Sustainable quick quantitative screening for the physiology of chitinases production by *Bacillus thuriengiensis gallariae* and detection *of Aedes aegypti* biopesticide activity

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Background
Chitin is considered the world's first and most abundant amino-carbon substrate.
Objective
The proposed method aims to speed up the detection and measuring of microbial chitinase production.
Materials and methods
Solid-state fermentation, plates, viscosity, and spectrophotometric methods were used to detect microbial chitinase activity. Analysis of variance was used for statistical analysis.
Results and conclusion
The trailed *Bacillus thuriengiensis gallariae* strain was differentiated from other tested strains of *Bacillus thuriengiensis* in terms of mosquito resistance, besides being superior to other studied *Bacillus sphaericus*. As anticipated, results from the clear zone plates method were not clear compared with the viscosity method where the strain exhibited the best. Worthy, the same result was reached in the color methods. The superlative incubation period for the physiological properties of chitinase production from *Bacillus thuriengiensis gallariae* was attained after 5 days. Results indicated that the best size of chitin particles was 1 mm, the best percentage of humidity of the growth was 67%, the best inoculum volume was 10 ml, and the best carbon source for the production of crude chitinases was glucose. The optimal concentration for the production of chitinases was 20% of the weight of the growth medium, yet 5% of the weight of the growth medium was the optimal percentage of glucose as a carbon source for the production of other protein metabolites. The proposed colorimetric method seemed to be an effective and rapid method to survey the aptitude of huge quantities of microorganisms to produce chitinases.

Keywords:
biocontrol, chitinases assay, colloidal chitin and Ostwald viscometer

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List of abbreviations: DNS, 3, 5- di-nitro-salicylic acid; NAG, N-acetyl-D-glucosamine

Introduction

The current contemporary trailed method is correlated to a method for industrially maximizing microbial chitinase production. In such a method, the speed of detecting and measuring microbial chitinase production is higher than the traditional method provided with preparing the traditional enzyme reaction substrate, colloidal chitin, while reducing the cost of production through a supply with a reaction material enzyme in traditional methods whether colloidal chitin or expensive chitin azure. It is also an environmentally friendly method through dispensing with concentrated acid chemicals releasing dangerous fumes in the preparation step of colloidal chitin in the traditional method.

Chitin is highly abundant in nature, more than 10^{10} tons/year, and is considered the world's first most abundant amino-carbon substrate [1] and the second carbon waste after cellulose [2]. It is insoluble in water, a polymer of ß-1.4 bonds of N-acetylglucosamine. As shown by radiography diffraction, it has a highly ordered crystalline structure [3], Chito-oligomers produced by enzymatic hydrolysis of chitin are useful in various fields such as in medical, agricultural, and industrial applications. It is considered an antibacterial, antifungal substrate, hypo-cholesterolemic, antihypertensive activity, and is frequently used as a food quality enhancer [4],

nitrogen fertilizer [5], biofuels and value-added chemicals [6], protoplast isolation, biopesticide, fungal biomass, morphogenesis and transgenes in plants [7], cosmetics [8], functional food [9] and insecticide, fungicide and single cell protein [10].

Sterilization of chitin by autoclaving or boiling releases D-glucosamine and N-acetylglucosamine from the macromolecule and these solubilized components function as the inducers for the biosynthesis of chitinase. The insoluble macromolecule is not an inducer of chitinase since sterilization by dry heat or chloroform will not bring about the release of the amino sugars or induction of the enzyme; whereas free glucosamine, N-acetylglucosamine, and chitobiose are all good inducers of chitinase. Most sustained synthesis of the enzyme occurs in an autoclaved chitin-salt medium [11].

The complete enzymatic hydrolysis of chitin that releases N-acetyl-D-glucosamine (NAG) is performed by a chitinolytic system, consisting of two hydrolases, the action of which is consecutive. The chitinase: poly-B-1,4- (2-acetamido-2-deoxy)-D-glueosideglycano-hydrolase, EC (3.2.1.14) hydrolyses the polymers of NAG, including tetramers and, to a lesser extent, trimmers three chitobiase (chitobioseacetamido, deoxy, glucohydrolase: EN 1 3.2.1.29) hydrolyses chitobiose (the dimer of NAG) and chitotriose [12].

Chitinase production is widely distributed among bacteria especially Streptomycetes, *Aeromonas, Chromobacterium, Klebsiella, Pseudomonas, Vibrio, Arthrobacter, Beneckea, Clostridium, Serratia* [13] and *Bacillus thuriengiensis* [14]. Chitinases are also produced by molds and fungi (especially *Lycoperdon)* and might be found in the emulsion of sweet almonds. Chitinases might be synthesized in the absence of substrate (constitutive enzyme) or its presence (adaptive enzyme); however, the addition of chitin to culture media greatly enhances enzyme production [12]. Chitinase activity had been assayed by various methods. The most common methods are viscos-metric, colorimetric, clear zone plates, and radiochemical methods [6], and Nephelo-metric (Turbidi-metric) method [15]. However, there are certain drawbacks to most of these methods, such as insensitivity at low range of sugar concentrations, lack of specificity, and difficulty in use in high-throughput screening [6].

Mosquitoes are considered among the insects that transmit human and animal diseases, so research trials targeted combating them with pesticides, some of which are chemical ones that harm the environment, while others are biological ones that are friendly to the environment. Among the most famous biocides are the bacteria Bacillus thuringiensis in general and sphaericus specifically for mosquitoes [16]. Recently some researchers discussed the mode of action of these bacteria as an insecticide, yet no specific factors have been reached on their lethal or weakening effect on insects at their various stages of growth [17].

While chitinase enzymes might be used as a direct insecticide, to enhance the effectiveness of other biocides, or to reduce the environmental adverse of chemical pesticides, the mechanistic mode of action theory of chitinase as an insecticide is that it targets the cuticle and the peritrophic matrix of the insect, that are chitin fibers linked to glycoproteins and proteoglycans and has an essential role in digestion physiology in the midgut of insects [18].

Numerous micro-organisms that can produce chitinases have not been discovered yet due to the difficulty of the experimental detection screening methods of microbial chitinases, so the suggestion of this method is considered to be a shortcut of some steps, tricks, and chemicals to facilitate the detection screening methods. Accordingly, it achieves the goals of sustainable development; as it saves the cost of chemicals in the stage of the enzyme reaction substrate, and also saves time and secures safety to researchers besides reducing the consumption of concentrated acids that have bad health impacts except under high laboratory safety and security conditions, such attain at the same time several sustainability goals preserving human health.

Materials and methods

Microbial Strains: *Bacillus thuriengiensis* sp. strains evaluated in the current work were got from different culture collections including HD: Howard Dulmage Collection, Cotton Insect Research Laboratory, ARS, USDA, Brownsville, Texas, USA; IP: Institute Pasteur Collection, Paris, France; NRRL: North Regional Research Laboratory, Peoria, Illinois, USA; and NRC: Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt), *Lysinibacillus sphaericus Amira* strain, Microbial Chemistry Department, NRC (Gen Bank accession number: KT361851.1) and fungal isolate from a rotting lemon at room temperature, were evaluated.

Colloidal chitin preparation: Colloidal chitin was prepared from pure chitin (Alpha Chemika, Mumbai 400 002, India, product no. AL-1161) [2].

Experimental methods

Plates assay method: According to Roshdy *et al.* [19] with some modification chitinolytic activity was assayed on plates containing 2% colloidal chitin (wet weight), 3% agar was added nutrient agar overnight, *Bacillus sp*. strains were inoculated on solid chitinase-inducing plates and incubated at 30°C for 72 h in JSR incubator. Chitin hydrolysis was determined by formation of a whitish, opaque halo (chitin polymer) around a translucent area (hydrolyzed chitin) surrounding the growing colony.

Solid state fermentation: As described by Roshdy *et al.* [19] with some modifications, the medium substrate was composed of two grams of pure chitin Alpha Chemika, Mumbai 400 002, India. Product no. AL-1161), was taken in a 250 ml Erlenmeyer flask, 10 ml distilled water (by Milli-Q), mixed thoroughly, and autoclaved at 121°C and 1.5 atm pressure for 15 min (in JSR autoclave). The flasks were cooled to room temperature, then inoculated with 1 ml of 24 h microbial culture under sterile conditions and incubated at 30°C temperature for 5 days. The supernatant fraction was harvested by adding 10 ml distilled water for every flask and shaking at 30°C, 100 rpm for 30 min, and centrifuged (4000g for 20 min at 4°C) (SIGMA 3–18 K) then the supernatant (crude extract) stored at 20°C until used for chitinases assays.

Viscosity: U-Tube capillary viscometer (Ostwald viscometer), was filled with the supernatant; until it reached the end of the capillary tube, then left to fall under the influence of gravity, and the time required was calculated at the end of the solution fall [20].

The viscosity unit is calculated by the following equation:

$$\eta = (\pi\ r4P\ t)/8\ V\ L\ \text{Poise},$$

Viscosity co-efficient for water at 27°C is 0.85 considering water as the reference liquid.

By applying this equation, the water took 4.14 s to exit the U-Tube capillary (Ostwald viscometer).

Ignoring the density of each supernatant (not available to measurement here), according to the principle of corresponding states (a dimensionless property of one substance is equal to that of another [21].

Substance when both are evaluated at the same reduced conditions), the degree of viscosity was calculated using the reference to the viscosity of distilled water, the unit of viscosity will be defined in the results under study by the following equation: the time it takes for the liquid to exit multiplied by the constant value 0.205314 (The product of dividing the viscosity coefficient of water in the experimental conditions by the time it takes to exit the capillary tube). The result will be the viscosity of the liquid is Poise. The relative viscosity was calculated by dividing the time it takes for the crude enzyme to fall by the time it takes for the distilled water to fall [22].

Chitinolytic spectrophotometric assay

The traditional chitinase activity DNS assay method

The supernatant used as crude chitinase enzyme (2.5 ml) was incubated with 2.5 ml 0.5% colloidal chitin in 2.5 ml 0.1 M ammonium acetate buffer pH 5.5 for 1 hour; The reaction was stopped by adding 2.5 ml of 10% 3, 5-di-nitro-salicylic acid (DNS), and heated in boiling water bath for 5 min, then centrifugated for 5 min at 4°C. The color of the filtrate by reduction of DNS in presence of the amino-sugar NAG released by crude chitinolytic enzymes activity, was measured at 540 nm (JASCO V-730 Spectrophotometer), the concentration of NAG produced by the enzymatic activity was measured from the NAG standard curve, [23].

The modified chitinases activity DNS assay (Roshdy method): with some modification to the method described by Jha and Modi [23], 2.5 ml of the supernatant of crude chitinases enzyme mix "immediately" with 2.5 ml of 10% DNS, and heating in a boiling water bath for 5 min, then centrifugation at 4°C for 5 min. The color of the filtrate by reduction of DNS in presence of the amino-sugar NAG released by crude chitinolytic enzymes activity, was measured at 540 nm (JASCO V-730 Spectrophotometer), the concentration of NAG produced by the enzymatic activity was measured from the NAG standard curve. Abdel-Salam *et al.* [24] determined NAG as chitinolytic hydrolyses final product. One unit (U) of chitinases activity was defined as the amount of enzyme required to release 1 μg of NAG/milliliter of chitinase enzyme solution/hour, under assay conditions.

Mosquito larvae biocontrol assay

The second-instar larvae of *Aedes aegypti* are used when diluting 10^{-3}, as described in detail by Afify *et al.* [25].

Production physiology of chitinases by *Bacillus thuringiensis gallariae* under chitin solid-state fermentation conditions: Production physiology parameters were determined with modification of Roshdy spectrophotometric method, by using chitin flakes instead of wheat bran [25].

Statistical analysis

Randomized block design in one way was used to arrange the majority of the experimental treatments. The two-way randomized blocks design was used only to analyze the data of the comparison experiment between the two spectrophotometric methods of chitinase detection (as a first factor) produced by various microorganism strains (as a second factor). Using CoStat software, an analysis of variance was performed on the study's data. Duncan's multiple range test at 0.01 probability level was used to compare the means of the treatments [26].

Results

Microbial chitinases detection methods

Clear zone plates method: Fig. 1, shows the lack of clarity of a transparent area around the microbial growth after 10 days of incubation, due to the insolubility of colloidal chitin in the agar. It is most likely that such lack of growth is due to the difficulty of producing chitinases in bacteria, where it needs to be induced, so the presence of carbon, nitrogen supplements, or minerals in the growth media might activate the production of chitinases, but the current study relies its theory on the presence of chitin alone in the microbial growth medium.

Viscometrical method: Results given in Table 1, show the variation of the fluid's viscosity by the U-Tube capillary viscometer (Ostwald viscometer), where the lowest viscosity was found in distilled water, reaching 0.85 Poise, followed by *Bacillus thuriengiensis* IP sotto 4A/4B, *Bacillus thuriengiensis* HD 133 suspensions, Control, *Bacillus thuriengiensis* 2245 NRRL, *Bacillus thuriengiensis* IP *thuriengiensis*, *Bacillus sphaericus* 2297, *Bacillus sphaericus* 2362, fungal isolate from lemon, *Lysinibacillus sphaericus Amira* suspensions while the suspension of *Bacillus thuriengiensis gallariae* was the slowest, being 0.94, 1.19, 1.24, 1.28, 1.36, 1.38, 1.40, 1.40, 1.44, and 1.62 Poise, respectively. The calculated viscosity units (Poise) were directly proportional to the values of the descent velocities, otherwise, it was calculated using the relative viscosity method as shown in Table 1.

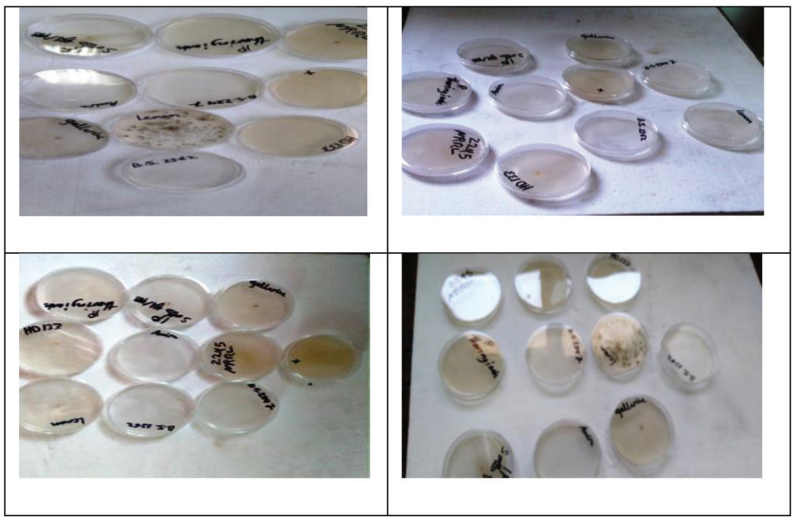
Spectrophotometric method: A comparison between traditional chitinases detection scheme method,

Table 1 Viscosity method values (Poise)

Micro-Organism	Time/s	Viscosity co-efficient unit (Poise)	Relative viscosity
Distilled water	4.14	0.85	1.00
Bacillus thuriengiensis IP sotto 4A/4B	6.06	0.94	1.46
Bacillus thuriengiensis HD 133	6.84	1.19	1.65
*Control	7.00	1.24	1.69
Bacillus thuriengiensis 2245NRRL	6.80	1.28	1.64
Bacillus thuriengiensis Ip thuriengiensis	6.70	1.36	1.62
Bacillus sphaericus 2297	6.64	1.38	1.60
Isolate from lemon	6.25	1.40	1.51
Bacillus sphaericus 2362	4.59	1.40	1.11
Lysinibacillus sphaericus Amira	5.79	1.44	1.40
Bacillus thuriengiensis gallariae	7.88	1.62	1.90

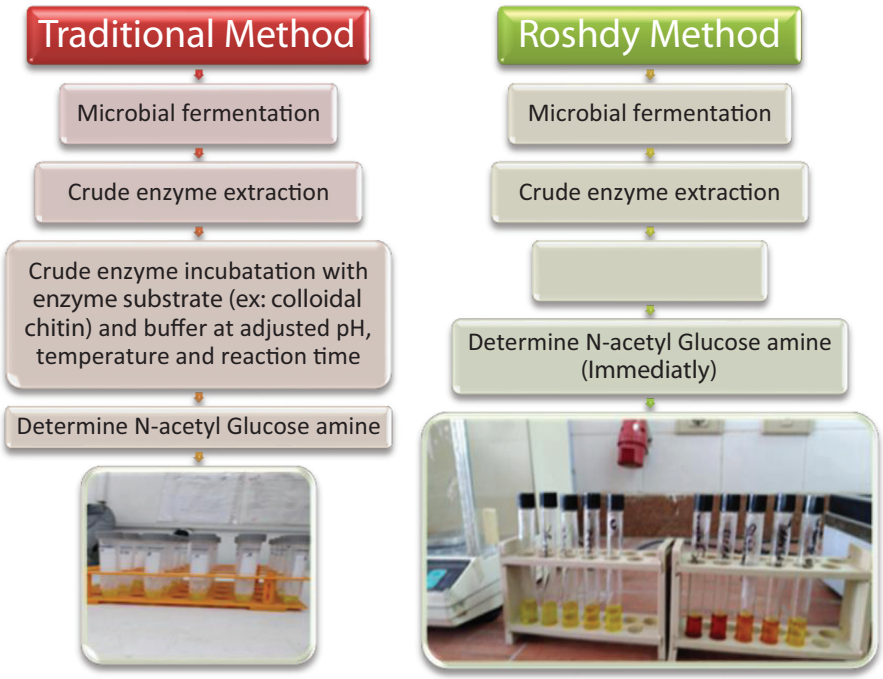
*Control represents a sample of growth media supernatant without microbial inoculum.

Figure 1



Clear zone plates method.

Figure 2



Comparison between traditional chitinases detection scheme method, and Roshdy modified scheme method.

Table 2 A two-way analysis of variance for effects of two spectrophotometric methods, various microorganism strains, and the interaction between them on chitinase

Source	*df*	Type III SS	MS	F	*P*
Blocks (Replicates)	2	8469.949745	4234.9749	1.613958	0.2
Methods	1	2541545.262	2541545.3	968.5882	0.0
Micro-Organisms	9	84533235.51	9392581.7	3579.532	0.0
Methods x micro-organisms	9	8736871.745	970763.53	369.96	0.0
Error	38	99710.81913	2623.9689		

and Roshdy modified scheme method is shown in Fig. 2.

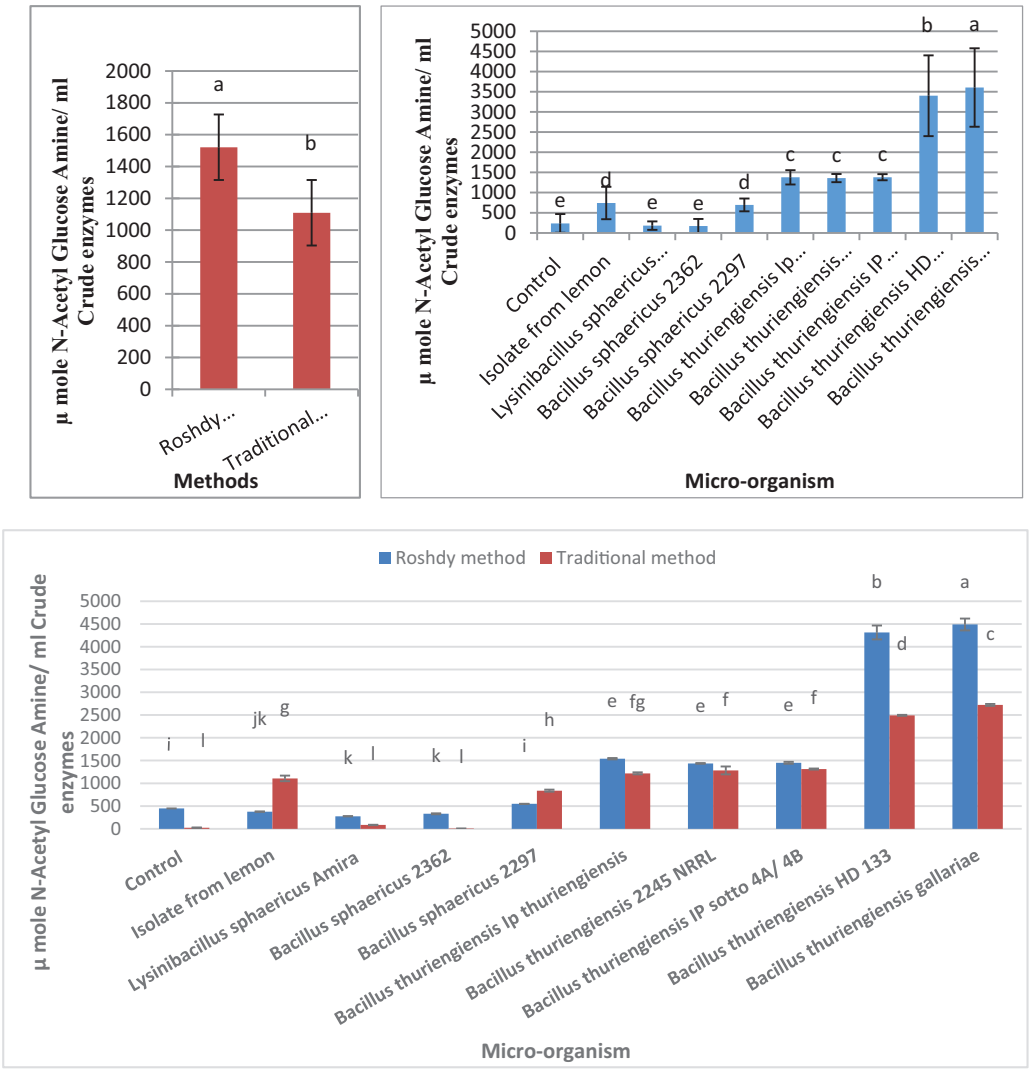
A two-way randomized blocks design was used to compare the two spectrophotometric methods of chitinase detection (the traditional and Roshdy method as a main factor) produced by various microorganism strains (the 9 strains mentioned in Table 1 as a second factor) and the interaction between them (Table 2).

Highly significant differences were recorded between the different two methods (Roshdy method > traditional method), the different strains, and the interaction between them. Irrespective of the method effect, the variations in microbial chitinase activity confirm the superiority of the two strains, Bacillus thuriengiensis HD 133 and *Bacillus thuriengiensis gallariae*, in the production of units of NAG at a reaction speed of 5 min. Regarding the interaction between methods and microorganisms, the highest value of NAG was produced by *Bacillus thuriengiensis gallariae* and determined by the Roshdy method, and the lowest value was produced by *Bacillus sphaericus 2362* and measured by the traditional method. These differences are confirmed as demonstrated in Table 2 and Fig. 3.

Comparison between mosquitocidal application against *Aedes aegypti* second instar larvae: It is clear in Fig. 4 that, the mosquito mortality percents of *Bacillus sphaericus* strains was superior to *Bacillus thuringiensis* strains because these bacteria are specialized as a biopesticide for mosquitoes in particular, it was 100% for all *Bacillus sphaericus* strains, while it was different from *Bacillus thuringiensis* strains and the fungal isolate from the lemon.

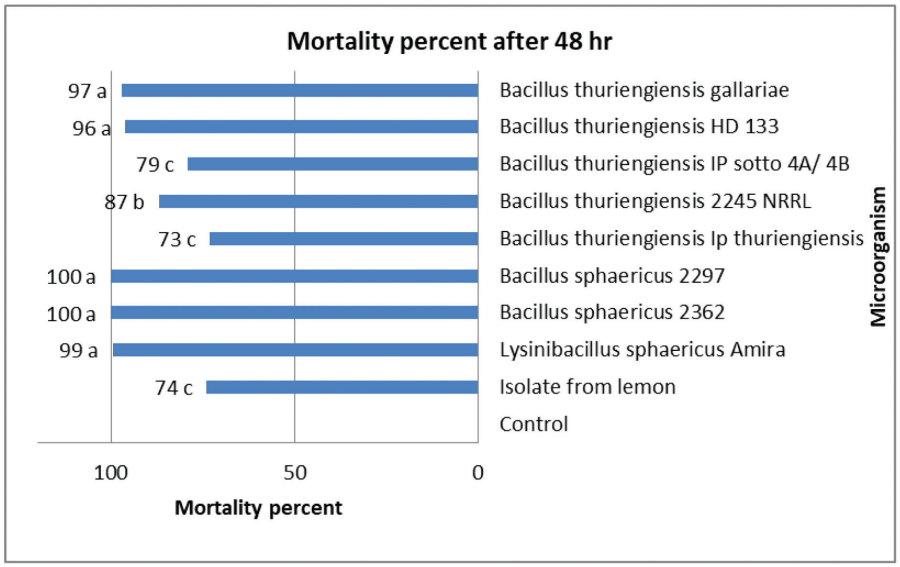
Statistically, *Lysinibacillus sphaericus* Amira, *Bacillus sphaericus* 2362, *Bacillus sphaericus 2297*, *Bacillus*

Figure 3



Comparison between different traditional and Roshdy microbial chitinases assay methods.

Figure 4



Mosquitocidal activities percent screening of selected microbial strains (dilution 10^{-3}) using full whole cultures supernatant against second instar larvae of *Aedes aegypti*.

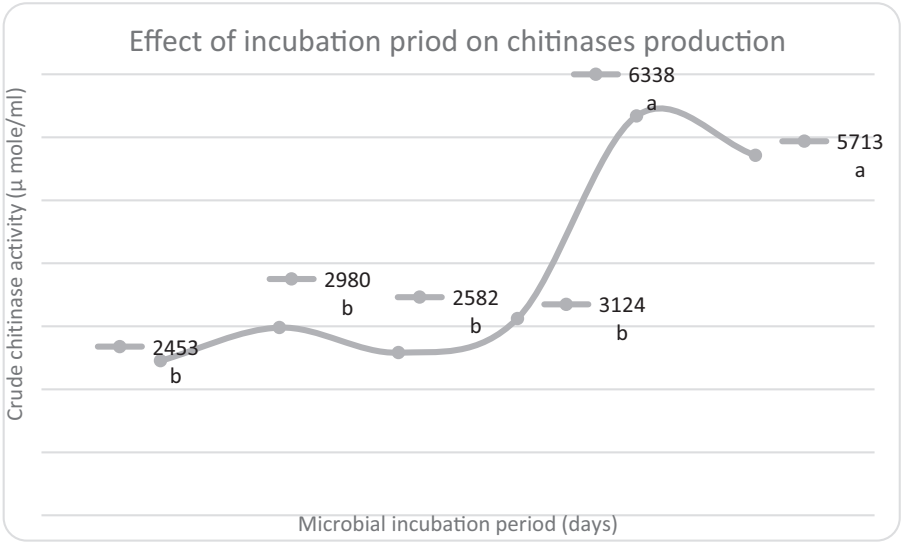
thuriengiensis HD 133, and Bacillus thuriengiensis gallariae strains increased the mortality percent after 48 h without significant difference between them. The mortality (%) values decreased significantly by *Bacillus thuriengiensis* 2245 NRRL treatment compared with the above-mentioned strains. Furthermore, no significant differences were detected between the strains isolated from lemon, *Bacillus thuriengiensis* IP *thuriengiensis, and Bacillus thuriengiensis IP sotto 4A/4B* in their effect on the mortality percentage of mosquito after 48 h.

Physiology of the production of chitinases by *Bacillus thuringiensis gallariae* under chitin solid-state fermentation conditions: *Bacillus thuringiensis gallariae* is the best strain in producing chitinases.

Effect of incubation period on crude chitinase production: It is clear in Fig. 5 that crude chitinase activity was significantly affected by the incubation period and followed the order: 5th greater than 6th without significant difference and 4th greater than 3rd greater than 2nd greater than 1st also without significant difference among them.

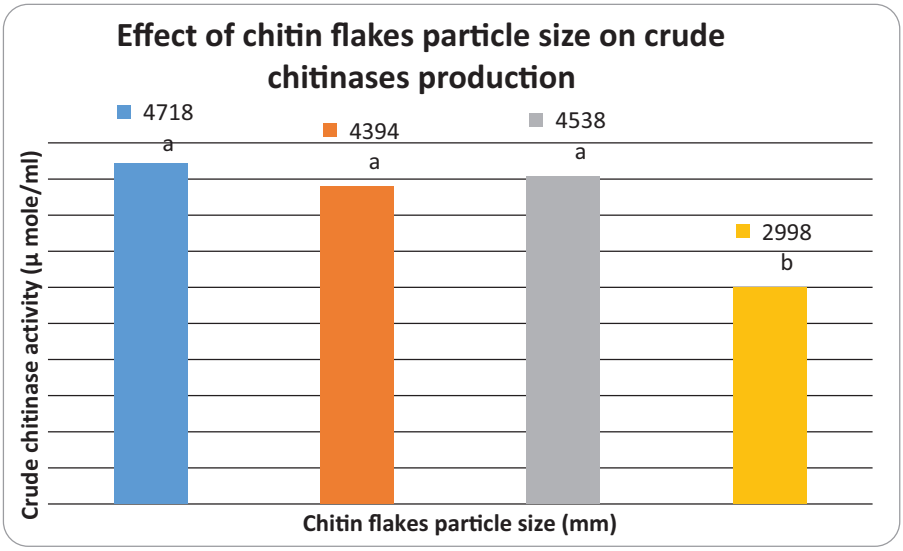
Effect of chitin flakes particle size on crude chitinases production: Results drawn in Fig. 6 showed that the best particle size of chitin flakes, whether for producing

Figure 5



Effect of incubation period on crude chitinase production.

Figure 6



Effect of chitin flakes particle size on crude chitinase production.

chitinases or other crude proteinous metabolites, was the smallest size (less than 1 mm) followed by the particle size from 1 to 2 mm without significant difference between them.

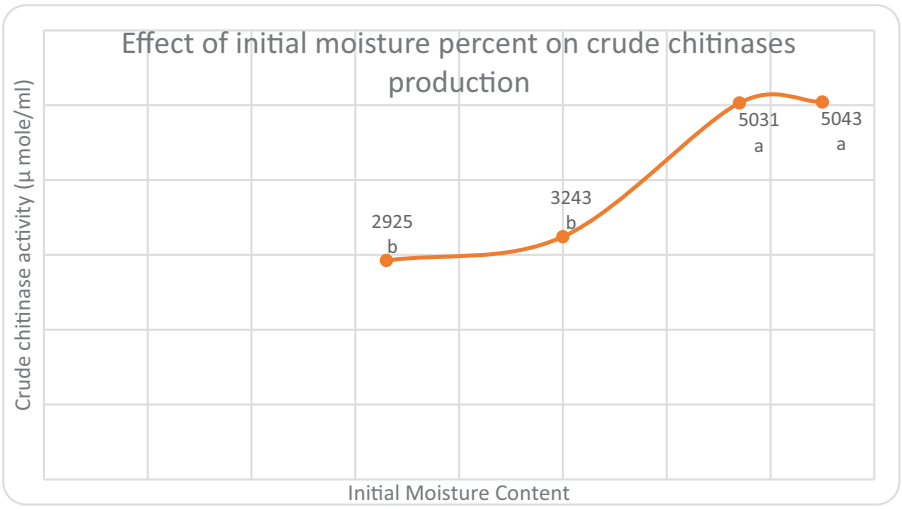
Effect of initial moisture content on crude chitinases production: Results given in Fig. 7 show an increase in the production of raw chitinases and other metabolites alike with increasing humidity up to about 70% yet the changes were not significant.

Effect of inoculum size on crude chitinase production: Results drawn in Fig. 8 also show that increasing the size of the inoculum negatively affects both chitinase production and other metabolites. No significant differences between neither the first three inoculum sizes and the last three inoculum sizes in their effect on crude chitinase production.

Effect of carbon source on crude chitinase production: Results in Fig. 9 graphically illustrate the effects of different carbon sources compared with using chitin as a single-growth medium. The results confirmed that the productivity of both chitinases and other metabolites was improved by adding a carbon source to the microbial growth environment. The best carbon sources for chitinases and metabolites were fructose and glucose without significant differences between them.

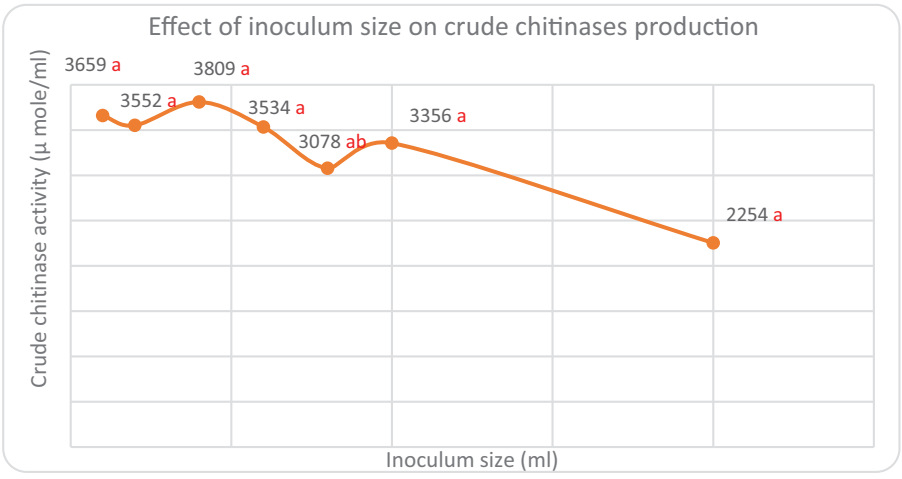
Effect of glucose concentration (%) on crude chitinase production: The concentration of added glucose at 5

Figure 7



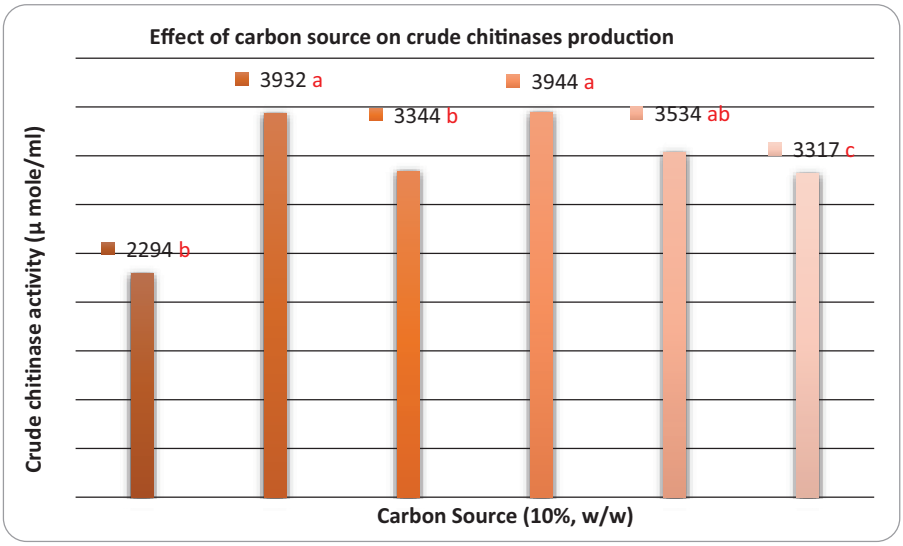
Effect of initial moisture content on crude chitinase production.

Figure 8



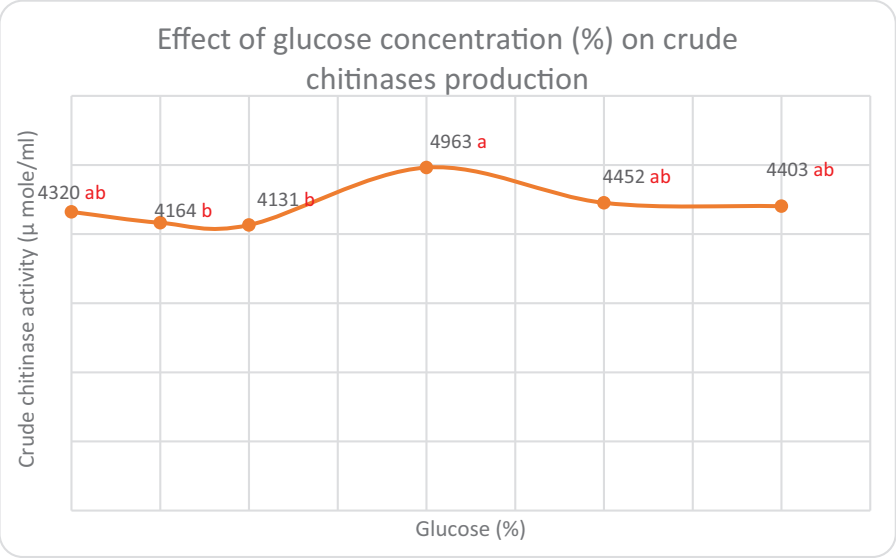
Effect of inoculum size on crude chitinase production.

Figure 9



Effect of carbon source on crude chitinase production.

Figure 10



Effect of glucose concentration on crude chitinase production.

and 20% (w/w) on the bacterial growth medium was the best for the production of metabolites and chitinase, respectively (Fig. 10).

Discussion

The current study revolves around the difficulty of methods of evaluating microbial chitinase production ability which is one of the most significant obstacles in the production of microbial chitinases; such repels researchers from expanding the discovery of microorganisms that produce this important enzyme. The current study compared some of the available methods with the proposed method to avoid the aforementioned difficulties.

Random available microbial strains were trailed regardless of their proven chitinase yield in previous studies; as the actual purpose of this study is to compare the productivity of microbial strains with large numbers suitable for screening survey studies. Improving the quality of research necessitates understanding the barriers confronting research, such as financial, facility-related, professional, administrative-organizational, scientific, and personal barriers [27]. It is necessary to expand the study of biochemical assays

of chitinolytic activity to enrich microbial biodiversity [6]. The most significant obstacles might be given as follows.

Difficulties of determination of microbial chitinases methods

Difficulty of enzyme substrate preparation: the most common microbial chitinase assay substrate is colloidal chitin, it is not homogeneous in distribution due to it is not soluble [28] which makes it difficult to weigh with an accuracy of part of a gram for enzymatic determination. Furthermore, the preparation of colloidal chitin takes a long time (several days), requires a lot of effort to prepare and concentrate harmful acids, and must be treated before disposal, which adds more burden to its preparation, and produces harmful fumes that the researcher inhales during colloidal chitin preparation, regardless of the occupational safety and health precautions that should be followed [2]. Despite this, it is considered one of the most common materials for the reaction of chitinase as enzymes enzyme reaction substrate and/or for the growth media of microorganisms that induce the production of chitinase, as some consider it cheaper, faster, and easier to prepare compared with others such as chitin glycol and swollen chitin, as their prices are very high [23].

Spectro-photometric method is considered the most accurate chitinase assay method, it uses the NAG Standard Curve to accurately measure the enzyme activity yield [29]; the defect of this method belongs to enzyme-substrate with its difficulties of preparation or providing; therefore, the method was modified to the method under study as follows:

Technical modification

The micro-organism grows on a sterile growth medium that consists only of chitin as a source of carbon and nitrogen; hence it will not be able to grow on this mono-source medium of carbon and nitrogen except by breaking down chitin linkages through microbial chitinases activity. The crude enzyme is extracted from fermented growth after a sufficient microbial incubation period (7 days). The free reducing sugar units are estimated directly in the extracted crude enzyme without adding enzyme substrate as usual in traditional methods, by the DNS reagent (Di, Nitro, Salselic), and then measured on a spectrophotometer along wavelength 400–450 nm. Thus, long steps to reach the step of estimating free sugar units are eliminated (Fig. 2).

Microorganism could grow on chitin as the only source of these two elements; accordingly, the presence of reduced free sugar units NAG is due only to the activity of microbial chitinases [5]. It is customary in the traditional methods of growing microorganisms, to add nutritional supplements to the medium for bacterial, fungal and streptomyces growth, as is the case in laboratory techniques book [29].

High costs and import procedures

Chitin Azure is an ideal alternative to avoid the problem of homogenous distribution in the enzymatic reaction solution; despite that Chitin Azure is an expensive enzyme substrate, as the price of one gram reached 526 euros. Chitin Azure also requires procedures and import expenses in some countries that do not produce it. While the use of Azure is a cheap method in addition to its speed (30 min) and accuracy [30]. The HPLC method is one of the accurate methods, but it is not suitable for microbial scanning due to its high cost and long estimate period [19].

Inaccuracy and lack of availability

The clear zone plates method is one of the descriptive methods because it is not that accurate, by scanning some strains of *Streptomyces* sp., Ekundayo *et al.* [2] found that *Streptomyces albus* had a wider clear zone than its counterparts on chitin agar plates, and it was active against all tested pathogenic fungi, namely: *Magnaportheoryzae, Fusariumgraminearum, Rhizoctoniasolani, Puccinia species* and *Botyrtiscinere* [2]. The justifications for the inaccuracy of viscosity measurement methods require a large database for each solution being measured, and this is very difficult in the case of mixed compounds (especially biological solutions) [21]. It had been shown that the accuracy of viscosity measurement methods ranges between 5 and 15%, as the fixed factors coefficients are difficult to generalize to different compounds. While, the radioactive method is not available for most research laboratories. Increasing the concentration of the reaction of the microbial alginate increases the time required for the solution to exit from the viscosity apparatus, and stresses the necessity of several graphic curves to reach the accurate arithmetic result of the viscosity in poise [22]. Viscosity is the resistance to flow for liquids, and the viscosity of a liquid may be due to the presence of hydrogen bonds in its molecules such as glucose (N-acetyl glucose amine units accordingly) [31]. There are two types of viscosity: absolute viscosity (dynamic viscosity) which is the fluid's resistance to flow and is measured in units of Poise and SI, Kinematic viscosity which requires knowledge of the density of the liquid and is measured in units of Stokes, this is the explanation for the inaccuracy of the viscosity method for

measuring enzymatic activity; as the increase of the monomer of the NAG units will increase the viscosity and the increase of the polymer of the chitin units will also increase the viscosity. This explains the inconsistency of the results of the viscosity experiment with the colorimetric experiment, where the strain *Bacillus thuriengiensis gallariae* excelled as well as the rest of the strains.

The length of time required for the experiment

While Shen *et al.* [30] considered that the use of Chitin Azure is a speed method (30 min). The colorimetric method (Ferrari) is one of the short and accurate methods at the same time [28]. Ferrari described it as fast as it takes only 15 min. It is also distinguished by its ability to measure chitinase enzymes and cellulase. When compared with the method under study, we find that Ferrari's method uses peroxidase enzymes to estimate the enzymatic activity of chitin and cellulose, while Roshdy's method avoids the step of the enzymatic reaction entirely. It is necessary to conduct industrial selection to survey the ability of large numbers of microbial strains to analyze industrial or biological polymers, (such as chitin) alike [32].

Application comparison as a biopesticide for *Aedes aegypti*

It is vital to use fungal biopesticides for the emergence of generations of insects resistant to biocides, and *Bacillus thuriengiensis gallariae* strain recorded the highest percentage among *Bacillus thuringiensis* strains. *Bacillus sphaericus* produces proteins that are toxic to different types of mosquito larvae during sporulation [33]. This protein consists of two types, which are 51 and 42 kDa, and are necessary to poison mosquito larvae that are rarely found together in most biopesticides, such as *Bacillus thuringensis*. The protein of 51 and 42 kDa is converted to the protein of 43 and 39 kDa, respectively, in the midgut of the insect, which greatly increases its toxicity. Comparisons of the difference in the susceptibility of larvae to infection with *Bacillus sphaericus* toxin indicate a possible difference like the target sites of the midgut epithelial cells and not in the absorption or processing of the toxin, in the midgut. Kumar *et al.* [34] found that the use of *Bacillus thuringiensis var. Kurstaki*, as a mosquitocide had a more effective lethal effect than the control group at the same concentration; for *Culex quinque fasciatus* and *Anopheles stephensi* types of mosquitoes, as the Kurstaki pesticide affected *Culex quinque fasciatus* (LC5 0.154%), while it affected *Anopheles stephensi* (LC5 0.372%). While Abu El-Ghiet *et al.* [35] found that lethal effect (EC 50) of *Bacillus thuringiensis* isolates JZ1 and JZ2 isolates, *Bacillus thuringiensis Kurstaki*, and the commercial chemical pesticide DiPEL 6.4 DF had a lethal effect on the second-instar larvae of *Aedes aegypti* as follows: 207, 932, 400, and 500 ppm, respectively, while their fatal effect on the first instar larvae of *Spodoptera littoralis* was as follows: 193.93, 589.7, 265.108, and 342.9, ppm respectively. Therefore, the researchers recommended introducing these bacterial isolates into programs for developing biocides for mosquitoes. There are different modes of action for bioinsecticides, such as insect growth, disturbance of its digestion system, and metabolism. Biocides change the nature of proteins, causing metabolic dysfunction, paralysis, activation of target poisoning, multiple sites of inhibition, and release of neuromuscular toxins [36]. It is also environmentally friendly as it is biodegradable and has a specific function with almost no negative impact on nontarget organisms. There is a direct relationship between bacterial chitinase productivity and the effect of the bacterial extract as a nematicide [24]. El-Bendary *et al.* [37] used several methods to study the recovery of spore toxin complex from *Bacillus thuringiensis* and *Bacillus sphaericus* to combat mosquitoes, such as the lyophilization method, flocculation by 0.1–0.2% ferric chloride and encapsulation by entrapment in calcium alginate beads, agar or polyacrylamide gel, or by adjusting the pH 2–5, or adding acetone, ethanol or acetic acid in different proportions, they recommended choosing the method based on the availability of equipment and the cost of production.

Through these discussions, along with the results of the bioinsecticide, viscosity and spectrophotometer results, we can nominate the *Bacillus thuriengiensis gallariae* strain as the best strain among the strains under study in producing chitinase enzymes. Just as the method could be used to survey the ability of some microorganisms, including bacteria and fungi, to produce chitinases, it could also be used to study the physiological properties of bacterial strain *Bacillus thuriengiensis gallariae*. So Roshdy's method is considered a promising method to facilitate knowledge of the microorganism's ability to produce chitinase in a faster manner, safe and secure, especially when scanning large numbers of microorganisms. It is also considered a more indicative method of the ability of a microorganism to produce intercellular chitinases.

Conclusions

In conclusion, the Roshdy method is superior to the traditional methods due to: I) Ease of the steps used to

discover the ability of the microorganism to produce chitinases compared with previous methods. II) The accuracy of estimating the ability of a microorganism to produce chitinase enzyme from the first experiment; because it is a quantitative method that uses a spectrophotometer. III) Save time, whether to import chitin azure or to prepare colloidal chitin (we do not need time to prepare colloidal chitin because we will not use it: only the growth period of the microbe, which ranges between 3 and 7 days, depending on the microorganism itself).

Providing hard currency by using local chemicals. IV) Preserving the environment; by not using concentrated acids. V) Quickly detecting microorganisms that decompose chitin residue, which is the second largest waste in the world after cellulose, and saving the efforts of researchers due to the lack of laboratory preparations. This, however, makes it easier to measure the production of microbial chitinases, whether in research organizations or specialized factories. We can also apply this to targeted pests.

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Declarations
Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Conflicts of interest
There are no conflicts of interest.

References

1 Kidibule PE, Santos-Moriano P, Ploub FJ, Fernández-Lobatoa M. Endo-chitinase Chit33 specificity on different chitinolytic materials allows the production of unexplored chitooligosaccharides with antioxidant activity. Biotechnol Rep 2020; 27:e00500.

2 Ekundayo FO, Folorunsho AE, Ibisanmi TA, Olabanji OB. Antifungal activity of chitinase produced by Streptomyces species isolated from grass and soils in Futa Area, Akure. Bull Nati Res Cent 2022: 46–95.

3 Fleuri LF, Kawaguti HY, Sato HH. Production, purification and application of extracellular chitinase from *Cellulosimicrobiumcellulans*191. Braz J Microbiol 2009; 40:623–630.

4 Kuddus M. Potenial applications of microbial chitinase: Recent Development. Biochem. Cell. Arch. 2014; 14:1–7.

5 Swiontek Brzezinska M, Jankiewicz U, Burkowska A, Walczak M. Chitinolytic microorganisms and their possible application in environmental protection. CurrMicrobiol 2014; 68:71–81.

6 Yan Q, Fong SS. Bacterial chitinase: nature and perspectives for sustainable bioproduction. Bioresources and Bioprocessing 2015; 2:3.

7 Singh M, Meena SC, Kumarjain R. Application of chitinase for sustainable agriculture. Adv Life Sci 2016; 5:11131–11138.

8 Pramesti E, Puspita ID. Optimization of colloidal chitin and inoculum concentration in chitinase production by streptomyces sp. PB2 using response surface methodology. E3S Web of Conferences 2020; 147:03011.

9 Taokaew S, Kriangkrai W. Chitinase-assisted bioconversion of chitinous waste for development of value-added chito-oligosaccharides products. Biology 2023; 12:87.

10 Sharma A, Arya SK, Singh J, Kapoor B, Bhatti BS, Suttee A, Singh G. Prospects of chitinase in sustainable farming and modern biotechnology: an update on recent progress and challenges. Biotechnol Genet Eng Rev 2023; 1:1–31.

11 Smith JR, Grula EA. Chitinase is an inducible Enzyme in *Beauveriabassiana*. J Invertebr Pathol 1983; 42:319–326.

12 Jeuniaux C. Chitinases. Chapter 111, in: Methods in Enzymology. 1966;8: 644–650.

13 Roopavathi AS, Vigneshwari R, Jayapradha R. Chitinase: production and applications. J Chem Pharm Res 2015; 7:924–931.

14 Martínez-Zavala SA, Barboza-Pérez UE, Hernández-Guzmán G. Chitinases of *Bacillus thuringiensis*: phylogeny, modular structure, and applied potentials. Front Microbiol 2020; 14:3032.

15 Jeuniaux C. Recherchessur les chitinase. I. Dosage nephelometrique et production de chitinase par des Streptomycetes. Arch Intern PhysiolBiochim 1958; 66:408–427.

16 Onen H, Luzala MM, Kigozi S, Sikumbili RM, Muanga CJK, Zola EN, *et al.* Mosquito-Borne Diseases and Their Control Strategies: An Overview Focused on Green Synthesized Plant-Based Metallic Nanoparticles. Insects 2023; 14:221.

17 Baranek J, Jakubowska M, Gabała E. Insecticidal activity of *Bacillus thuringiensis* towards Agrotisexclamationis larvae-A widespread and underestimated pest of the Palearctic zone. PLoS ONE 2023; 18: e0283077.

18 Berini F, Casartelli M, Montali A, Reguzzoni M, Tettamanti G, Marinelli F. Metagenome-aourced microbial chitinases as potential insecticide proteins. Front. Microbiol 2019; 10:1358.

19 Roshdy AM, Shata HM, Ali SM, Youssef AM, Foda MS, Kahil T. Commercial extraction of applicable alkaline protease and chitinase by *Bacillus thuringiensis dendrolimus* IP 4A/4B. J Mod AgricBiotechnol 2022; 1:21.

20 Boller T, Gehri A, Mauch F. VÓ§geli U. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 1983; 157:22–31.

21 Mehrotra AK, Monnery WD, Svrcek WY. A review of practical calculation methods for the viscosity of liquid hydrocarbons and their mixtures. Fluid Phase Equilibria 1996; 117:344–355.

22 Stevens RA, Levin RE. Viscometric Assay of Bacterial Alginase. Appl Environ Microbiol 1976; 31:896–899.

23 Jha SC, Modi HA. Comparative Analysis of Chitinase Activity by Four Different Assay from Soil Born Actinomycetes. In 4th International Conference on Multidisciplinary Research & Practice, Ahmedabad, India, 2017. 185–190

24 Abdel-Salam MS, Ameen Hoda H. Soliman, Gaziea M, Elkelany U S, Asar Amira M. Improving the nematicidal potential of Bacillus amyloliquefaciens and *Lysinibacillus sphaericus* against the root-knot nematode Meloidogyne incognita using protoplast fusion technique. Egyptian. J Biol Pest Cont 2018; 28:31.

25 Afify A, Aboul-Soud M, Foda M, Sadik M, Kahil T, Amira R, *et al.* Production of alkaline protease and larvicidalbiopesticides by an Egyptian Bacillus sphaericus isolate. Afr J Biotechnol 2009; 8:3864–3873.

26 Gomez KA, Gomez AA. Statistical Procedures for Agricultural Research. New York, USA: Jon Willey and Sons Inc; 1984:2.

27 Karimian Z, Sabbaghian Z, Salehi A, Sedghpour BS. Obstacles to undertaking research and their effect on research output: a survey of faculty members' views at Shiraz University of Medical Sciences. EMHJ 2012; 18:1143–1150.

28 Ferrari AR, Gaber Y, Fraaije MW. A fast, sensitive and easy colorimetric assay for chitinase and cellulase activity detection. Biotechnolr Biofuels 2014; 7:37.

29 Senthilkumar M, Amaresan N, Sankaranarayanan A. Plant-Microbe Interactions Laboratory Techniques, Springer Protocols Handbooks 2021 https://doi.org/10.1007/978-1-0716-1080-0_58. Science Business Media, LLC, part of Springer Nature.

30 Shen C, Chen Y, Yang C, Chen J, Liu C. Colloid chitin azure is a dispersible, low-cost substrate for chitinase measurements in a sensitive, fast, reproducible assay. J Biomol Screen 2010; 15:213–7.

31 Klassen J. MSU: CHMY 362 Eelements of Physical Chemistry. Montana State University in Bozeman 2023.

32 Wright RJ, Gibson MI, Christie-Oleza JA. Artificial selection of microbial communities to enhance degradation of recalcitrant polymers 2018.

33 Baumann P, Clark MA, Baumann L, Broadwell AH. *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. Microbiol Rev 1991; 55:425–36.

34 Kumar AS, Moorthi M, Ramakrishnan N, Dhanapakiam P, Ramasamy VK, Rajeswari AR. Efficacy of *Bacillus thuringiensis var*. Kurstakiin the control of two mosquito species (*Anopheles stephensi* and *Culexquinquefasciatus*). J Environ Biol 2009; 30:433–5.

35 Abu El-Ghiet UM, Moustafa SA, Ayashi MM, El-Sakhawy MA, Ateya AAE, Waggiallah HA. Characterization of *Bacillus thuringiensis* isolated from soils in the Jazan region of Saudi Arabia, and their efficacy against *Spodoptera littoralis* and Aedes aegypti larvae. Saudi J Biol Sci 2023; 30:103721.

36 Ayilara MS, Adeleke BS, Akinola SA, Fayose CA, Adeyemi UT, Gbadegesin LA, *et al.* Biopesticides as a promising alternative to synthetic pesticides: A case for microbial pesticides, phytopesticides, and nanobiopesticides. Front. Microbiol 2023; 16:1040901.

37 Foda MS, Ali SM, Youssef AM, Kahil T, Hoda MS, Roshdy AM. Production Physiology of Alkaline Protease by Bacillus thuringiensisspp. under Solid-State Fermentation Conditions. J Appl Sci Res 2013; 9:1975–1984.