Ekundayo *et al.*
Bulletin of the National Research Centre (2022) 46:95
https://doi.org/10.1186/s42269-022-00782-4

Bulletin of the National Research Centre

RESEARCH

Open Access

Antifungal activity of chitinase produced by *Streptomyces* species isolated from grassland soils in Futa Area, Akure

Fatuyi Olanipekun Ekundayo, Abisola Eniola Folorunsho*, Tope Abraham Ibisanmi and Olayinka Bukola Olabanji

Abstract

Background: This study was carried out to investigate the antifungal activity of chitinase produced by *Streptomyces* species isolated from grassland soils against selected pathogenic fungi, namely: *Magnaporthe oryzae*, *Fusarium graminearum*, *Rhizoctonia solani*, *Puccinia* species and *Botyrtis cinerea*. Chitinase produced by *Streptomyces albus* was purified consecutively with dialysis, gel filtration and ion- exchange chromatography, the test isolate can be exploited for large scale production of chitinase.

Results: Seven *Streptomyces* species (*Streptomyces rimosus, S. albus, S. riseoflavus, S. fumosus, S. spiralis, S. aureofaciens and S. flavogriseus)* isolated from three different grassland soils were screened based on the morphology and their ability to produce chitinase. *S. rimosus*, *S. fumosus*, *S. albus* and *S. flavogriseus* were able to produce chitinase, although the enzyme chitinase was found to be higher in *Streptomyces albus*. The effect of environmental factors were determined on *Streptomyces albus* being the best chitinolytic producing isolate. Temperature of 30ºC was favourable for *Streptomyces albus* and pH 6.0 was a growth factor for maximum chitinase production. The activity of the enzyme was stimulated by the addition of metal ions like Mn^{2+}, K^{+}, Na^{+}, Mg^{+}, Fe^{2+}, Ca^{2+} but Zn^{2+} inhibited chitinase activity. Chitinase activity was favoured by maltose as carbon source and $NH_4 (SO_4)_2$ as nitrogen source. Culture filtrate of *Streptomyces albus* was able to inhibit the growth of all the selected pathogenic fungi namely *Magnaporthe oryzae*, *Fusarium graminearum*, *Rhizoctonia solani*, *Puccinia* species and *Botyrtis cinerea* whereas *Streptomyces rimosus* has the least inhibitory activity.

Conclusions: *Streptomyces albus* has the highest ability to produce chitinase which can act as antifungal for degradation of the cell wall of many phytopathogenic fungi.

Keywords: Chitinase, *Streptomyces*, Grassland, Soils, Pathogenic

Background

Chitin is a linear polymer that is made up of β-1, 4-N-acetylglucosamine, this chitin is the second most abundant biopolymer on this planet earth after cellulose. Chitin can be found in the outer skeleton of algae, crabs, shrimps, insects, yeasts, fungi and lobsters, also in the internal structures of other invertebrates (Shahidi and Abozaytoun 2005). Chitin comprises 22–44% of cell walls of fungi. Chitin is a hard, inelastic polysaccharide, white, and is a major contributor to pollution in coastal areas. Chitin contain high percentage of nitrogen(6.89%) which makes it an effective chelating agent. Chitin and its associated materials have a broad usage in in waste water treatment, wound healing, drug delivery and dietary fiber. Chitin exists in two allomorphic forms, i.e., α-chitin and β-chitin, these 2 allomorphic forms of chitin vary in polarities and packing of adjacent chains in the

*Correspondence: Abisolaeniolafolorunsho@gmail.com
Department of Microbiology, School of Sciences, Federal University of Technology, P.M.B. 604, Akure, Ondo State, Nigeria

succeeding sheets. Chitin can be degraded by chitinase (Bussink et al. 2007).

Chitinases are glycosyl hydrolases with the sizes ranging from 20 kDa to about 90 kDa (Bhattacharya and Gupta 2007) Chitinases are a huge and diverse group of enzymes that show differences in their molecular structure, substrate specificity, and catalytic mechanism. Chitin contain important degrading enzyme call Chitinase, which is involved in bioconversion processes, physiological reactions of wastes from crustacean chitin, in chitin mineralization from soil environments and marine and in plant protection by preserving them from chitin, containing parasites, e.g. fungi which are used by insects to degrade the structural polysaccharide "chitin" during the molting process (Zhang et al. 2002).

Biological control of pests can also be easily carried out by using chitinase. Among the microorganisms that contain chitin, approximately 90–99% of the chitinolytic populations are actinomycetes. Actinomycetes, particularly *Streptomyces* sp. have been a widely exploited group of microorganisms in the production of secondary metabolites and enzymes of commercial importance in medical and agricultural applications (Kumar and Gupta 2006). Chitin can be degraded directly by chitinase to low molecular weight chitooligomers, which serve a broad range of medical functions, industrial and agricultural. Chitinase is known to lyse the cell wall of both live and dead fungi. Chitinase from Streptomyces exhibit other antifungal activities like inhibition of spore germination, germ tube elongation, bursting of spores, etc. *Streptomyces* are present in soils abundantly, especially in not too acid and dry, and soil rich in organic matter; here, in propagule numbers, they frequently exceed the combined counts of all other bacteria (Watve et al. 2001). They decompose plant and animal residues, for the addition of these residues to soil greatly increases the activity and size of the *Streptomyces, Streptomyces* as a genus of Gram-positive bacteria that grow with a filamentous form similar to fungi in various environments. Hence, this study was carried out to investigate the antifungal activity of chitinase produced by *Streptomyces* species isolated from grassland soils against selected pathogenic fungi, namely: *Magnaporthe oryzae*, *Fusarium graminearum*, *Rhizoctonia solani*, *Puccinia* species and *Botyrtis cinerea*.

Methods

Collection of samples

Soil samples were collected from three different sources of grassland soils at a depth of 10 cm at Obanla, Federal University of Technology, Akure, (FUTA) in Ondo State, Nigeria using soil auger. The above soil samples were used for the isolation of *Streptomyces* species after been transported in sterile polyethylene bags to the Department of Microbiology laboratory, Obakekere, FUTA.

Determination of physicochemical properties of the soil samples

The physicochemical properties of the soil used for the isolation of *Streptomyces* were determined. The parameters measured included pH, Calcium, Magnesium, Sodium, Organic Carbon, Organic matter, Potassium, Sodium, Phosphorus and Nitrogen at O.B.C. laboratory, Akure, Ondo State.

Determination of the pH of soil samples

Twenty (20) grams of each soil sample was weighed and put in a 100 ml beaker. A 20 ml of distilled water was added to the sample. The suspension was stirred for fifteen minutes thrice using a stirrer, the electrode was rinsed with the distilled water and wiped with tissue paper, and the pH meter was calibrated using buffer 4 and 7. The pH of the suspension was read twice from the pH meter and the average was calculated (AOAC 2007).

Determination of metal concentrations

Air-dried, ground and sieved soil sample (0.5 g) was accurately weighed into a digestion tube and 6 ml aqua regia and 1.5 ml hydrogen peroxide were measured and added into the digestive tube. The digestion tubes were placed on digestive furnace (Model:KDN-20C, China) and heated at a temperature for 3 h at 180 °C. All the digests were cooled and filtered through Whatman filter paper in to 50 ml volumetric flask. Lanthanum chloride solution (1%) was added to the filtrate and the flask containing the filtrate was made up to the mark with double distilled water. Each sample was digested in replicates of five and transferred to acid washed stoppered glass bottle, labelled and kept for metal analysis. The concentrations of Calcium and Magnesium in the filtrate were determined by using flame atomic absorption Spectrophotometer, sodium and potassium was determined by flame photometer. Final concentration of the element present in soil samples was calculated as:

$$\text{Concentration of the element in soil (mg/kg)} = \frac{Conc.\,(\text{mg/l}) \times (\text{ml})}{(\text{g})}$$

where Conc.: concentration of the element obtained (mg/l), V is the final volume of the digested solution (50 ml) and W is the weight of the soil sample (0.5 g).

Determination of organic carbon and organic matter of soil samples

One gram finely ground soil sample was passed through 0.5 mm sieve without loss and was put into 500 ml

conical flask, to which 10 ml of 1 N potassium dichromate and 20 ml conc. H_2SO_4 were added with measuring cylinder. The contents were shaken for a minute and allowed to stand for 30 min. Then 200 ml distilled water, 10 ml orthophosphoric acid and 1 ml diphenylamine indicator were added. The solution was titrated against 0.5 N ferrous ammonium sulfate till the colour changes from blue-violet to green after which the blank titration was carried at the beginning without soil. The results were calculated by making use of the following formulas:

$$\text{Organic carbon \%} = \text{N} \times (\text{V1} - \text{V2}) \times 0.39 \times \text{mcf} \qquad \text{S}$$

where N is the Normality of ferrous ammonium sulfate.

V1 is the Volume of 0.5 N ferrous ammonium sulfate required to neutralize 10 ml of 1 N $K_2Cr_2O_7$ i.e., blank reading (ml).

V2 is the Volume of 0.5 N ferrous ammonium sulfate needed for titration of soil sample (ml).

S = Weight of air-dry sample (g).

$0.39 = 0.003 \times 100\% \times 1.31$ (0.003 is the milliequivalent weight of carbon in g). only 77% of the organic matter content is oxidized and a fraction of $100/77 = 1.31$

Organic matter (%) is equal to Organic carbon(%) × 1.724.

1.724 is the average content of carbon in soil organic matter is equal to 58%)

Determination of moisture content

Using oven drying method as described by (AOAC 2007), ten (10) g of composite soil sample was taken. The samples were oven dried at 105 °C for 24 h. Dry weight of the sample was taken till it showed its constant weight. The amount of water present in the soil sample is equivalent to the amount of weight lost during the process. The formula below was used to calculate the percentage of moisture content in each of the soil samples (Joel and Amajuoyi 2009).

$$\text{Moisture content (MC)(\%)} = \frac{\text{Loss in weight on drying(g)}}{\text{Initial sample weight (g)}} \times 100.$$

The corresponding moisture correction factor (mcf) for analytical results is calculated as following: *X* Moisture correction factor (mcf) = 100 + %moisture100.

Determination of available phosphorus of soil samples

5 g of air-dried soil samples was weighed into a baker and 35 ml of Phosphorus extracting solution, NH_4Cl was measured and added to the content of the baker. The mixture was well stirred for 5 min before filtered using 11 µm Whatsman filter paper of which 4 ml of the filtrate was pipetted into a test tube and 4 ml of ascorbic acid was also added. The resulting mixture was allowed to stand for 30 min on a test tube rack for colour development. The colour developed was blue and the procedure was repeated for the other samples. The standard was also prepared by measuring 0.5 ml of 100 ppm Phosphorus standards and adding 4 ml of indicator M and R solution. Twenty-five (25) milliliters of distilled water were added. The prepared solution was transferred into another test tube, after which a blank was prepared by measuring 4 ml of the ascorbic acid reagent and 25 ml of distilled water into another test tube, after which the available soil Phosphorus absorbance at 660 mm wavelength was read by making use of coming colorimeter model 253 (AOAC 2007).

Determination of total nitrogen content of soil samples

Soil sample (1 g), which previously has been grinded and sieved in a 2 mm wire mesh was weighed and transferred into a 500 ml micro-kjeldahl flask and 20 ml distilled water was added, the mixture was shaken gently for a few minutes and was allowed to stand for about 30 min. A twenty (20) ml concentrated copper oxide catalyst was added to the mixture. The flask was then transferred to a mantle for mechanical heating, the heater was placed in the fume cupboard connected to the electrical outlet socket and was switched. The flask was then left to boil for about 5 h in the fume cupboard. After the digest has been observed to be clear of H_2SO_4 fumes in the flask, the heater was then switched off. The micro-kejdahl flask was then removed from the heater and allowed to cool, follow by decanting the digest into another flask after which 100 ml of distilled water was added to the content of the flask. The micro-kejdahl flask was then attached to the distillation apparatus. After which a fifty (50) ml boric acid consisting of indicator solution was transferred into 25 ml conical flask. The flask was then placed under the condenser of the distillation apparatus. The tip of the condenser was positioned such that it was about 4 mm above the surface of the boric (H_3BO_3) solution in the conical flask. The digest was then distilled by allowing hot steam to pass from the steam pot into the digest in the micro-Kjedahl flask, thereby causing the digest to boil and distill over into boric acid. After about 150 ml of the distillate had been collected in boric acid, the distillation was stopped. After which distilled water was titrated with 0.5 m standard hydrogen chloride, colour change at the end of the reaction was from green to pink (AOAC 2007).

Sterilization of glass wares and culture media

Test tubes, beakers, conical flasks and other glass wares used were thoroughly washed, rinsed and drained. Prepared media were corked with cotton wool and foil paper. The glass wares and the media were then sterilized using an autoclave at 121 °C for 15 min in order to avoid contamination. The water used for serial dilution was also sterilized using the autoclave. Inoculating loops were

thoroughly and appropriately flamed using a Bunsen burner. Work benches were also swabbed using cotton wool and ethanol to make it free of contamination (Olusola-makinde et al. 2021).

Isolation of *Streptomyces* species from grass land soils

Ten-fold serial dilution was carried out on each of the soil samples, starch casein agar used for the isolation of Streptomyces species from grass land soils was prepared according to manufacturer's specification and was sterilized in an autoclave at 121 °C for fifteen minutes, after sterilization, it was allowed to cool. The diluent from dilution factor of five was inoculated into the plates; 20 ml of the cooled agar was then poured into the inoculated plates and was allowed solidified after which the plates were incubated at 28 °C for 5 days.

After incubation, the growth of the organisms was observed, sub culturing was done until pure cultures were obtained, the pure isolates were kept on starch casein agar slants for the screening of chitinase production ability.

Biochemical tests

Biochemical tests were carried out to identify, differentiate and characterize organisms. Gram staining technique was carried out to ascertain the morphology and gram reaction behavior of the isolates, other biochemical tests carried out include: sugar fermentation test, catalase and citrate (Olusola-makinde et al. 2021).

Gram staining

A drop of water was put on a grease free slide using a sterile syringe; an inoculum from a cultured plate was picked using a flamed inoculating loop and mixed with the water on the slide to make a smear. The smear was allowed to dry and passed through flame thrice in order to heat fix the slide. Crystal violet was added for one minute and then rinsed off with water. lugol´s Iodine was added which is the mordant and left for 60 s, then washed with water and allowed to dry. 70% ethanol was then added to decolorize and was washed with water immediately. Counter staining was done with safranin for one minute. It was rinsed with water and then air dried. Immersion oil was added which was then viewed with × 100 lens of the light microscope (Ibisanmi and Aribisala 2022).

Catalase test

A drop of hydrogen peroxide was added on a clean glass slide using a sterile syringe; an inoculum of the test organism was picked from the cultured plate using a flamed inoculating loop and dropped on the hydrogen peroxide. Production of bubbles indicates that the test organism is catalase positive while absence of bubbles indicates that the organism is catalase negative (Olusola-makinde et al. 2021).

Citrate utilization test

Simmons citrate agar was prepared following manufacturer´s specification, i.e., 24 g in 1 L of distilled water; it was sterilized in an autoclave at 121 °C for 15 min. Citrate slope was prepared in sterilized bijou bottle, few colonies of the test organism was picked using a sterile wire loop and inoculated into the citrate slope, It was incubated at 37 °C for 24 h and observed for change in colouration, If the organism has the ability to use citrate, the medium changes its colour from green to blue (Ibisanmi and Aribisala 2022).

Sugar fermentation test

Sugar fermentation test was carried out using maltose, sucrose, fructose and glucose sugars. Peptone broth was prepared following the manufacturer´s specification (i.e., 15 g in 1L of distilled water) and poured into different conical flasks. 1 g of each was measured from carbohydrate into the prepared peptone water then phenol red was added as indicator for acid production, the solution was prepared into universal bottles, Durham tubes were inserted in an inverted position and the media were sterilized. After sterilization, each bottle was aseptically inoculated with the test microorganism using a flamed inoculating loop and incubated at 37 °C for 72 h. Colour change from orange to yellow indicates that there is a drop in the pH because of the production of acid by the fermentation of the sugar present in the media making it positive. gas production is observed as bubble in the inverted Durham tube and noted as a positive result, absence of bubble in the inverted Durham tube and no colour change indicating the bacteria cannot ferment that particular carbohydrate source present in the media which shows it is negative (Olusola-makinde et al. 2021).

Production of colloidal chitin

30 g of chitin flakes were suspended in 200 ml concentrated hydrochloric acid and then incubated in rotary shaker at room temperature for 2 h until the chitin flakes are completely dissolved. With rigid stirring, the resulting solution was poured into 1 l deionized water to form precipitates of colloidal chitin, which were subsequently collected by centrifugation at 7000 rpm for ten minutes at 4 °C. The precipitates were washed with sterile distilled water (30 ml each) several times to bring the pH value of the colloidal chitin suspension to 2.0–3.0. the suspension was then neutralized with 1 m sodium hydroxide and centrifuged again at 7000 rpm for ten minutes and washed with sterile water for two to three times to obtain

the low-salt colloidal chitin, the colloidal chitin was then dried in an oven at 60 °C for 24 h in order to obtain chitin pellets. The chitin pellet was kept at 4 °C for further application (Wu et al. 2009).

Screening of *Streptomyces* isolates for chitinase

Different isolates were screened for the production of chitinase by plate assay method. Nutrient agar medium enriched with 0.1% colloidal chitin as sterilized after which the organism was introduced and incubated at 30 °C for 4 days. Following incubation period, 0.1% Congo red solution was fed over the plates after which it was distained with 1% NaCl, the plates were observed for zone of clearance. Formation of clear utilization zone around the organism was considered as positive (chitin utilizers) (Wu et al. 2009).

The basis of their chitinase activity determined quantitatively (chitinase assay) and qualitatively (Measuring clearance zone) was used in the selection of working strain. Good visible growth of the organism was observed after incubation for 7 days, they showed clear zone around the colonies which indicates extra cellular chitinase production, the specie with the widest zone of inhibition was selected for chitinase production and assayed for the effect of cultural conditions such as carbon source, incubation, pH, temperature, etc.

Production of chitinase

Minimal synthetic medium (MSM) was supplemented with chitin as the carbon source, after that the pH was adjusted to 6.3 with 50 mM phosphate buffer and autoclaved at 121 °C, 15 atm for 20 min, the medium was also subjected to the required environmental factors for maximum chitinase production. The medium was inoculated with a suspension of the best chitinolytic *Streptomyces* species to give a final suspension and placed in a rotary shaker at 4000 rpm for 20 min and then incubated at 30 °C for 48 h. The culture was harvested at the 36th hour of incubation (Duzhak et al. 2012).

Antifungal activity of the crude enzyme

Antifungal activity of the crude enzyme was determined by agar well diffusion method as described by (Olutiola et al. 2000) Potato dextrose agar (PDA) was prepared following the manufacturer's specification, it was sterilized and allowed to cool. The mycelium of the test fungi was placed in the center of the petri plates containing PDA and wells were bored at equidistant from each other in the agar plates with the use of a cork borer (diameter 10 mm). The wells were then filled with 0.1 ml of the culture filtrate and 50% mancozeb. Distilled water was used as negative Control. The plates were allowed to stand for one hour to allow diffusion of the metabolite in the filtrate, then incubated at 25 °C for 72 h and observed for zones of inhibition. Three replicates of the experiment were performed and the diameters of the inhibition zones were measured and recorded (Narayana and Vijayalakshmi 2009).

Assay and optimization for chitinase production

Chitinase activity was assayed using a mixture containing 1 ml of 0.5% pure chitin (sigma suspended in 50 Mm acetate buffer pH of 5.2) and 1 ml of enzyme solution. The reaction mixture was then incubated at 37^0C for 1 h with shaking and was stopped by centrifugation (5000 g/min) for 10 min, 1 ml of dinitrosalicylate (DNS) reagent was added followed by heating at 100 °C for five minutes. Absorbance of mixture was measured at 540 nm against the blank prepared with sterile production medium without the enzyme (Sadasivam and Manickam 2014).

Effect of temperature on chitinase production

A 50 ml of production medium was prepared, sterilized and 2 ml inoculum was added then incubated at different temperatures at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C and 80 °C after which culture filtrate was harvested for every 24 h. The enzyme activity and protein content were studied for up to 6 days (Gao et al. 2012).

Effect of pH on chitinase production

A 50 ml of production medium was prepared and pH of the medium was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. The sterilized production media were inoculated with 2 ml suspension and incubated under the shaking condition. The enzyme activity and protein content study were done for every day up to 6 days (Gao et al. 2012).

Effect of incubation time

To determine the optimum incubation period for chitinase production, inoculated flasks were incubated in a rotary shaker in 100 rpm at room temperature for about 6 days. Every 24 h, the culture filtrate was harvested and checked for the enzyme activity as well as the total protein content (Gao et al. 2012).

Determination of the effect of carbon source on chitinase production

50 ml of production medium was prepared and then sterilized after which 2 ml inoculum was introduced and incubated for each carbon source. The culture filtrate was harvested for every 6 h each for 2% glucose, fructose, maltose, galactose and sucrose, then the enzyme activity was studied for 36 h (Gao et al. 2012).

Table 1 Physicochemical analysis of grassland soils collected in Obanla, FUTA

Soil sample	pH	OC (%)	OM (%)	N (%)	MC (%)	P (mg/kg)	K (cmol/kg)	Na (cmol/kg)	Ca (cmol/kg)	Mg (cmol/kg)	Percentage Clay	Percentage Sand	Percentage Silt
Grassland soil 1	5.06 ± 0.07^{a}	1.62 ± 0.01^{a}	2.84 ± 0.01^{a}	0.23 ± 0.06^{a}	12.40 ± 0.12^{c}	9.25 ± 0.08^{b}	0.37 ± 0.22^{a}	0.40 ± 0.07^{a}	3.01 ± 0.01^{b}	1.20 ± 0.000^{a}	18.97 ± 3.52^{a}	15.87 ± 3.71^{a}	10.14 ± 2.51^{a}
Grassland soil 2	5.06 ± 0.02^{a} 6.43 ± 1	2.48 ± 0.03^{b} 4.31 ±	4.24 ± 0.06^{b}	0.39 ± 0.09^{c}	11.63 ± 0.01^{b}	11.83 ± 0.08^{c}	0.41 ± 0.01^{b}	0.49 ± 0.04^{a}	3.15 ± 0.05^{b}	1.30 ± 0.01^{b}	18.49 ± 2.00^{a}	27.70 ± 2.69^{b}	19.19 ± 1.15^{b}
Grassland soil 3	$.70^{b}$	1.99^{c}	5.56 ± 1.67^{c}	0.30 ± 0.20^{b}	8.36 ± 1.62^{a}	0.21 ± 0.25^{a}	42.38 ± 2.08^{b}	16.60 ± 2.20^{b}	2.93 ± 1.40^{a}	3.56 ± 1.61^{c}			

OC organic carbon, *OM* organic matter, *N* nitrogen, *MC* moisture content, *P* phosphorus, *K* potassium, *Na* sodium, *Ca* calcium, *Mg* magnesium. Values are expressed in mean ± SEM. $P < 0.05$ was considered to be statistically significant

Table 2 Morphological characteristics of the *Streptomyces* species isolated from grassland soils sample collected in Obanla, FUTA

Probable organism	Colour	Spore Shape	Texture	Growth	Elevation
Streptomyces rimosus	Yellow	Filamentous	Rough	Good	Flat
Streptomyces albus	White	Oval	Rough	Good	Flat
Streptomyces fumosus	Yellow	Filamentous	Rough	Good	Flat
Streptomyces riseoflavus	Yellow	Oval	Rough	Very good	Flat
Streptomyces spiralis	Yellow	Round	Rough	Moderate	Flat
Streptomyces flavogriseus	Yellow	Round	Rough	Good	Flat
Streptomyces aureofaciens	Yellow	Round	Rough	Very good	Flat

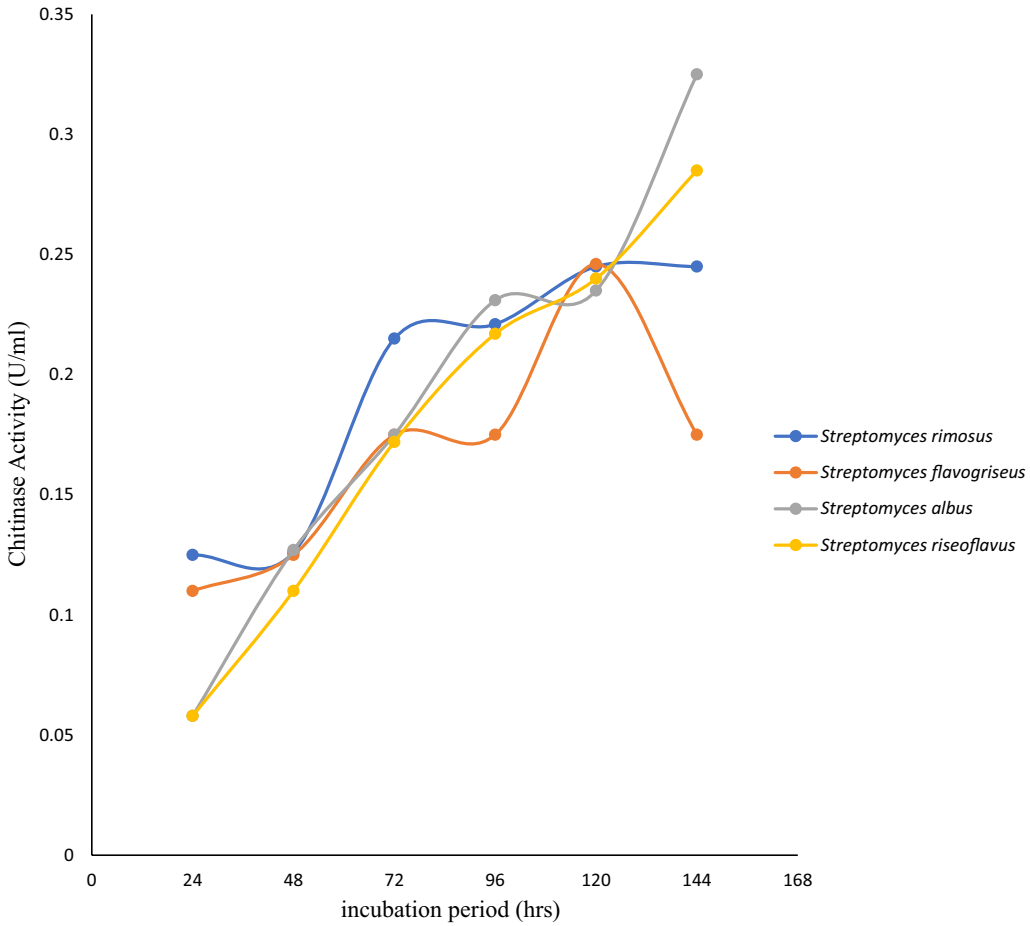

Fig. 1 Growth pattern of the *Streptomyces* isolates that were able to produce chitinase at different incubation period

Determination of effect of nitrogen source on chitinase production

50 ml of production medium was prepared and then sterilized after which 2 ml inoculum was introduced and incubated for each nitrogen source. The culture filtrate was harvested for every 6 h for NH_4Cl, NH_4CO_3, NH_4NO_3, $NH_4(SO_4)_2$ $NH_4(NO_3)_2$ and $(NH_4)PO_4$, then the enzyme activity was studied for 36 h (Gao et al. 2012).

Effect of metal ion on chitinase production

50 ml of production medium was prepared and sterilized after which 2 ml inoculum was added and then incubated

Table 3 Biochemical characteristics of the *Streptomyces* species isolated from grassland soils sample collected in Obanla, FUTA

Probable organism	Gram stain	CAT	CIT	GLU	SUC	FRU	MAL
Streptomyces rimosus	+	+	+	+	−	+	+
Streptomyces albus	+	+	+	−	−	−	+
Streptomyces fumosus	+	+	−	−	−	−	+
Streptomyces riseoflavus	+	+	−	−	−	−	+
Streptomyces spiralis	+	+	+	+	−	−	+
Streptomyces flavogriseus	+	+	−	−	+	−	+
Streptomyces aureofaciens	+	+	−	−	+	−	+

CAT catalase, *CIT* citrate, *GLU* glucose, *SUC* sucrose, *FRU* fructose, *MAL* maltose

Table 4 Zone of inhibition of the *Streptomyces* species isolated from grassland soils sample on chitin mineral salt medium

Isolates	Clearance zones(mm)
Streptomyces rimosus	1.81 ± 0.2^{bc}
Streptomyces flavogriseus	1.42 ± 0.08^{b}
Streptomyces albus	2.00 ± 0.15^{c}
Streptomyces riseoflavus	1.67 ± 0.22^{b}
Streptomyces fumosus	0.00 ± 0.00^{a}
Streptomyces spiralis	0.00 ± 0.00^{a}
Streptomyces aureofaciens	0.00 ± 0.00^{a}

for the different for metal ions. The culture filtrate was harvested for every 6 h each for the following metal ions; KCl, $CaCl_2$, $FeCl_3$, NaCl, $MgCl_2$, $ZnSO_4$ and $MnCl_2$, then the enzyme activity was studied for 36 h (Gao et al. 2012).

Purification of crude chitinase

Dialysis of the crude chitinase

The crude enzyme was precipitated by adding ammonium sulphate up to 60% saturation. The mixture was centrifuged at 10,000 rpm for 15 min at 4 °C. The precipitate was then re-suspended in 50 mM Tris–HCl, pH 8.0and dialyzed against the same buffer overnight at 4 °C with three buffer changes. To get concentrated enzyme free from salt and metal ions, Dialyzed enzyme solution was concentrated with 4 M sucrose solution. Then the sample in dialysis bag is used as protein source for further work (Matsumae et al. 2000).

Table 5 Purification steps of crude chitinase produced from *Streptomyces albus*

Steps	Vol	Chittinase activity µmol/min/ml	Protein conc. mg/ml	Total chittinase activity µmol/min	Total protein mg	Specific activity µmol/min/mg	Yield %	Purification fold
Crude extract	150	28.443	19.228	4266.467	221.894	1.479	100	1
Ammonium ppt	40	73.753	36.524	2950.1	80.772	2.019	69.146	1.365
Ion exch	26	95.210	42.747	2475.449	57.910	2.227	58.021	1.506
Gel filtration	16	11.876	13.991	190.02	13.581	0.848	4.454	0.574

Table 6 Inhibitory zones of chitinase (crude enzyme) against pathogenic fungi

Streptomyces species/chemical fungicide	Test fungi/zones of inhibition (mm)				
	Magnaporthe oryzae	*Fusarium graminearum*	*Rhizoctonia solani*	*Puccinia* species	*Botrytis cinerea*
Streptomyces rimosus	5.12 ± 1.45^{c}	4.23 ± 0.22^{c}	2.17 ± 0.17^{b}	4.25 ± 2.20^{c}	1.31 ± 0.08^{b}
Streptomyces albus	10.19 ± 0.75^{c}	10.74 ± 0.29^{d}	9.22 ± 2.07^{d}	10.06 ± 3.52^{d}	8.10 ± 1.25^{d}
Streptomyces riseoflavus	0.56 ± 0.47^{a}	4.04 ± 0.04^{c}	5.01 ± 2.01^{c}	3.21 ± 1.02^{c}	4.28 ± 2.10^{c}
Streptomyces flavogriseus	2.80 ± 0.22^{b}	2.11 ± 0.03^{b}	1.42 ± 0.09^{b}	1.22 ± 0.11^{b}	1.60 ± 0.56^{b}
Mancozeb	17.14 ± 0.15^{d}	18.95 ± 0.07^{e}	13.09 ± 0.34^{e}	12.21 ± 0.12^{d}	10.78 ± 0.26^{d}
Negative control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are expressed in mean ± SEM. $P < 0.05$ was considered to be statistically significant

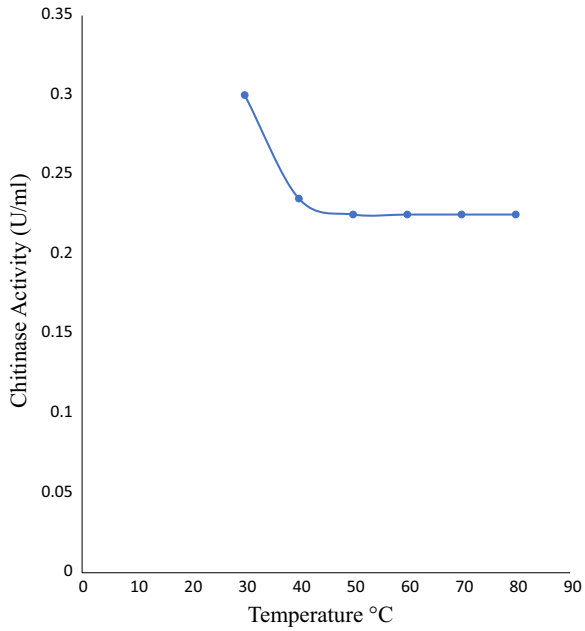

Fig. 2 Chitinase producing activity of *Streptomyces albus* at different temperature

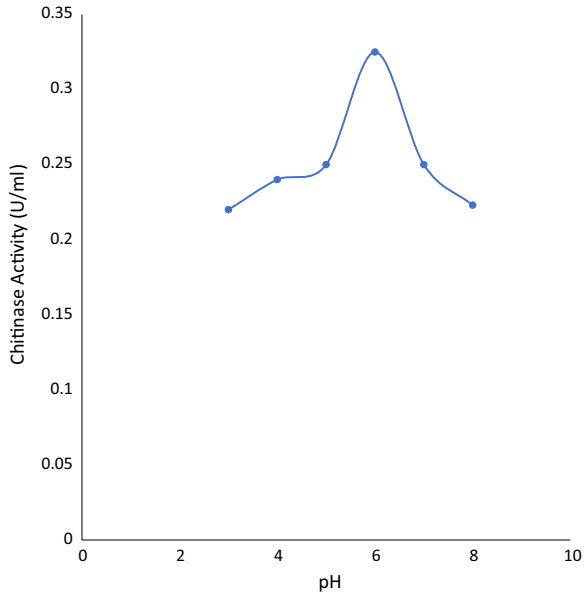

Fig. 3 Chitinase producing activity of *Streptomyces albus* at different pH

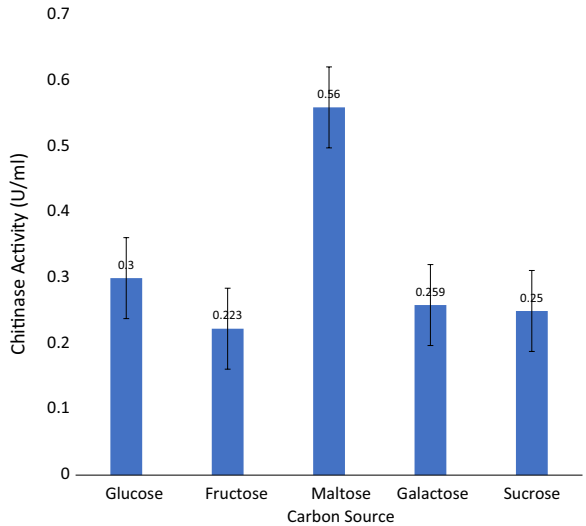

Fig. 4 Chitinase producing activity of *Streptomyces albus* at different carbon sources

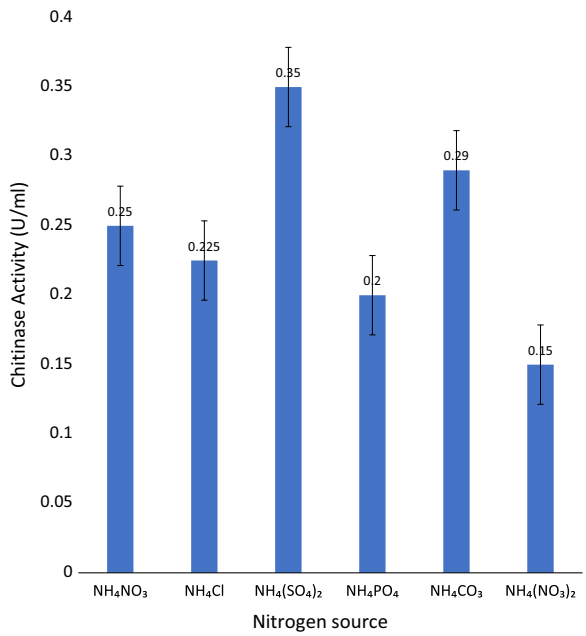

Fig. 5 Chitinase producing activity of *Streptomyces albus* at different nitrogen sources

Ion exchange chromatography

The dialyzed sample was washed with the acetate buffer after it was loaded on a pre-equilibrated DEAE-cellulose column chromatography (2.6 × 20 cm). The proteins were eluted in a stepwise gradient on NaCl (0–1.0 M) at a flow rate of 24 ml/h and the absorbance was read at 280 nm in a spectrophotometer. The fractions with chitinase activity were combined, dialyzed against the acetate buffer (50 mM, pH 5.0), and concentrated by lyophilization (Matsumae et al. 2000).

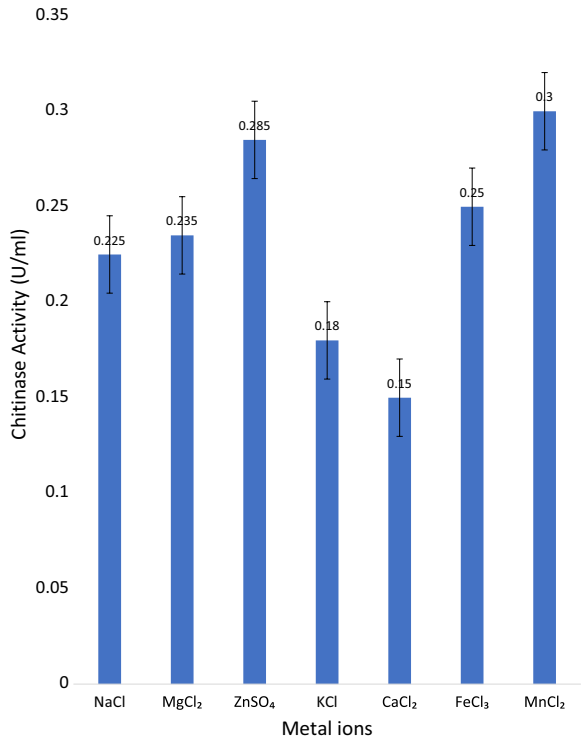

Fig. 6 Chitinase producing activity of *Streptomyces albus* at different metal ion

Sephadex G-100 Gel Filtration Chromatography

After dialysis was loaded onto a column of Sephadex (G-100 (1.5 × 24 cm) (St Louis, Sigma-Aldrich, MO)) equilibrated with phosphate buffer (20 mM, pH 6.5), the protein pellet obtained. The column was eluted at a flow rate of 60 ml/h with a1:1 volume gradient from 0.1 to 1 M NaCl in the same buffer. The elution fraction (2.5 ml) was collected and assayed for chitinase activity and those fractions which showed high activity was collected and used for further analysis (Matsumae et al. 2000).

Statistical analysis

Data are presented as mean ± standard error (SE). Significance of difference between different treatment groups was tested using one-way analysis of variance (ANOVA) on SPSS window 8 version 21 software. For all the tests, the significance was determined at the level of $P < 0.05$.

Results

Physicochemical analysis of soil samples collected from FUTA

The result indicated that the pH of grassland soil ranges between 5 and 6.50. The organic carbon content ranges between 1.60 and 4.31, the other physicochemical analysis results are shown in Table 1.

Morphology and characteristics of *Streptomyces* species

As shown in Table 2, all the *Streptomyces* have a distinct yellow colour except for *Streptomyces albus*. Some of the isolates obtained were hard, chalky while some were leathery and dry. Microscopic study showed that cell arrangement was either scattered or clustered. All the *Streptomyces* isolates formed spores ranging from round to oval form and they all have rough textures with flat elevations. Seven species of *Streptomyces* were isolated from grassland; *Streptomyces rimosus*, *Streptomyces albus*, *S. flavogriseus*, *S. riseoflavus*, S. *spiralis*, *S. aureofaciens,* and *S. fumosus* which grew well on starch casein agar except *S. spiralis* which grew moderately. Figure 1 shows growth pattern of the *Streptomyces* isolates that were able to produce chitinase at different incubation period.

Biochemical characteristics of the *Streptomyces* species isolated from the soil samples

(Table 3) All the *Streptomyces* species isolated from grassland soils were Gram positive, catalase positive and they all utilized maltose.

Screening of *Streptomyces* isolated for chitinase production

Streptomyces albus was found to have the widest zone of inhibition on chitin mineral salt medium after 7 days, the zones measured were recorded in Table 4.

Table 5 shows the purification steps of crude chitinase from *Streptomyces albus.*

Antifungal activity of the crude enzyme

Out of the four *Streptomyces* species tested for, the culture filtrate of *Streptomyces albus* was active against all the selected pathogenic fungi namely *Magnaporthe oryzae*, *Fusarium graminearum*, *Rhizoctonia solani*, *Puccinia* species and *Botyrtis cinerea*. However, *Fusarium graminearum* was also susceptible to the activity of *Streptomyces flavogriseus*, *Streptomyces rimosus* has the least inhibitory activity. Mancozeb inhibited all the test fungi and it was more effective than culture filtrate of the isolates. The negative control had no effect on the test isolates (Table 6).

Effect of physicochemical parameters on chitinase production

Figures 2, 3, 4, 5 and 6 show the Chitinase producing activity of *Streptomyces albus* at different temperature, pH, carbon sources, nitrogen sources and metal ion.

Chitinase production was initially found after 24 h of incubation and reached maximum levels after 6 days of cultivation. Chitinase synthesis was found to decline as the incubation time increased. Maximum yield of chitinase by *Streptomyces albus* was found after 6 days of

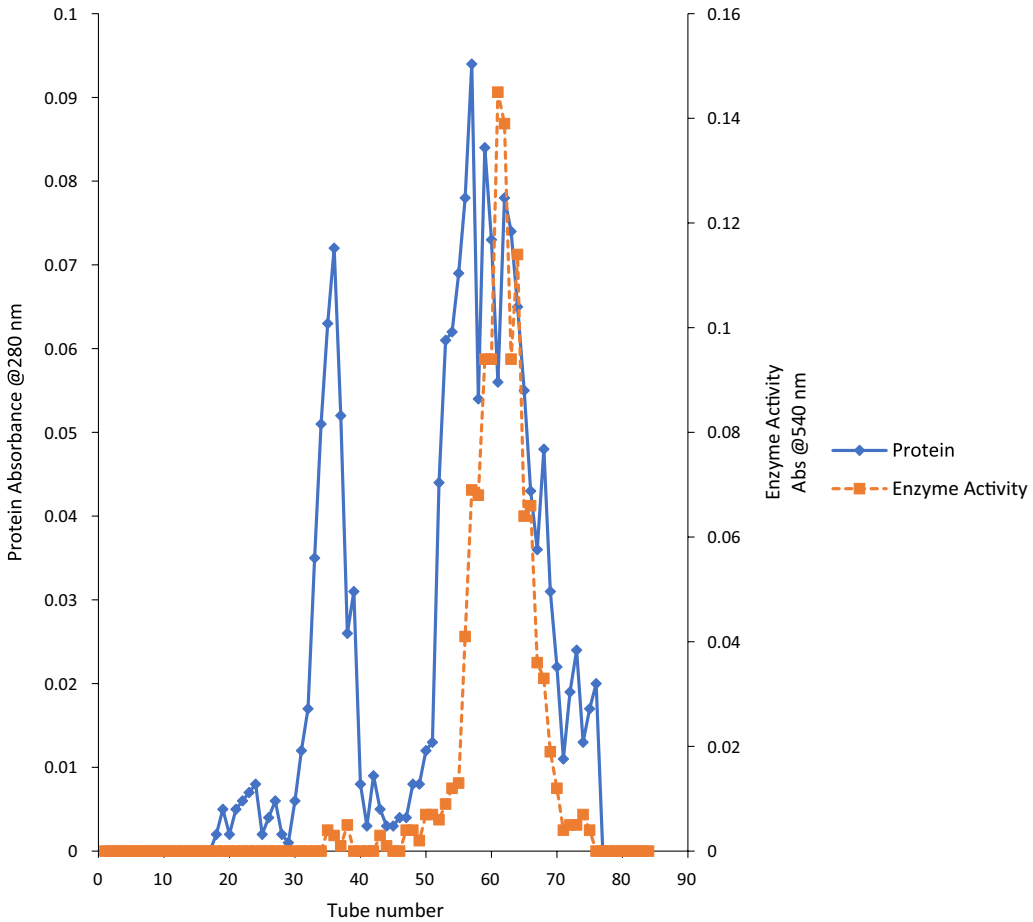

Fig. 7 Elution profile of chitinase produced by *Streptomyces albus* on gel filtration

incubation. Chitinase production varied as medium pH changed between 4 and 8. A high level of chitinase activity was observed in the culture medium with pH 6 and optimum temperature for chitinase production was at 30 °C. Enhanced chitinase production was found in medium enriched with maltose, also, there was an increase in chitinase production in medium enriched with NH_4 $(NO_3)_2$ and $MnCl_2$ metal ion. Figure 7 shows the elution profile of chitinase produced by *Streptomyces albus* on gel filtration. Figure 8 shows Elution profile of chitinase produced by *Streptomyces albus* on ion exchange chromatography.

Discussion

Streptomyces rimosus, S. albus, S. flavogriseus, S. fumosus, S. riseoflavus, S. spiralis and S. aureofaciens were isolated from grassland soils in FUTA. It can be observed that there is greater number of *Streptomyces* in the dry soil than all other sources; this has been linked to the ability of *Streptomyces* to bridge soil spaces and form spores using mycelium, together with their ability to use a broad-Spectrum of suitable inorganic nitrogen and carbon sources, enable long term survival. This is confirmed by the work of Ekundayo et al. (2014).

Chitinases are group of enzymes that decompose chitin and are produced by a diverse range of life forms. Among the microorganisms, 90 to 99% of the chitinolytic populations are actinomycetes (Vijay and Shyam 2006). The chitinase produced by microorganisms is inducible in nature. It is influenced by medium components such as carbon and nitrogen sources, substrates and parameters like pH, temperature and incubation period (Dahiya et al. 2006). Colloidal chitin (0.4%) induced more chitinase production on sixth day when compared to other substrates and other concentrations of colloidal chitin unlike the results of Kavi Karunya et al. (2011) they observed maximum production with 0.3% of colloidal chitin. The role of temperature in inducing the production of extracellular chitinase is significant. All *Streptomyces* isolates screened for chitinase production produced the enzyme in varying amounts, similar result was observed

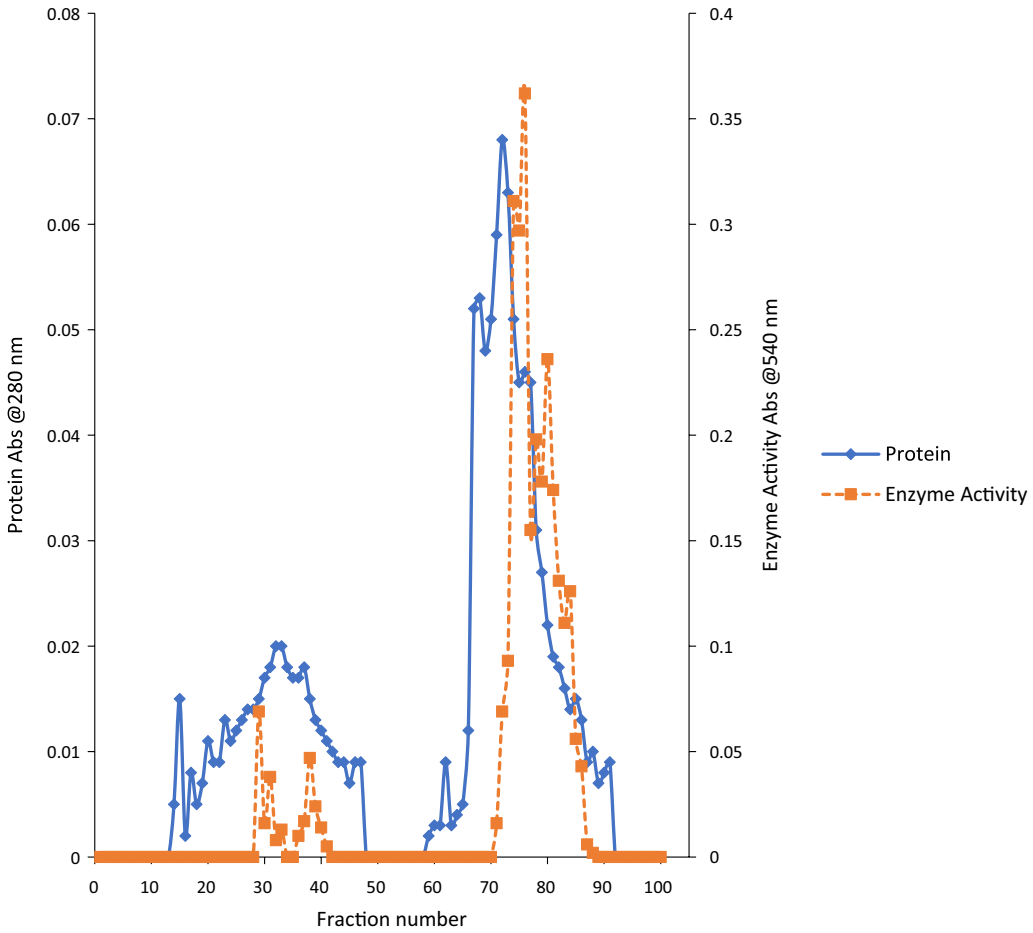

Fig. 8 Elution profile of chitinase produced by *Streptomyces albus* on ion exchange chromatography

by Narayana and Vijayalakshmi (2009). The qualitative screening showed *Streptomyces albus* with the highest zone of inhibition. *Streptomyces albus* isolated from soils was screened for maximum production of chitinase by observing clear zone in Petri dishes and was selected for further studies.

According to the results obtained chitinase activity of *Streptomyces albus* was found to be most active at temperature 30^0 C. There was a decrease in chitinase production at 50^0 C, 60^0 C, 70^0 C and 80^0 C, the production was extremely reduced. This indicates that the organism's activity decreased with increase in temperature, pH 6 was extremely favourable, the production was also lowered at acidic pH which means that the organism produces chitinase best in an alkaline pH. Among the carbon sources tested, maltose induced maximum chitinase production on sixth day and for the nitrogen source; NH_4 $(SO_4)_2$ and metal ion; $MnCl_2$.

Out of four *Streptomyces* species tested for, the culture filtrate of *Streptomyces albus* was active against all the selected pathogenic fungi namely *Magnaporthe oryzae*, *Fusarium graminearum*, *Rhizoctonia solani*, *Puccinia* species and *Botyrtis cinerea*. However, *Fusarium graminearum* was also susceptible to the activity of *Streptomyces flavogriseus* and Streptomyces *rimosus* has the least inhibitory activity. Similar result was reported by Tirumurugan et al. (2015) in which antagonistic activity of chitinase produce by isolate *Streptomyces sp* was effective in restricting the radial growth of phytopathogens such as *F. oxysporum* and *Alternaria sp.* At the same concentration mancozeb inhibited all the test fungi and it was more effective than culture filtrate of the isolates. The negative control had no effect on the test isolates. This may be as a result of refined materials used in the production of antibiotics. Oladunmoye (2007) reported that antibiotics have high degree of purity; conventional antibiotics and other pharmaceutical products are usually prepared from synthetic materials.

Production of chitinase and antifungal ability can always be correlated since; the cell wall of fungi is 80%

chitin. Thus, they can be considered as effective biocontrol agents of plant pathogens (Kamil et al. 2007) Chitinases are involved in the defense mechanism of plants and vertebrates. Human serum chitinase activity has recently been described, the possible role suggested is a defense against fungal pathogens (Ramesh et al. 2011; Zervakis and Papadopoulou 2010). Most of these chitinases are induced in vegetative plant organs by infection but some are also present in seeds (Ramyasmruthi et al. 2012). Soil bacteria also produce chitinolytic enzymes.

Conclusions

Streptomyces albus isolated from the grassland soil sample of FUTA was evaluated for chitinase production. Maximum chitinase production was observed on day 6 of incubation, pH 6, temperature 30 °C, culture medium with colloidal chitin, maltose and $MnCl_2$ metal ion influenced chitinase production. Crude chitinase exhibited antifungal activity against wide range of pathogenic organisms. The result concluded that *Streptomyces albus* has the highest ability to produce chitinase which can act as antifungal for degradation of the cell wall of many phytopathogenic fungi. The test isolate can be exploited for large scale production of chitinase.

It is recommended that grassland would be looked more into for potential Streptomyces species with chitinase and antifungal activities; this could be an important source of biocontrol agents that can either serve as substitute or enhancement for commercial fungicides. Further research is required to boost this potential. It is therefore suggested that broader clinical studies be done to measure this novel advantage, in order to convert it to improved clinically applicable outcomes.

Abbreviations

FUTA: Federal University of Technology Akure; PDA: Potato dextrose agar; H_2SO_4: Hydrogen sulphate; $K_2Cr_2O_7$: Potassium dichromate.

Acknowledgements

The Department of Microbiology, School of Life Sciences, Federal University of Technology Akure, Akure Nigeria, is acknowledged for providing the basic infrastructure for carrying out the research work.

Author contributions

AEF, TAI and OBO: conceived and designed the experiments, contributed to sample preparation, carried out the experiment, processed the experimental data, performed the analysis, wrote the manuscript, drafted the manuscript, designed the figures and contributed to the interpretation of the results. FOE: involved in planning and supervised the work, contributed to the interpretation of the results, other contribution. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declare that he has no competing interest.

Received: 26 January 2022 Accepted: 29 March 2022
Published online: 08 April 2022

References

Association of Official Analytical Chemists (AOAC) (2007) Official methods of analysis, 18th edn. Published by Inc.; 2200 Wilson Boulevard; Arlington, Virginia 222013301, USA.

Bhattacharya DA, Gupta RK (2007) Bacterial chitinases: properties and potential. Crit Rev Biotechnol 27(1):21–28

Bussink AP, Aerts JM, Boot RG (2007) Evolution of mammalian chitinase (-like) members of family 18 glycosyl hydrolases. Genetics 177:959–970

Dahiya N, Tewari R, Hoondal G (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 71:773–782

Duzhak AB, Panfilova ZI, Vasyunina EA (2012) Isolation and characterization of chitinase from wild type V-10 and mutant M-1 *Serratia marcescens* strains. Appl J Biochem Microbiol 36(4):344–352

Ekundayo FO, Oyeniran KA, Adedokun AD (2014) Antimicrobial activities of some streptomyces isolated from garden soil samples and fish pond water in FUTA. J Biolaogical Sci 22:21–29

Gao L, Xu H, Li J, Liu Z (2012) Optimization of *Serratia marcescens* chitinase production. J Microb Biotechnol 31:525–530

Ibisanmi T, Aribisala O (2022) Evaluation of antioxidant, phytochemicals and antibacterial potential of *Mormordica charantia* (linn) against pathogenic bacteria isolated from ready to eat food sold in akure metropolis, nigeria. Bact Emp 5(1):e389

Joel OF, Amajuoyi CA (2009) Determination of selected physiochemical parameters and heavy metals. J Appl Environ Sci 13(2):27–31

Kamil Z, Rizk M, Saleh M, Moustaf S (2007) Isolation and identification of rhizosphere soil chitinolytic bacteria and their potential in antifungal biocontrol. Glob J Mol Sci 2(2):57–66

Kavi Karunya S, Reetha D, Saranraj P, John D (2011) Optimization and purification of chitinase produced by *Bacillus subtilis* and its antifungal activity against plant pathogens. Int J Pharm Biol Arch 2(6):1680–1685

Kumar D, Gupta RA (2006) Biocontrol of wood rotting fungi. Indian J Biotechnolol 5:20–25

Matsumae H, Kafini Y, Shibatani T (2000) Purification and characterization of the chitinase from *Serratia marcescens,* Sr41-8000. J Ferment Bioeng 77:152–158

Narayana JP, Vijayalakshmi M (2009) Chitinase production by *Streptomyces* sp. ANU 6277. Braz J Microbiol 40:725–733

Oladunmoye MK (2007) Comarative evaluation of antimicrobial activities of leaf extract of *Mirabilis jalapa*, microbial toxins on some pathogenic bacteria. Trends Med Res 2(2):108–112

Olusola-makinde O, Olabanji OB, Ibisanmi TA (2021) Evaluation of the bioactive compounds of Vernonia amygdalina Delile extracts and their antibacterial potentials on water-related bacteria. Bull Natl Res Centre 45(1):191

Olutiola PO, Famurewa O, Sonntag HG (2000) An introduction to general microbiology; a practical approach, 2nd edn. Heldelberg, Nigeria. pp 51–68, 71, 131

Ramesh R, Joshi A, Ghanekar P (2011) *Pseudomonads*: major antagonistic endophytic bacteria to suppress bacterial wilt pathogen, *Ralstonia*

solanacearum in the eggplant (*Solanum melongena* L.). World J Microbiol Biotechnol 25:47–55
Ramyasmruthi S, Pallavi S, Pallavi K, Tilak Y, Srividya S (2012) Chitinolytic and secondary metabolite producing *Pseudomonas fluorescens* isolated from *Solanaceae* rhizosphere effective against broad spectrum fungal phytopathogens. Asian J Plant Sci Res 2(1):16–24
Sadasivam S, Manickam A (2014) Biochemical methods, 2nd edn. New Age International Publishers, pp 116–117
Shahidi F, Abozaytoun R (2005) Chitin, chitosan and co-products: chemistry, production, applications and health effects. Adv Food Nutr Res 49:93–135
Tirumurugan A, Sankari D, Vijayakumar (2015) Screening of chitinase production and antifungal activity of streptomyces SP. ACT7 from east coast region, south India. Int J Pharm Pharm Sci 7(5):38–41
Vijay B, Shyam S (2006) Characterization of chitinases from microorganisms isolated from Lonar Lake. Indian J Biotechonol 5:357–363
Watve M, Tickoo R, Jog M, Bhole B (2001) How many antibiotics are produced by the genus *Streptomyces*? Arch Microbiol 176:386–390
Wu YJ, Cheng CY, Li YK (2009) Cloning and expression of chitinase A from *Serratia marcescens* for large-scale preparation of N, N-diacetyl chitobiose. J Chin Chem Soc 56(4):688–695
Zervakis GI, Papadopoulou KK (2010) Antagonistic bacteria of composted agro industrial residues exhibit antibiosis against soil-borne fungal plant pathogens and protection of tomato plants from *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Plant Soil 333:233–247
Zhang H, Huang X, Fukamizo T, Muthukrishnan S (2002) Site-directed mutagenesis and functional analysis of an active site tryptophan of insect chitinase. Insect Biochem Mol Biol 32(11):1477–1488. https://doi.org/10.1016/S0965-1748(02)00068-1

Publisher's Note