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Full Length Research Paper

Production of alkaline protease and larvicidal biopesticides by an Egyptian *Bacillus sphaericus* isolate

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One strain, isolated from Egyptian soil, was identified as *Bacillus sphaericus* with powerful larvicidal toxicity against *C. pipiens* and extra-cellular production of alkaline protease (AP) in the growth medium. The pH adjustment of the growth medium between 6.0 and 7.5 resulted in the highest AP activity, peaking at pH 6.5. AP crude preparations exhibited optimum activity at pH 10 and at temperature range of 65 - 70ºC. Interestingly, the highest AP activity was observed with 3% fodder yeast, as the sole component of the medium. Moreover, optimal AP yield was attained by using strong aeration at 9:1 (air : medium) ratio. Similarly, the highest AP enzyme level was consistently achieved 3-day after aerobic growth in shake flasks at 30ºC, without significant decrease in AP yield upon extended incubation periods up to 6 days. Notably, maximum proteolytic activity was achieved with casein as a substrate followed by skim milk, gelatin and bovine serum albumin. The crude AP enzyme activity exhibited quasi-linear response with enzyme concentrations up to 0.25 mg ml^{-1}. The isolated *B. sphaericus* might be employed for the economic production of important products such as biopesticides and proteolytic enzymes.

Key words: Biopesticides, Larvicidal activity, Alkaline protease, *Bacillus sphaericus*, Fodder yeast.

INTRODUCTION

The genus *Bacillus* constitutes a diverse group of rod-shaped, gram-positive bacteria characterized by their ability to produce robust endospores as a survival mechanism in response to adverse environmental conditions (Gibson and Gordon, 1974; Schallmey et al., 2004). The members of the genus *Bacillus* are widely distributed in soil, water and air and could be isolated from almost every kind of environment including desert sands, tropical and temperate zones of soils, air, snow, cold and thermal waters, both fresh and preserved foods of all varieties, milk, feces of man and domestic animals, birds, amphibians and fish. *Bacillus* species could also be recovered in large numbers from stored plant and animal products (Gibson and Gordon, 1974; Rizkallah, 1991). Accumulating recent studies clearly indicate that *Bacillus* species continue to be dominant workhorses in various industrial fermentation processes including production of biopesticides, vitamins, antibiotics therapeutic agents, flavoring compounds and various types of enzymes. In addition, such enzymes include amylases, proteases, cellulases, glucanases, sugar isomerases, xylanases, N-acyl-homoserine lactonases and chitosanases (Senesi et al., 2001; Dong et al., 2002; Santos and Martins, 2003; Choi et al.,

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2004; Schallmey et al., 2004).

Some of the most important applications of bacilli are their utilization in production of biopesticides and enzymes. Biopesticides are now considered the safest insecticides and constitute safe and environmentally-friendly substitute of the harmful chemically synthesized insecticides. Two *Bacillus* species namely, *B. thuringiensis* and the mosquitocidal *B. sphaericus* strains, are now produced commercially as bioinsecticides, and used extensively in the field for the control of insect pests and vectors of human disease (Poopath and Tyagi, 2004). Specifically, *B. sphaericus* has been successfully used for the biological control of mosquito-borne diseases such as malaria (Barbazan et al*.,* 1998; Mulla et al*.,* 2001), a major killer disease that threatens about 40% of the world's population (Sharma, 1999).

B. sphaericus is capable of producing powerful binary toxin (Bin) during its sporulation phase and less powerful mosquitocidal toxins (Mtx) during vegetative growth (Yang et al., 2007 and references therein). The powerful binary toxin produced during sporulation are crystalline protein endotoxins that are initially produced by those bacilli as inactive protoxins. They are partially hydrolyzed and converted by some proteases in the midgut of the susceptible insect species to the active bioinsecticidal toxins that are lethal to the target insects **(**Kumar and Venkateswerlu, 1998; Rukmini et al., 2000; Zouari et al., 2002; Promdonkoy et al., 2003). For example, some strains of *Bacillus sphaericus* synthesize a parasporal crystal containing 2 major protein protoxins of 51 and 42 kDa with potent larvicidal activities against a variety of mosquito species; both are required for toxicity. Following ingestion of spore/crystal toxins by mosquito larvae in the midgut, the binary protoxin 42 and 51 kDa proteins are proteolytically hydrolysed into 39 and 43 kDa active toxins, targeting primarily the midgut epithelial cells (Baumann et al*.,* 1991 and references therein). This is followed by binding of active toxins to specific receptors present in the midgut brush border membrane and putative internalization of toxin and cell lysis (Poopathi and Tyagi, 2004).

The significant role of proteases (peptidohydrolases group EC 3.4.4.1 - 3.4.4.20), produced by *B. thuringiensis* and *B. sphaericus,* in the physiology and regulation of sporulation and pathogenicity of those organisms is well documented (Chen et al., 2003 and 2004; Fedhila et al., 2003). Moreover, their significant importance in the cell metabolism and endotoxin reactions are clear in terms of their roles in protoxin activation (Rukmini et al., 2000). Thus, novel research trends have advocated the possible utilization of bioinsecticides-producing bacilli in the production of some industrially important proteases in addition to their bioinsecticides production (Tyagi et al., 2002). Among proteases produced by some bacilli, alkaline proteases (AP) that work efficiently at alkaline pH range (8-10) has been successfully used for many Industrial applications. These applications include their use in detergents industries, leather tanning and in food industries. Several reports have shown that some biopesticides-producing bacilli, e.g. *Bacillus thuringiensis* and *B. sphaericus* produce appreciable levels of enzymes in their growth media (El-Bendary et al., 2002; Chen et al., 2004; Tyagi et al., 2002; Moharam et al., 2003; Fedhila et al., 2003; Rizkallah, 1991).

The aim of the present work was to investigate the possible utilization of a locally isolated Egyptian strain, which was identified as *B. sphaericus*, for the simultaneous production of industrially important microbial products such as biopesticides and AP enzyme. The results are discussed in the light of optimal production conditions and efficient use of low-cost local agroindustial by-products such as fodder yeast to cut down the production costs.

MATERIALS AND METHODS

Isolation of target bacterial strains and culture conditions

Unless otherwise stated, all media were purchased from Difco and all chemicals were purchased from Sigma. Soil samples were obtained from selected sites in 4 Egyptian governorates, namely: Menofeya, Sharkia, Gharbia and Qena. About 10 g of muddy soils were collected from each site and suspended in 50 ml sterile water, well-shaken and was then pasteurized at 65ºC for 15 min. Next, the pasteurized muddy paste was used as a source of inocula for plates containing standard nutrient agar or NYSM agar media containing: 5 g l^{-1} peptone, 3 g l^{-1} beef extract, 0.5 g l^{-1} yeast extract, 0.01 g l^{-1} $MnCl_2$, 0.1 g l^{-1} $CaCl_2$, 0.2 g l^{-1} $MgCl_2$ and 1.5% (w/v) agar. Minimal media used contained (g l^{-1}): 8 $(NH_4)_2$ SO_4, 5.6 K_2HPO_4, 2.4 KH_2PO_4, 0.8 $MgSO_4$, 0.3 $MnCl_2$, 0.14 $FeCl_2$, 0.2 $ZnSO_4$ and supplemented with 0.5 yeast extract, 0.2 thiamin and 2 x 10^{-6} biotin. A medium composed of 1% yeast extract in sterile water was also used. After incubation at 30ºC for 48 h, the resulting bacterial colonies were picked and purified with repeated streaking on agar plates before being kept on slants of appropriate media. Media based on agro-industrial by-products and legumes seeds were used as mono-component media at 3% (w/v) prepared in dH_2O.

Growth conditions for bioinsecticides and AP enzyme production

The isolated bacilli cultures were grown aerobically in shake flasks incubated on rotary shaker (100- 150 rpm) at 30ºC. Both types of media (standard and industrial types) were used to test the formation of the mosquitocidal biopesticides and AP enzyme by the selected isolates of bacilli obtained from soil samples. Unless otherwise indicated, 2 successive transfers of the selected cultures were implemented. Thus, actively growing cultures (24 h - old) were used to inoculate 250 ml conical flasks containing 25 ml of the medium understudy. Next, cultures were incubated under shaking for 3 days at the indicated temperature. At the end of the incubation period, appropriate ten-fold dilutions (10^{-1} to 10^{-8}) of the full whole cultures (FWC) were tested for the presence of the larvicidal activity against second instar larvae of *Culex pipiens* the most common mosquitoes species in Egypt. On the other hand, production of AP enzyme was tested in the culture supernatants after centrifugation of full-grown cultures at 4000 rpm for 30 min to separate the bac-

terial cells. The clear supernatant was subsequently used as a source of crude alkaline protease. To test the effect of temperature on AP activities, standard reaction mixtures were incubated in water baths set at different temperatures in the range between 30 to 80ºC for 20 min. The reactions were then stopped and the liberated solubilized proteins were determined and expressed as enzyme units, where 1 U = µg tyrosine released min^{-1} ml^{-1} culture (El-Refai et al., 2005).

AP enzyme activity assay

The AP activity was essentially determined as previously described (El-Refai et al., 2005; Adinarayana et al., 2003; Mabrouk et al., 1999), with some modifications. The standard reaction mixture composition, contained 0.5 ml of enzyme source (diluted culture supernatant), 1 ml of glycine-sodium hydroxide buffer (0.1 M) in which 1% casein was dissolved as substrate; in some instances skim milk was used instead. The reaction mixture was incubated at 40ºC for 20 min after which the enzymatic reaction was stopped by addition of 1 ml trichloroacetic acid (20%) with thorough mixing. Then, the stopped reaction mixture was centrifuged at 3000 rpm for 15 min. Approximately, 0.5 ml of the clear solution was carefully pipetted in clean tubes and 1 ml of NaOH (1 M) was added to each tube followed by 0.5 ml of diluted (1:2) Folin phenol reagent slowly with continuous mixing. After 15 min, the optical density (OD) of solutions was measured at 600 nm. The OD values at time zero cleared reaction mixtures were subtracted from those of the experimental tubes. The OD values were subsequently converted to the equivalent µg of tyrosine using standard curve of authentic tyrosine concentrations (5-50 µg ml^{-1}) that were treated similarly to experimental enzyme-catalyzed reaction mixtures. The enzyme activity was calculated and expressed as units (1 U is defined as the amount of enzyme that liberates 1 µg tyrosine per min under specified reaction condition) as follows:

$$\frac{\mu g Tyro\sin e(released)xF}{T(\min)}$$ = µg tyrosine min^{-1} m^{-1} cluture supernatant;

Where F is the enzyme dilution factor and T is the reaction time in min.

Bioassay of bacterial larvicidal activity against mosquitoes larvae

Bioassays of the locally isolated *Bacillus* cultures including *B. sphaericus* were essentially carried out as described by de Barjac (1990). Toxicity was determined with laboratory reared *C. Pipiens*. Serial 10-fold dilutions in distilled water were tested in a preliminary toxicity screen. The range of concentration of final whole culture, which killed 50 and 90% of the larvae, was identified. Then, further toxicity tests were carried out in the observed ranges in order to precisely evaluate the LC_{50} and LC_{90} values for each putative candidate isolate with potent larvicidal activity, respectively. In this context, bacterial dilutions were placed in small cups, in duplicates, along with 10-second-instar mosquito larvae. Appropriate controls were run simultaneously using distilled water instead of cultures. The cups were, then, covered with muslin and kept at room temperature 27 ± 2ºC. The mortality percentage was recorded by counting the number of living larvae and corrected by using appropriate control and applying the following Abbott's formula:

$$Cm(\%) = \frac{Om(\%) - Cm(\%) \times 100}{100 - Cm(\%)}$$

Where *Cm* is corrected mortality and *Om* is observed mortality (Abbott, 1925).

The *Cm* (%) was then plotted against culture dilution of cells ml^{-1} on semi-log paper to determine LC_{50} and LC_{90} values. The bacterial cultures exhibiting significant activities against mosquito larvae were kept on slants and stored at 4ºC for further characterization. The isolated bacilli that showed no activities against mosquitoes larvae were discarded, whereas, those cultures with significant larvicidal activities were further examined microscopically and the characteristics of their colonies on the agar plates were equally recorded. Moreover, additional studies on their potencies against mosquitoes larvae were conducted using the dilution methods for Full Whole Cultures (FWC) approach after their growth for 3 days in shake flasks using nutrient broth medium under shaking conditions at 30ºC (El-Bendary, 1999; Foda et al., 2003).

RESULTS

Isolation and characterization of larvicidal *B. sphaericus* from soils of Egyptian governorates

We have isolated and purified a total of 57 bacilli isolates from 4 governorates, namely: Menofeya, Gharbia, Qena and Sharkia and their mosquitocidal activities against larvae of *C. pipiens* were examined. In a primary screen, at least 16 *Bacillus* isolates gave potent activities at low dilutions (10^{-1} dilutions) of FWC within 48 h of the bioassay (data not shown). In order to study the potential of larvicidal activities of these strains, they were further subjected to a secondary round of screening at higher dilutions ((10^{-1} to 10^{-3}). The objective of this experiment was to select the *Bacillus* isolates that produce the highest levels of mosquitocidal toxins for further studies. Secondary screening resulted in the identification of 2 isolates namely No.2 and No.5, that were initially isolated from Menofeya governorate, possessing a highly potent and persistent level of larvicidal activity at higher dilutions (10^{-3}) (Table 1). These 2 isolates were selected for further studies and characterization. Interestingly, comparative evaluation revealed that strain No.5 exhibits a 20% increase in larvicidal activity at dilution 10^{-5} when compared to strain No.2 (Table 2). Accordingly, phenotypic and biochemical characterization was primarily focused on Isolate No.5. The LC_{50} for the toxicity exhibited by strain No.5 was calculated by plotting the log mortality (%) versus the dilution of FWC tested. According to Table 3, the LC_{50} value falls in the range between 6-8 x 10^{-6} concentrations, indicative of a *Bacillus* strain with a highly potent toxicity against *C. pipiens* larvae.

Morphological examination revealed that isolate No.5 form beige-colored and medium-sized colonies when grown on nutrient agar. Moreover, their texture is smooth

Table 1. Identification of isolates from Menofeya governorate with potent larvicidal activity over high dilution range of FWC.

Strain number	Culture concentrations	Mortality (%) at 24 h	Mortality (%) at 48 h
Control (dH_2O)	0	0	0
2	10^{-1} 10^{-2} 10^{-3}	100 100 80	100 100 100
4	10^{-1} 10^{-2} 10^{-3}	20 20 0	20 20 20
5	10^{-1} 10^{-2} 10^{-3}	100 100 100	100 100 100

Table 2. Comparative analysis of larvicidal activity of 2 highly potent isolates No.2 and No.5 at very high dilutions of FWC§.

Strain number	Culture concentrations	Mortality (%) at 24 h	Mortality (%) at 48 h
Control (dH_2O)	0	0	0
2	10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}	100 100 60 60 40 25	100 100 100 100 60 25
5	10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}	100 100 60 60 50 25	100 100 100 100 80 25

§Strains were grown for 3 days in nutrient broth.

Table 3. Determination of LC_{50} value for the toxicity of *B. sphaericus* (No.5) against *C. pipiens* larvae.

Culture concentrations x 10^{-6}	Mortality (%) at 24 h	Mortality (%) at 48 h
Control (dH_2O)	0	0
10.0	50	80
8.0	50	55
6.0	40	45
4.0	35	40
2.0	30	30
1.0	25	25

and semi-glistening with round margin and their appearance is shiny, with little elevation and flat. Furthermore, microscopic examination of the cells revealed the rod-shaped morphology of the vegetative cells; sporulated

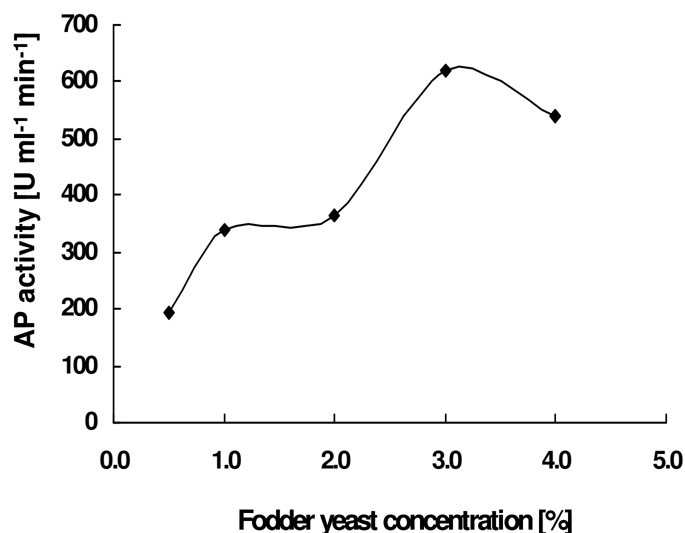

Figure 1. Effect of fodder yeast concentration on AP activity in the culture of *B. sphaericus* (No.5). The experiments were run in 3 replicates with independent cultures. Values of each data point are the average of 3 replicates.

cells (sporangia) with subterminal spores that are round in shape giving the sporangia club-shaped appearance (data not shown). These characteristics along with high toxicity against mosquitoe larvae suggest that the isolate No.5 is a strain of *B. sphaericus* (El-Bendary, 1999).

Optimization of alkaline protease production by *B. sphaericus* (No.5)

The possible co-production of extracellular proteases and highly potent mosquitocidal toxins by *B. sphaericus* isolate (No.5) was investigated. In this context, factors affecting enzyme yield and activity were thoroughly studied.

Growth media types

Special emphasis was given to the production of alkaline protease (AP) activities on various types of growth media due to its industrial importance. Several growth media types were tested for the simultaneous production of AP and mosquitocidal toxin activities. These media included standard NYSM medium and some agro-industrial protein-rich by-products such as cottonseed meal and fodder yeast, in addition to powdered legumes seeds available in the local market, namely horse beans soybeans, kidney beans, black eye beans and lentils. Each substrate was tested separately at 3%, as a complete growth medium for protease production under shaking conditions (150 rpm) for 3 days at 30ºC. Interestingly, *B. sphaericus* isolate No.5 Exhibited, on average, a powerful AP activity (expressed as $U.ml^{-1}$) in all tested media, with the highest levels obtained with fodder yeast (718) followed by soybeans (625), kidney beans (591) and black-eyed beans (559). Thus, we tested the presence of various concentrations of fodder yeast in the growth media on AP enzyme activity levels. The effect of different concentrations of fodder yeast (0.5 - 4%) on the level of AP enzyme activities is graphically illustrated in Figure 1. The highest AP activity was obtained upon using fodder yeast at 3% final concentration in the growth medium. Notable decrease in enzyme activity was observed at higher fodder yeast concentration (Figure 1). On the other hand, while the supplementation of fodder yeast medium with 1% of various carbohydrates had no significant effect on the level of AP activities, the addition of organic salts such as sodium acetate and sodium formate drastically decreased AP enzyme activity levels (data not shown).

pH and temperature

The effect of initial growth medium pH on AP enzyme activity was examined. To this end, 2 buffers having different pH ranges were incorporated into the fodder yeast medium. The obtained results indicate that *B. sphaericus* exhibited appreciable levels of AP activities in an initial pH range of 6.0-8.0 (Figure 2A). Moreover, the highest enzymatic levels were obtained with initial pH values between 6.0 and 7.0 (Figure 2A). On the other hand, the organism failed to produce high levels of the enzyme in media with initial pH values less than 5.5 (Figure 2A). Subsequently, the optimal pH for crude AP activity produced by *B. sphaericus* (No.5) was examined. The obtained results are graphically illustrated in Figure 2B. The results show that the AP enzyme maintained a highly stable activity over a wide range of alkaline pH values (8 - 12), with an optimal activity specifically observed at pH 10 (Figure 2B). It was evident that the fodder yeast medium with initial pH < 5.5 did not support production of AP activity (Figure 2A). Moreover, the utilization of phosphate-buffer in the growth medium may not be economically feasible due to the increasing costs of production. Thus, the pH adjustment of the medium to the desired values, favorable to enzyme production with sufficient amounts of NaOH to overcome and neutralize the acidic nature of fodder yeast cellular proteins, was tested as a low-cost alternative to the incorporation of buffers into the growth medium. Table 4 shows the level of AP activities obtained in fodder yeast media upon using buffers as compared to those obtained using distilled water in the presence and absence of pH adjustment with NaOH to pH value 6.5. This approach correlated well with high levels of AP activities comparable to those obtained using phosphate buffers. Furthermore, the utilization of fodder

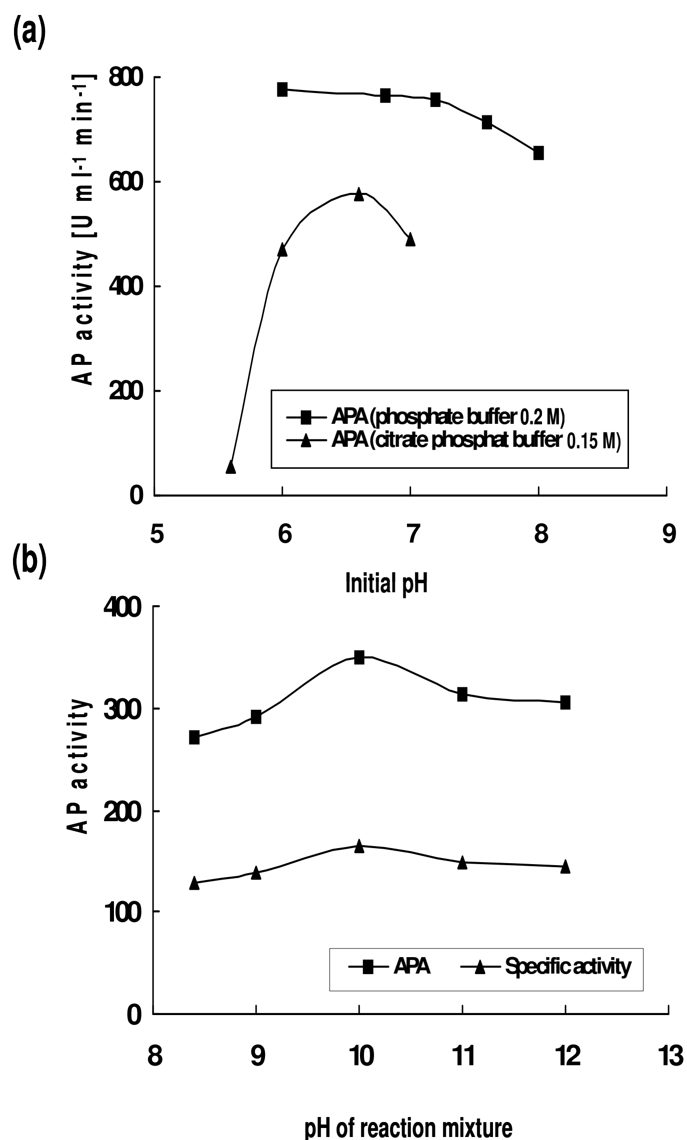

Figure 2. Effect of pH of buffered initial medium (A) and pH of enzyme reaction mixture (B) on crude AP activity produced by *B. sphaericus* (No.5). The experiments were run in 3 replicates with independent cultures. Values of each data point are the average of 3 replicates.

yeast suspended in distilled water without prior pH adjustment to pH 6.5 failed to support AP activity production (Table 4). Next, the impact of reaction temperature on the reaction rate of AP produced by *B. sphaericus* (No.5) was studied. Several temperature ranges (30-80°C) were employed and results are graphically illustrated in Figure 3. The reaction rate proportionally increased with increasing incubation temperature, peaking at 65ºC and then followed by a rapid decline in AP activity at 80ºC (Figure 3).

Aeration rate and inoculum size

The extent of aeration (air: medium ratio) in the fermentation flasks were varied in the range between 9.0:1 and 0.6:1 air: medium, respectively; the AP activity was determined and the final pH values obtained in each case were measured. The highest AP level (258 U mg^{-1}) was obtained with the highest air: medium ratios (that is 9:1) (data not shown). The AP activity decreased gradually with decreasing the above-mentioned ratio by increasing the media volumes in the experimental flasks. Likewise, the final pH at harvest time recorded the highest values in the presence of the highest levels of aeration followed by gradual decrease in the final pH as a function of lowering the aeration levels and the corresponding AP activity level (data not shown). Next, the effect of both the incubation period and inoculum size on AP enzymatic activity was explored. Table 5 shows the production pattern of AP activity during 6 days of growth of *B. sphaericus* (No.5) cultures in fodder yeast medium under shaking conditions. A gradual increase in AP activity was evident, peaking at 4 days and stabilizing thereafter, without appreciable changes for an extended incubation period up to 6 days. Moreover, a range of inocula size [0.5-6.0% (v/v)] was tested, for the inoculation of experimental flasks containing fodder yeast medium under shaking and AP activities were assayed. The results indicated that inoculum size exerted no significant effect on AP activity levels over the range tested.

Substrate and enzyme concentration

The effect of both substrate and enzyme concentration on AP reaction rate was investigated. Figure 4A represents a Michaelis-Menten plot drawn as a function of reaction rate and substrate concentration. Thus, the maximum velocity (V_{max}) and Michaelis constant (K_m) could be determined. The crude AP activity of *B. sphaericus* (No.5) exhibited a V_{max} value of approximately 330 µg tyrosine min^{-1} ml^{-1} and an apparent K_m about 0.015% for casein in the reaction mixture. According to Figure 4B, the reaction rate exhibited a quasi-linear response with increasing enzyme concentrations up to 0.26 mg ml^{-1} of reactions mixture, leveling off with a plateau in the reaction rate with no further increase at higher enzyme concentrations. To test the scope of substrate specificity of crude AP enzyme, standard reaction mixtures were prepared but with the replacement of casein substrate with different proteins including gelatin, skim milk bovine serum albumin, bovine fibrin, keratin and collagen. As a control, casein was used as a standard substrate for comparative purposes. The highest value of enzyme activity [U ml^{-1} min^{-1}] was obtained with casein (235) followed by skim milk (147), gelatin (135) and bovine serum albumin (78). No activity was recorded using the insoluble substrates namely, collagen, keratin and bovine fibrin.

Table 4. Effect of adjustment of initial medium pH to the optimum pH (6.5) with NaOH on AP production by *B. sphaericus* (No.5).

(Fodder yeast 3%) Suspended in:	Initial pH		pH adjustment to 6.5 BA	Final pH[§]	Enzyme [U ml^{-1}]	Soluble protein [mg ml^{-1}]	Specific activity [U mg^{-1}]
	BA	AA					
dH_2O adjusted to pH 6.5	5.5	5.35	OK	8.60	519	2.64	197
Phosphate Buffer [0.1 M] pH 6	5.7	4.75	OK	8.25	426	1.76	242
Phosphate Buffer [0.1 M] pH 8	6.5	5.00	OK	8.50	513	2.19	234
dH_2O without pH adjustment	5.5	5.35	Not done	5.40	125	2.12	59

BA, Before autoclaving; AA, After autoclaving; **§** Final pH of bacterial culture grown in medium pH-adjusted to 6.5 with NaOH (0.05 M).

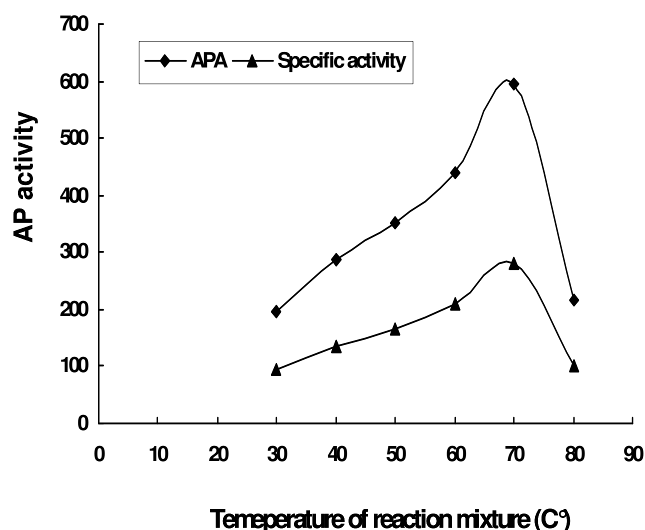

Figure 3. Effect of incubation temperature of enzyme reaction mixture on activity of crude AP produced by *B. sphaericus* (No.5). The experiment was run in 3 replicates with independent cultures. Values of each data point are the average of 3 replicates.

DISCUSSION

In this study, a large number of soil-inhabiting bacilli were isolated from selected Egyptian governorates and they were subsequently screened for potential mosquitocidal biopesticide production. One culture, isolate No.5 obtained from Menofeya, that produced the highest level of the biopesticide (Tables 1 and 2), was thus further characterized and identified as a strain of *B. sphaericus* pathogenic to mosquitoes larvae. The species of *B. sphaericus* is well known to include some strains that form parasporal bodies made of proteins that are highly toxic to mosquitoes larvae. This group of bacilli were first reported by Kellan et al. (1965) and since then, more than 300 strains have been isolated and identified world-wide (Poopathi and Tyagi, 2004). In this report, the Egyptian strain of *B. sphaericus* (No.5) has been shown to produce potent mosquitocidal toxin with LC_{50} value (Table 3) in the vicinity of those produced by the *B. sphaericus* strain IN 2362 and other highly potent cultures (El-Bendary, 1999; El-Bendary et al., 2002).

In parallel, the Egyptian strain of *B. sphaericus* (No.5) was able to extracellularly produce high levels of AP on a variety of media including standard media used for biopesticides production, e.g. NYSM and nutrient broth, as well as media composed of locally available agroindustrial by-products and powdered legume seeds. The highest AP enzyme yields were obtained upon using a medium composed solely of dried fodder yeast, a by-product of ethanol fermentation industry, at 3% (w/v) final concentration in tap water (Table 1). The production of the AP enzyme on agroindustrial byproduct is desirable to decrease the production costs and increase the economic feasibility of this application. This trend is adopted by other scientists (Mabrouk et al., 1999; El-Bendary et al., 2002; Moharam et al., 2003; Foda et al., 2003; El-Refai et al., 2005) for the production of extracellular proteases.

The use of fodder yeast cells as a sole component of medium proved to be highly efficient since the yeast cells contain appreciable levels of all components required for microbial growth including proteins, carbohydrates, lipids, mineral salts as well as Vitamins, all in balanced levels as to support growth of the desired organism (Salama et al., 1983a, b; Foda et al., 2003). It is also noted that supplementation of the fodder yeast medium with different carbohydrates did not result in marked increase of the AP enzyme yield. This result is in agreement with previously published data indicating that *B. sphaericus* (No.5) relies heavily on the protein present in growth media, since this bacterial species does not utilize sugars as source of carbon and energy (Russel et al., 1989). Although, the fodder yeast medium was highly efficient in production of AP enzyme by *B. sphaericus* isolate (No.5) (Figure 1), the growth medium required adjustment of the initial pH values to 6.0-7.5, to insure high AP enzyme productivity. The pH adjustment required either the incorporation of

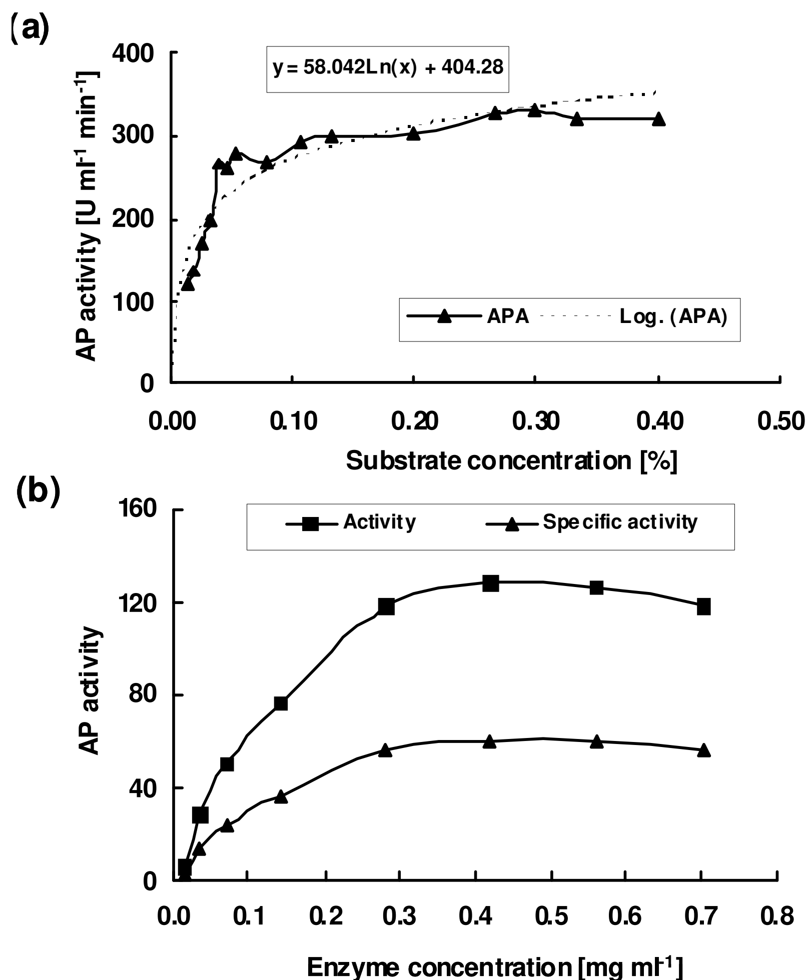

Figure 4. Effect of substrate (casein) and enzyme concentrations in reaction mixture on activity of crude AP produced by *B. sphaericus* (No.5). (A) Effect of casein concentration of AP activity. Standard reaction mixtures were prepared with varying casein concentration between 0.13 and 2.0% in reaction mixture. (B) Effect of enzyme concentration on AP activity. APA denotes AP activity expressed as U ml^{-1} min^{-1}, while specific activity is expressed as U mg^{-1}. The enzyme concentrations tested were in the range between 0.02 - 0.66 mg ml^{-1} of protein in the reaction mixture. The experiments were run in three replicates with independent cultures. Values of each data point are the average of three replicates.

suitable buffers (e.g. phosphate buffer) or the adjustment with NaOH of the suspending water to pH value of 6.5 (Figure 2A and Table 4). The obtained results showed that fodder yeast cells lower the pH to values around 5.5, which is not favorable for growth and enzyme production by *B. sphaericus* isolate No.5. In fodder yeast medium, the pH drops in this medium to values around 5.5 that was due to the high buffering capacity of proteins of the yeast cells on the acidic side, thus requiring addition of NaOH to the growth medium (El-Bendary et al., 2002). The effect of initial pH of the growth medium on the production of proteases seems to be variable among different species of bacilli. Thus, El-Refai et al. (2005) reported that *B. pumilus* FH9 required high initial pH of growth medium to produce the highest levels of proteases and keratinolytic activities and the enzyme production was depressed at lower pH values. Moreover, the investigation of the biochemical properties of the AP enzyme produced by *B. sphaericus* (No.5) revealed that the enzyme exhibited optimum activity at pH 10 with slight decrease at pH 11 and 12 (Figure 2B). These findings are in agreement with other reports stating that the optimum pH of AP produced by *B. sphaericus* strain BSE 18 was 10.2 (Dumusois and Priest, 1993). Furthermore, it

Table 5. Effect of incubation period on AP production in fodder yeast medium by *B. sphaericus* (No.5).

Incubation period (days)	Final pH	Enzyme [U ml^{-1}]	Soluble protein [mg ml^{-1}]	Specific activity [U mg^{-1}]
1	7.50	256	1.88	136
2	7.10	357	2.08	172
3	8.05	627	2.21	284
4	8.00	633	2.08	305
5	7.90	623	1.97	316
6	8.00	616	2.02	305

was reported that optimum pH of AP obtained from *B. sphaericus* strains IS2362 and NRC 69 was about 8.5 (El-Bendary et al., 2002). Taken together, these results advocate the superiority of this enzyme for possible future applications in detergents industry and dehairing of hides. On the other hand, the presence of good levels of aeration in the fermentation flasks was a prerequisite for highest AP enzyme production by *B. sphaericus* (No.5). Similar results were recorded by El-Bendary et al. (2002) who reported that highest levels of the enzyme produced by strains 2362 and 69 were obtained when the medium volume occupied only 5% of the fermentation flask volume under shake culture conditions.

Several studies have been conducted on *Bacillus thuringiensis* cultures that proved their ability to produce extracellular proteases including alkaline protease (Donovan et al., 1997; Reddy et al., 1998; Oppert, 1999). Such studies may indicate the possible role of those types of proteases in the insecticidal activities of both *B. sphaericus* and *B. thuringiensis*. Moreover, it has been shown that a sfericase (serine protease type) is produced during stationary phase of growth of *B. sphaericus* cultures (Almog et al., 1994). Wati et al. (1997) could identify a protease that is related to sfericase enzyme and produced by *B. sphaericus*. The pH for AP optimal activity has been shown to be organism-dependent. In this context, previous studies reported that the optimum pH for protease isolated from *Pseudomonas pseudomallei* was pH 8.0 (Moharam et al., 2003), whereas AP from *Myxococcus xanthus* worked optimally at pH 8.2 (Dumont et al., 1994). Moreover, the optimum pH for the AP isolated from the moderate halophile *Halomonas* sp. ES 10 was pH 11 (Kim et al., 1992).

In this work, it was shown that the AP enzyme of *B. sphaericus* (No.5) could efficiently hydrolyze casein followed by skim milk, gelatin, and bovine serum albumin with little or no activity on insoluble proteins (e.g. keratin, fibrin and collagen). The enzyme catalyzed proteolysis of casein proceded optimally at temperature 65-70ºC with notable decrease at high incubation temperatures (Figure 3). In this context, Moharam et al. (2003) reported that purified AP enzyme from 2 *B. sphaericus* cultures IS2362 and NRC-69 exhibited maximum activity at 60ºC. Similar optimum temperature of 60ºC for AP of some other cultures of *B. sphaericus* with mosquitocidal activities were previously reported for AP enzyme from *Bacillus* sp. mutant 533-F13 (Moharam et al., 2003). A highly thermostable AP enzyme was obtained form an alkalophilic and thermophilic *Bacillus* sp. strain AH-101 that exhibited maximum activity at 80ºC (Takami et al., 1989).

Concluding remarks

The present study was devoted to investigate the possible utilization of an indigenous organism isolated from the Egyptian soils for production of biopesticides active against mosquitoes as well as the production of alkaline protease enzyme highly needed for its wide industrial applications. The simultaneous production of both microbial products in the same fermentation process from one organism is envisaged to increase the feasibility of the industrial application rendering the process cost-effective and highly applicable in the future under local conditions. Further investigations to explore the biochemical properties of the AP enzyme of *B. sphaericus* isolate (No.5) are required and will be claudicated after the purification and characterization of the AP enzyme in subsequent future studies.

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