14N1 extracted under		*Bti* extracted under	
	Static	Shaking	Static	Shaking
Tap water	63±3 a	58±4 a	60±6 a	62±4 a
Distilled water	55±3 a	50±6 abc	57±3 a	53±3 abc
Ethanol/5%	25±3	17±3	33±3 cde	40±0
Acetone/5%	20±0	23±3	57±3 a	40±6
Omo blue/0.5%	37±3 b	20±6	20±0	33±3
Omo pink/0.5%	35±3 b	23±3	37±3 cd	40±0
Tween20/0.5%	33±3bc	17±3	53±3 a	50±6 bcde
Tween40/0.5%	40±0 b	35±3	57±3 a	47±3
Tween60/0.5%	40±6 b	40±0 cde	53±3 a	57±3 abc
Tween80/0.5%	33±3 bc	33±3	35±3 cd	40±0
Glycerol/5%	57±3 a	50±6 abc	40±0 bc	57±3 abc
Triton X 100/0.5%	17±3	23±3	33±3 cde	60±0 ab
Lactose/5%	18±2	40±0 cde	37±3 cd	40±6
$CaCl_2$/0.1M	20±6	23±3	17±3	17±3
KCl/0.1M	13±3	20±0	23±3	13±3
NaCl/0.1M	20±0	12±2	40±6 bc	43±3
$MnCl_2$/0.1M	35±3 b	35±3	50±6 ab	53±3 abcd
$MgCl_2$/0.1M	17±3	23±3	40±6 bc	40±6
Na_2SO_4/0.1M	33±3 bc	20±0	37±3 cd	37±3
Acetate buffer pH 4	17±3	12±2	17±3	12±2
Acetate buffer pH 4.5	23±3	43±3 cde	20±6	18±2
Acetate buffer pH 5	17±3	40±0 cde	25±3	15±3
HCl-KCl buffer pH 1.5	13±3	13±3	15±3	20±0
HCl-KCl buffer pH 2	17±2	17±3	25±3	17±3
Carbonate buffer pH 9.5	13±3	40±0 cde	15±3	23±3
Carbonate buffer pH 10	17±3	23±3	60±6 a	53±3 abcd
Carbonate buffer pH 10.5	10±0	13±3	40±6 bc	23±3
Glycin-HCl buffer pH2.5	13±3	10±0	15±3	38±4
Citrate buffer 3.5	20±0	40±6 cde	12±2	10±0

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Citrate buffer 4	17±3	40±6cde	57±3 a	43±3
Citrate buffer 4.5	13±3	37±3	37±3 cd	27±3
Citrate buffer 5	20±0	37±3	18±2	20±0
Citrate buffer 5.5	7±3	55±3 ab	20±0	20±0
Phosphate buffer pH 7	13±3	42±2 cde	20±0	57±3 abc
Phosphate buffer pH 7.5	17±3	40±6 cde	35±3 cd	38±4
Glycin-NaOH buffer pH 10	20±0	22±2	17±3	17±3
Succinate buffer pH 5.5	23±3	23±3	18±2	13±3
Tris-HCl buffer pH 7.5	17±3	47±4 bcd	57±3 a	40±6
Tris-HCl buffer pH 8	17±3	17±3	40±6 bc	40±0
Tris-HCl buffer pH 8.5	17±3	17±3	42±2 bc	38±4

Mortality % is expressed as mean value ± standard error. Values for each treatment followed by different letters are significantly different at P = 0.05.

Extraction time

The contact times studied were 10, 20, 30, 60, 120, 180 min and 24 h under static condition.

Extraction temperature

The studied temperatures were 5, 20, 30, 40 and 50 °C for 10 min under static condition.

Repeat extraction

Consecutive extractions (2 times) using the initial fermented culture (1 g) and renewing the solvent (100 mL each) were carried out for 10 min at room temperature under static condition. Also, more concentrated toxins extraction was studied through 4 consecutive extractions using 10 mL solvent in each step.

Scaling up concentrated toxin extraction

In this experiment, crude toxins in 1 kg of fermented solids of each tested organism were extracted under optimized conditions by consecutive applying tap water (500 mL) and decantation to separate sand and renewing the solvent up to 2 L solvent (tap water).

Formulation

Formulation of extracted mosquitocidal toxins was according to Zidack and Quimby (2002) with some modification. Talcum powder was added to concentrated toxins at 3:1 (w/v) and mixed well then dried in a laminar flow hood for 48 h at room temperature. The mosquitocidal activities of these formulations were tested for 1 year.

Bioassay of mosquitocidal activity

Bioassay of mosquitocidal activity of the crude toxins extracted from fermented cultures produced under SSF was adopted from Ampofo (1995) with some modification. Toxicity was determined with laboratory reared second instar larvae of *C. pipiens*. Serial dilutions were prepared from final fermented culture extracts and were placed into 100 mL beakers in triplicate along with 10 larvae each of *C. pipiens*. Control was run simultaneously using tap water only. About 10 mg of ground fish meal was added to each cup. The beakers were covered with muslin and kept at 26±2 °C with 10 h light/14 h dark cycle. The mortality percentage was calculated by counting the number of living larvae after 48 h and adopting Abbott's (1925) formula. Each bioassay was repeated two times in different days. All controls in bioassay tests showed mortalities around 0-10%.

Statistical analysis

Data were statistically analyzed according to SPSS system using one-way analysis and the Duncan's multiple range tests to determine the significance between means (Duncan, 1955). Data were expressed as mean values ± standard errors.

RESULTS AND DISCUSSION

Evaluation of different solvents for extraction of crude mosquitocidal toxins from fermented cultures of *L. sphaericus* 14N1 and *Bti*

Recovery of microbial products from fermented solids is one of the most challenging problems in solid-liquid separation (Luna-Finkler and Finkler, 2012). Therefore, suitable solvent selection is a critical factor for the recovery of the microbial products from the fermented mass (Castilho *et al.*, 2000).

Forty solvents were tested for extraction and recovery of crude mosquitocidal toxins from fermented cultures. Among them, tap water, distilled water and glycerol (5%) were the most efficient solvents for extraction of toxins from fermented culture of *L. sphaericus* 14N1 under static and shaking conditions (50-63% mortality at 2×10^{-5} final fermented culture dilution) as shown in Table 1. For *Bti*, tap water, distilled water, Tween 60, $MnCl_2$ and carbonate buffer pH 10 were the most efficient solvents for toxin extraction under both static and shaking conditions (50-62% mortality at 2×10^{-5} dilution). In addition, acetone (0.5%), Tween 20, Tween 40, citrate buffer pH 4 and Tris-HCl buffer pH 7.5 were good solvents for extraction of toxins of *Bti* under static condition (53-57% mortality at 2×10^{-5} dilution) however, glycerol (5%), Triton X-100 (0.5%) and phosphate buffer pH 7 were suitable for

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extraction of toxins under shaking conditions (57-60% mortality at 2×10^{-5} dilution).

For economic reasons, in the following experiments, tap water was used for extraction of toxins for both tested cultures under static conditions.

Effect of contact time

Contact time is one of the crucial factors affecting the recovery of metabolites from solid mass in SSF (Castilho *et al.*, 1999). No significant differences in the recovery of crude toxin of *L. sphaericus* 14N1 and *Bti* from fermented solids were observed at all tested contact times (Table 2). Leaching of toxins in short contact time (10 min) in this study is an advantage and may be related to using an inert, non water absorbed material like sand as a carrier. With other carriers the microbial products diffuse throughout the solid material therefore, long extraction times may be required for complete recovery (Mussatto *et al.*, 2012). It was mentioned that short leaching times maximize the volumetric productivity of the leaching equipment (Lonsane and Krishnaiah, 1992). In the next experiments the extraction time for both cultures was 10 min.

Table 2: Effect of contact time.

Contact time (min)	Mortality % of *C. pipiens* larvae at 2×10^{-5} dilution after 48h	
	*L. sphaericus*14N1	*Bti*
10	63±3 a	57±3 a
20	57±3 a	57±3 a
30	60±0 a	53±3 a
60	67±3 a	60±6 a
120	57±3 a	60±0 a
180	60±0 a	57±3 a
1440	63±3 a	53±3 a

Mortality % is expressed as mean value ± standard error. Values for each treatment followed by different letters are significantly different at $p = 0.05$.

Effect of extraction temperature

Temperature is one of the most important factors in the extraction of microbial products from fermented solids in SSF (Castilho *et al.*, 2000). For both cultures, all tested temperatures showed good extraction for toxins from their fermented mass (Table 3).

Singh *et al.* (1999) reported that an ideal solvent would extract the microbial product selectively and completely at room temperature with minimal contact time and, preferably, at the pH of the cultivated substrate.

Effect of repeated extraction and scaling up concentrated toxin extraction

Crude mosquitocidal toxins of tested cultures were extracted completely in the first extraction (100 mL).

Table 3: Effect of extraction temperature.

Extraction temp (°C)	Mortality % of *C. pipiens* larvae at 2×10^{-5} dilution after 48h	
	L. sphaericus 14N1	*Bti*
5	60±0 a	53±3 a
20	60±6 a	50±6 a
30	57±3 a	57±7 a
40	53±3 a	60±6 a
50	60±6 a	57±3 a

Mortality % is expressed as mean value ± standard error. Values for each treatment followed by different letters are significantly different at $p = 0.05$.

However, no significant mosquitocidal activities were detected in the second extraction (Data not shown). In SSF system free flowing solvent is very much limited. Thus adequate amount of solvent is required to leach out the microbial product from fermented solids (Palit and Banerjee, 2001). Efficient recovery of microbial products with low volume of solvent might be desired to have concentrated extract (Lonsane and Krishnaiah, 1992). Therefore, effect of successive repeat extraction using smaller volumes of solvent was studied. For both cultures most of toxins extracted very well in extraction 1 and 2 (20 mL) (about 60% mortality at 2×10^{-5} dilution) and no significant activities found in extraction 3 and 4 (0% mortality at 5×10^{-2} dilution) (Table 4).

Concentrated extraction of crude mosquitocidal toxins efficiently obtained from one kilogram fermented solids by 2 L tap water added consecutively in 4 times (500 mL each) at room temperature (25 °C) for 5 min for each time under static conditions.

It was reported that the leaching efficiency is affected by a number of factors such as reprocessing of the fermented solids, efficiency of solvents, additives to solvents, diffusivity of solute as well as solvent, retention of solvent by solids, mixing of solids and solvent, the ratio of solids to solvent, contact time, temperature and pH of the system and techniques used for separation of the leachate (Castilho *et al.*, 2000).

Table 4: Effect of repeated extraction.

Extraction run no.	Dilution at	Mortality % of *C. pipiens* larvae after 48h	
		L. sphaericus 14N1	*Bti*
First	2×10^{-5}	63	60
Second	5×10^{-2}	0	0
	2×10^{-1}	30	95
Third	4×10^{-1}	100	100
	5×10^{-2}	0	0
Fourth	2×10^{-1}	35	80
	4×10^{-1}	100	100

Mortality % is expressed as mean value ± standard error. Values for each treatment followed by different letters are significantly different at $p = 0.05$.

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Formulation

A large number of factors potentially affect the economic feasibility of biological control product. One of them is the formulation (Brar *et al.*, 2006). Both talcum powder formulations (*L. sphaericus* 14N1 and *Bti* toxins) showed stable toxicities against *C. pipiens* larvae over 8 months period storage at room temperature (Figure 1) after that the activity significantly decreased.

Talcum powder is used as an anti-sticking agent, an anti-caking agent, a carrier, a thickener, smooth filler and an adsorbent (EUROTALC 2017). Talcum powder was reported as a good carrier material for formulation of many bio-pesticides (Boyetchko *et al.*, 1999; Brar *et al.*, 2006; Sarma *et al.*, 2011; Ritu *et al.*, 2012; Tripathi *et al.*, 2015; Private Pesticide Applicator Safety Education Mannual, 2015).

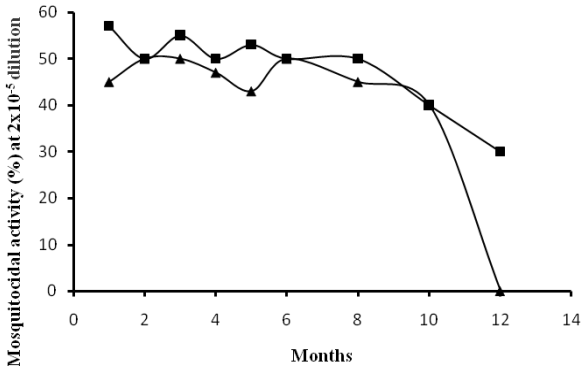

Figure 1: *L. sphaericus* 14N1 (■) and *Bti* (▲) formulations activities through 12 months storage.

CONCLUSION

It is very important to study the operating conditions for mosquitocidal toxins extraction from SSF and formulation. The extraction efficiency under mild conditions is critical factor influencing the economic feasibility of SSF.

Efficient recovery of crude toxins from fermented solids of Ls14N1 and Bti was obtained by using tap water as solvent under static conditions at 5-50 °C for 10 min. Also, crude toxins in 1 kg efficiently extracted by 4 consecutive addition of 500 mL tap water to have 2 L of concentrated toxins. These toxins were efficiently formulated by adding talcum powder and their activities were stable for 8 months. This study contains economic strategy for recovery of mosquitocidal toxins from SSF for application in the field or for further toxin downstream or formulation processing.

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