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Full Length Research Paper

Purification and molecular characterization of chitinases from soil actinomycetes

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Microbial extracellular chitinases are used in agriculture as effective biocontrol agents and in waste degradation, pharmaceutical and food industry. Actinomycetes are widely tapped group for production of extracellular chitinases. In the present study, approximately 260 actinomycetes were isolated from various ecological habitats was subjected to primary analyses and screened for production of chitinase by plate assay method. Diameter of zones of hydrolysis ranged from 8 to 16 mm. Based on the results, isolates 130, 194, 184, NRRLB 24916 (*Streptomyces mexicanus*) and NRRLB 16746 (*Streptomyces albidoflavus*, positive control) were selected for secondary screening and purification. Enzyme activity was estimated in crude cell free extract and partially purified samples. Activity ranged from 7.16 to 14.12 IU/ml (in crude extracts) and 12.1 to 23.10 IU/ml (in partially purified samples). In case of highest chitinase producing isolate 130, effect of various fermentation conditions (pH, temperature and substrate concentration) was studied in crude extract. Furthermore, complete purification of isolate 130 was done by column chromatography and the activity in purified fraction was found to be 32.12 IU/ml. The K_m and V_{max} values of the purified fractions for isolate 130 were 2.11 µg/ml/min and 53.11mg/ml respectively. This shows that the enzyme has high affinity for the substrate. SDS gel electrophoresis of the purified fraction showed presence of single band of approximately 65 to 70 kDa. Analyses of purified chitinase were done using MS/MS technique. N-terminal sequence corresponded to chitinase, the gene encodes a protein of 453 amino acid residues. Comparison of deduced amino acid sequence to other chitinases in the database indicated that enzyme showed 70% similarity with chitinase from *Streptomyces plicatus* and belongs to glycoside hydrolase family 18. Homology modeling showed that the enzyme was folded into a domain of $(\alpha/\beta)_8$ barrel structure. Identification of secondary structure was done by CD spectroscopy. Isolate 130 was capable of degrading biodegradable wastes such as crustacean shells.

Key words: Actinomycetes, extracellular chitinase, primary screening, secondary screening, purification, MS/MS analyses, homology modelling, protein structure, biodegradation.

INTRODUCTION

Chitinases are enzymes that hydrolyse the β-1,4 linkage of N-acetyl glucosamine present in chitin chains. Due to vast availability, low cost, high stability and productivity, microbial chitinase is attaining prominence for waste management, pest control in agriculture, and human health care (Das et al., 2015, 2016; Rathore and Gupta, 2015). Improving the yield of the enzyme and consequent cost reduction depends on the selection of strains, optimization of fermentation conditions, genetic improvement of strains and kinetic studies of enzyme (Andualem,

2014, Fentahun and Kumari, 2017).

Classical approaches in extracellular chitinase characterization include isolation and screening of bacterial groups for their ability to produce chitinase enzyme. Wide range of bacteria in the environment is efficient producers of extracellular chitinases. Actinomycetes are well known producers of chitinases (Kumar and Singh, 2013; Mohanta, 2014). Researchers are exploring diverse untapped habitats in an attempt to discover new actinomycete strains for producing novel chitinase enzyme, having applications in various industries (Gurung et al., 2013; Anbu et al., 2015). The next step is fine-tuning of fermentation processes, aimed specifically at the production of purified, well characterized enzymes from selected strains on a large scale (Bui, 2014; Yassien et al., 2014; Kumar et al., 2016).

Developments in biotechnology, such as protein engineering and directed evolution, revolutionized the probability of producing novel enzymes by introducing or modifying the capability of specific genes (Sandgren et al., 2013; Chen et al., 2014; Walia et al., 2015; Enkhbaatar et al., 2016; Castillo et al., 2016). Advances in biotechnology is providing a plethora of enzymes displaying new activities and having adaptability to a range of conditions leading to their increased adoption for industrial purposes (Diaz et al., 2004; Brzezinska et al., 2013; Sriyapai et al., 2013; Munar et al., 2013).

With the advancement in industrialization and urbanization, dumping of enormous amount of materials as wastes has become a nuisance (Akhtar, 2014). Conventional techniques for management of biodegradable wastes are becoming increasingly expensive and energy inefficient. Secondly, the chemical treatment methods are hazardous to both environment and humans (Wilts et al., 2016). As a result, search for more sustainable approaches becomes important for conversion of wastes into by-products that can be directly used for commercial purposes (Benhabiles et al., 2013). One such approach is bioremediation, which makes use of the enzymatic potential of micro-organisms present in the environment for effectual degradation of biodegradable wastes (Karigar and Rao, 2011). Waste contains substances like cellulose, starch and lignin, which are susceptible to microbial degradation. It is an eco-friendly process which decomposes the wastes into useful raw materials (Ilangumaran et al., 2017).

The purpose of the current investigation was to screen actinomycete isolates for production of chitinase enzyme followed by optimization of fermentation parameters (pH, temperature and substrate concentration) for improving the yield of the enzyme. Next step was the use of molecular techniques to characterize the enzyme for identification of the type of protein and active site residues. This in turn will form the basis for protein engineering of enzymes and at a later stage will allow specific manipulation of the associated amino acids for desired enzymatic properties. From the application point view, role of the selected isolate in biodegradation of chitin present in crustacean/shrimp waste samples was also studied.

MATERIALS AND METHODS

Collection of soil samples and isolation of actinomycetes

Soil samples were collected from various ecological habitats (Table 1) and actinomycete isolates were isolated by plate dilution method. Single isolates were purified by restreaking on yeast extract-malt extract agar plates and stored as glycerol stocks at -20ºC/-80°C deep freezers (Vestfrost/Sanyo, Model- MDF-U55V) (Khanna et al., 2011; Solanki et al., 2011).

Primary screening of isolates for production of chitinase

Colloidal chitin was prepared by adding 5 gof chitin powder (HiMedia) in 60 ml conc. HCl. The mixture was then kept at room temperature overnight with vigorous stirring. It was then filtered through Whatman no.1 filter paper and the residue remaining on filter paper was added to 200 ml of 95% ethanol and stirred vigorously overnight. The mixture was centrifuged at 5,000 rpm for 20 min at room temperature. Precipitate was transferred to a glass funnel containing Whatman no. 1 filter paper and washed with sterile distilled water until the pH of the sample became neutral. The chitin that was retained on filter paper was removed and stored in dark at 4°C (Priya et al., 2011). Chitin agar medium supplemented with 1% colloidal chitin (pH 8.0) was prepared and autoclaved. The cultures were spot inoculated on the medium plates and incubated at 28°C for 14 to 21 days until the zone of chitin hydrolysis was observed around the isolates. The clear zone diameter was measured by subtracting the inoculum size from the total zone diameter used as an indicator for chitinase activity (Gadelhak et al., 2005; Priya et al., 2011).

Secondary screening or quantitative analyses of chitinase activity

Strains showing maximum zones of clearance during primary screening were selected for subsequent secondary screening (Das et al., 2015, 2016).

Standard inoculum preparation under submerged fermentation process

Isolates showing maximum zone of clearance were inoculated in 25 ml of 148G medium (composition (g/L^{-1}) Glucose 22, Beef extract 4, Bacto peptone 5, Yeast extract 0.5, Tryptone 3, NaCl 1.5, (pH 7.5) (Schupp and Divers, 1987) respectively. The flasks were incubated at 28°C on a rotary shaker (New Brunswick Scientific, Excella E24R) at 200 rpm for 5 days. After incubation,

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Table 1. Number of isolates from different ecological habitats.

S/N	Habitat
1	Agricultural soils Agricultural soil, Dhanaura, Uttar Pradesh Agricultural soil, Yamuna Bank, Delhi Agricultural soil, Nainital, Uttarakhand Agricultural soil, Kashipur, Uttarakhand
2	Industrial soils Sugar Plant, Dhanaura, Uttar Pradesh Chemical Plant, Faridabad
3	Landfill soils Dumping site, Sarai Kale Khan, Delhi
4	River/lake soils Yamuna Bank, Delhi Lake soil, Purana Quila, Delhi
5	Diversity park soils Great Himalayan National Park, Teerthan Valley, Himachal Pradesh

absorbance was measured at 600 nm in each case. Culture broths were serially diluted as follows in 148G medium going up to a dilution of 10^{-7}.

(a) 100 µl of culture broth + 900 µl of 148G medium (10^{-1} dilution)
(b) 100 µl of 10^{-1} dilution + 900 µl of 148G medium (10^{-2} dilution)
(c) 100 µl of 10^{-2} dilution + 900 µl of 148G medium (10^{-3} dilution)
(d) 100 µl of 10^{-3} dilution + 900 µl of 148G medium (10^{-4} dilution)
(e) 100 µl of 10^{-4} dilution + 900 µl of 148G medium (10^{-5} dilution)
(f) 100 µl of 10^{-5} dilution + 900 µl of 148G medium (10^{-6} dilution)
(g) 100 µl of 10^{-6} dilution + 900 µl of 148G medium (10^{-7} dilution)

The respective dilutions were plated on Yeast Extract Malt Extract medium (YM) and incubated at 28°C till the appearance of colonies. CFU's/ml were then calculated (El-Sersy et al., 2010; Shanmugapriya et al., 2012; Shaikh et al., 2013). The inoculum having an average viable count of 10^5 to 10^7 CFU's/ml was transferred to production broth (Chitin broth) (Tweddell et al., 1994). The respective media flasks were incubated at 28°C for 5 to 6 days on rotary shaker at 200 rpm.

Estimation of chitinase activity in culture broths (crude enzyme)

After incubation, culture broths were centrifuged at 10,000 rpm for 10 min at 4°C and the cell free supernatants were used as a source of crude enzyme. Chitinase activity was measured in each case, using p-dimethylaminobenzaldehyde (p-DMAB) method (Tweddell et al., 1994). Culture filtrate (0.5 ml) was added to 1.5 ml of colloidal chitin (10 mg/ml) prepared in 50 mM sodium acetate buffer (pH 6.8) in a test tube. To this, 0.1 ml potassium tetraborate was added and incubated at 100°C for 3 min. Tubes were cooled under tap water and 3 ml p-DMAB reagent was added and incubated at 36 to 38°C for 20 min. Tubes were cooled and OD was recorded at 585 nm using spectrophotometer (Elico, Model-SL-160). By using a calibration curve for N-acetylglucosamine, enzyme activity (U = 1 unit of chitinase) is defined as the amount of enzyme that releases 1 µmol of NAGA/ml (Reissig et al., 1995; Gadelhak et al., 2005). Enzyme activity was calculated by using the formula:

$$\text{Enzyme activity (IU/ml/min)} = \frac{\text{Concentration of NAGA} \times \text{dilution factor}}{\text{Time of incubation (min)} \times \text{volume of enzyme}}$$

Where,

$$\text{NAGA concentration} = \frac{\text{Actual absorbance (OD)}}{\text{Slope from graph}}$$

and actual OD = Test OD- (Enzyme blank OD + Substrate blank OD).

Estimation of protein content in cell free culture extract

Protein concentration in crude enzyme was determined by Lowry's method with BSA (Bovine serum albumin) as a standard (Lowry et al., 1951; Das et al., 2016).

Optimization of fermentation parameters (pH, temperature and substrate concentration) for enzyme production in crude extract

Various culture conditions like pH of medium (6 to 9), incubation temperature (30 to 55°C) and substrate concentration (0. 25 to 2.5%; w/v) were optimized for enhanced production of enzyme in submerged fermentation process (Kuddus and Ahmad, 2013; Karthik et al., 2015).

Statistical analyses of enzymatic activity using SPSS software

Data obtained after optimization of fermentation conditions (pH, temperature and substrate concentration) for isolate 130 was statistically analysed using one way ANOVA and multiple comparison test (Post-Hoc test) at significance level of $p < 0.05$. Both the tests were performed by using IBM SPSS Statistics 19 software (Gangwar et al., 2016).

Purification of enzymes and enzyme assay of purified products

Ammonium sulphate saturation and dialysis for chitinase enzyme

Crude extracts from the highest enzyme producers were subjected to partial purification. Crude enzyme solutions (250 ml) of the cultures were saturated by sequential addition of ammonium sulphate followed by dialysis and concentration. Enzyme activity and protein content were estimated in ammonium sulphate saturated, dialyzed and concentrated samples of isolates. The concentrated sample of isolate 130 was purified further by ion exchange chromatography using DEAE Bio-Gel A (Sigma) column (BioRad, 1.3×16 cm). Both unbound and bound fractions were tested for chitinase activity. Active fraction was used as purified enzyme solution. Enzyme activity as well as protein content was estimated in purified fractions (Karthik et al., 2015; Gangwar et al., 2016). The fractions were loaded on SDS-PAGE gel.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

For observing the protein profile of enzyme samples and determining the molecular weight of purified fractions, denaturing SDS- PAGE was used. A broad range prestained standard marker (BioRad, 15-210 kDa) was used in this case (Sowmya et al., 2012; Castillo et al., 2016).

Analysis of kinetic parameters

The effect of colloidal chitin on the chitinase activity of isolate 130 was evaluated by ranging the colloidal chitin concentration from 0.5 to 10 mg/ml. A Lineweaver-Burk plot was obtained by plotting 1/v against 1/s. Kinetic parameters (K_m and Vmax) were estimated by linear regression from Lineweaver-Burk plot (Nagpure and Gupta, 2013; Rahman et al., 2014).

Characterization of enzyme by molecular approach

Protein identification by mass spectrometry analyses

Slices of interest containing the protein bands from the semi-denaturing PAGE were cut and subjected to trypsin digestion. The final samples were submitted for MALDI-MS (ABI SCiex 5800 TOF/TOF system) and LC-ESI-MS/MS (Waters SYNAPT G2 with 2D nano ACQUITY system) analyses respectively. Raw data from both the analyses were transformed in mz data format and used to query non-redundant protein databases with a licensed version of MASCOT 2.1 (Matrix Science, Boston, USA) (Rashad et al., 2017).

Analysis of structure and catalytic sites

The peptide sequence of isolate 130 obtained after MS/MS analysis was used to investigate the structure and catalytic sites. The sequence was matched against the NCBI database and the FASTA protein sequence database. pBLAST was performed to study the homology with chitinases from related *Streptomyces* species.

For constructing the structure for the enzymes, N-terminal sequence of isolate 130 was submitted in SWISS-MODEL. The software searches the database for similarity with the query structure. The best suited structure was chosen by the SWISS-MODEL and based on the sequence of that model, a tentative three dimensional structure was made (Bienert et al., 2017).

Homology modeling approach was adopted for structural and functional study of catalytic site of isolate 130. The pBLAST analyses of amino acid sequence revealed the three dimensional structure of chitinase (Altschul et al., 1990). The most suitable high-resolution protein structure was selected as the template protein. Multiple sequence alignment of target and template protein sequences was performed by ClustalW. Modeling was performed with the help of MODELLER version 9.11 (Schwede et al., 2003; Arnold et al., 2007; Bienert et al., 2017). After aligning, the target and template sequences were used as input files in MODELLER, the software automatically calculates and gives a model containing all non-hydrogen atoms (Ubhayasekera and Karlsson, 2012; Hamid et al., 2013).

The final 3D model of chitinase was verified by the Structural Analysis and Verification Server (SAVES) which used PROCHECK software. Ramachandran Plot was constructed and analysed to check the percentage of residues present in most favored, allowed, generously allowed and disallowed regions respectively (http://nihserver.mbi.ucla.edu/SAVES). Compatibility of the 3D model with its own amino acid sequence (1D) was done by VERIFY 3D program. Identification of active sites that are responsible for substrate binding was done by using Catalytic Site Atlas (CSA) database of European Bioinformatics Institute (http://www.ebi.ac.uk/thorntonsrv/databases/CSA/) (Hoell et al., 2006).

Circular dichroism for protein secondary structure analysis

Circular dichroism measurements were performed on a Chirascan spectropolarimeter (Applied Photophysics). The CD spectra were recorded from 190 to 260 nm. The results were analyzed by Graphpad Prism processing software (Gangwar et al., 2016; Berini et al., 2017).

Role of extracellular enzymes in bioremediation of wastes

Collection of waste samples from various sites

Biodegradable waste samples such as fishery wastes (crustacean (prawns, shrimp and crab) shells) were collected (Table 2). Furthermore, raw wastes were pretreated to convert it into powdered substrates (Table 2) (Kumar and Sharma, 2017).

Primary and secondary screening of isolates for degradation of wastes

For screening of isolate 130 for its ability to degrade wastes, the culture was spot inoculated on chitin agar medium supplemented with 0.4% crustacean (prawns, shrimp and crab) shells powdered substrate. The clear zone diameter was measured by subtracting the inoculum size from the total zone diameter in order to observe the potential of isolate for degradation of chitin (Subramaniam et al., 2012; Setia and Suharjono, 2015). For quantitative screening, enzyme activity was estimated in crude extracts under solid state fermentation. Isolate was inoculated in 25 ml of 148G medium. The culture flask was incubated at 28°C on a rotary shaker at 200 rpm for 5 days. After incubation, absorbance was measured at 600 nm in each case. Culture broth was serially diluted in 148G medium going up to a dilution of 10^{-5}. The respective dilutions were plated on Yeast Extract Malt Extract medium (YM) and incubated at 28°C till the appearance of colonies. CFU's/ml were then calculated. Inoculum having an average viable count of 10^4 to 10^5 CFU's/ml was transferred in mineral salt broth supplemented with specific substrate and incubated at 28°C for 5 to six days on rotary shaker at 200 rpm.

Table 2. Details regarding collected biodegradable waste sample.

Waste material used as substrate	Collection site	Collected by	Pre-treatment procedure followed
Crustacean (prawns, shrimp and crab) shells	Ghazipur fish market, New Delhi	Undergraduate student, working under Delhi University Innovation Project)	Crustacean shells were treated using chemical method.

Table 3. Clear zone produced by isolates due to production of chitinase.

Strain	Clear zone diameter (mm)
Isolate 130 (Sugar plant, Dhanaura, U.P.)	16
NRRLB 16746 *Streptomyces albidoflavus* (chitinase control)	13
Isolate 194 (Dumping site, Sarai Kale Khan, Delhi)	12
Isolate 184 (Chemical plant, Faridabad, Delhi)	10
NRRLB 24916 *Streptomyces mexicanus (*xylanase control)	8

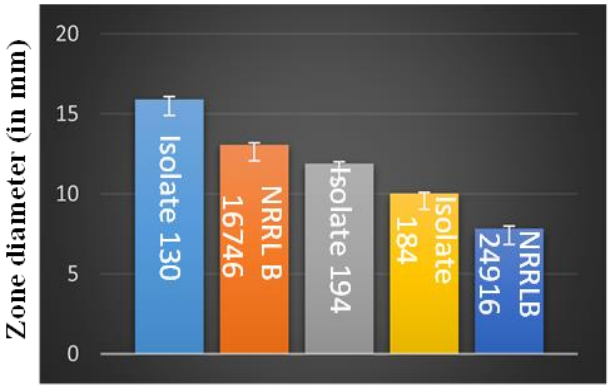


Figure 1. Comparison of activity of chitinase producing isolates (colloidal chitin was used as substrate). *Error bars presented mean values of ± standard deviation of triplicates of three independent experiments; SD determined was in the range of 0.05 to 0.1.

Enzyme activity was estimated in crude cell free extract by p-dimethylaminobenzaldehyde (p-DMAB) method. Culture filtrate (0.5 ml) was added to 1.5 ml of colloidal chitin (10 mg/ml) prepared in 50 mM sodium acetate buffer (pH 6.8) in a test tube. To this, 0.1 ml potassium tetraborate was added and incubated at 100°C for 3 min. Tubes were cooled under tap water and 3 ml p-DMAB reagent was added and incubated at 36 to 38°C for 20 min. Tubes were cooled and OD was recorded at 585 nm using spectrophotometer. By using a calibration curve for N-acetylglucosamine, enzyme activity (U = 1 unit of chitinase) is defined as the amount of enzyme that releases 1 µmol of NAGA/ml (Reissig et al., 1995; Gadelhak et al., 2005). Enzyme activity was calculated by using the formula as mentioned earlier (Hoang et al., 2011; Brzezinska et al., 2014).

RESULTS AND DISCUSSION

Collection of soil samples and isolation of actinomycetes

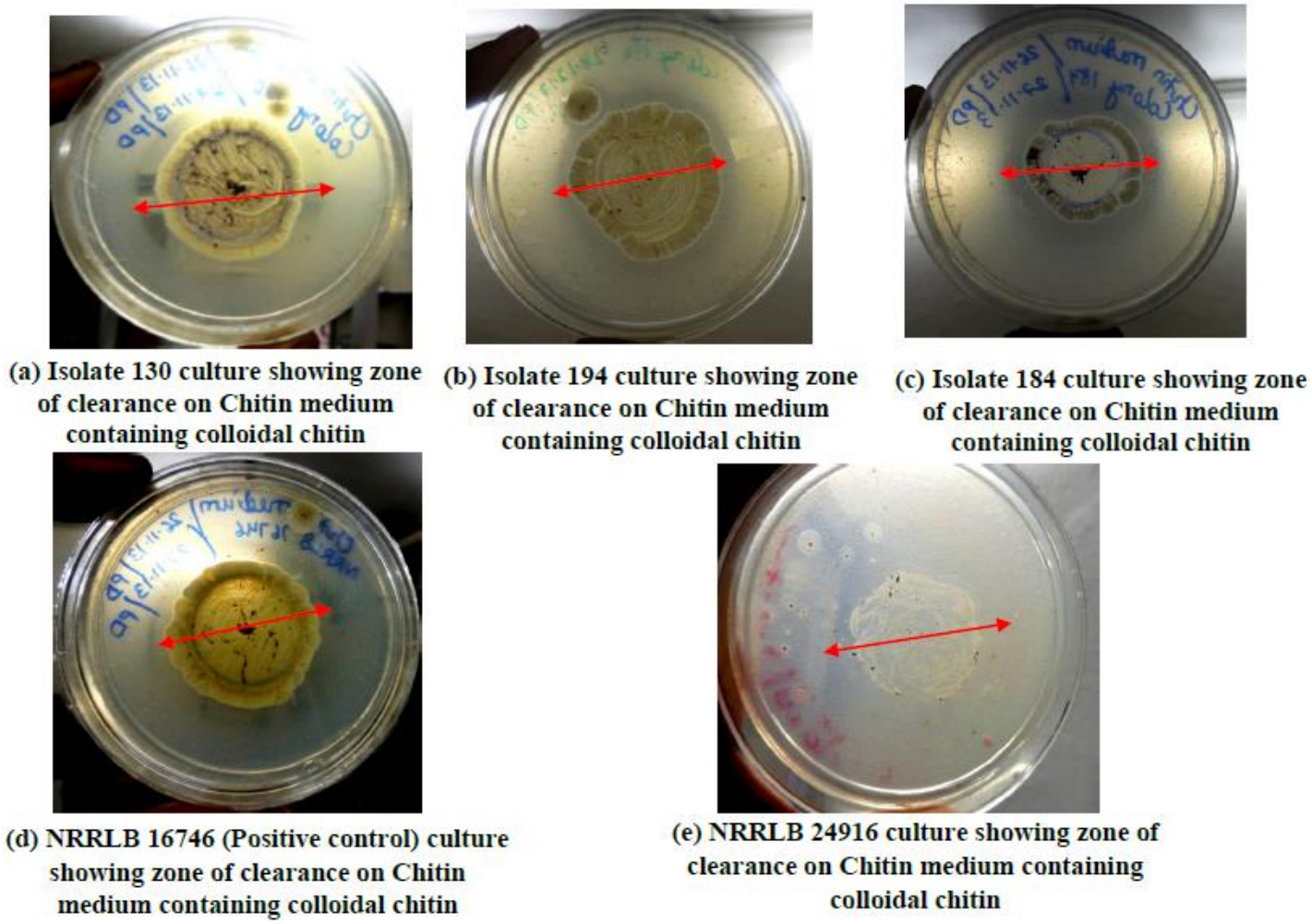
In the course of our study, a total of 260 actinomycete bacterial isolates were isolated. Isolates from diverse ecological habitats were subjected to primary screening to select actinomycetes capable of producing comercially important extracellular chitinase. Isolation of actinomycetes from varied ecological environments has also been reported by researchers for identifying producers of extracellular enzymes (Lekshmi et al., 2014; Mohanta, 2014).

Primary screening of isolates for production of chitinase

Among the 105 strains tested, 72% were found to be chitinase producers. Based on the results of primary screening, isolate no 130, 194, 184, *Streptomyces albidoflavus* (NRRLB 16746) and *Streptomyces mexicanus* (NRRLB 24916) that showed high chitinase activity and representing different ecological habitats were selected for further analyses. Comparison of chitinase activity of isolates is shown in Table 3 and Figure 1. The zone of clearance produced due to hydrolysis of chitin is shown in Figure 2.

The results obtained during primary screening were correlated to data reported in literature. Priya et al., 2011 isolated 36 *Streptomyces* strains and screened them for production of chitinase. Out of these, only 10 isolates showed clear zones of hydrolysis on colloidal chitin agar medium.

The strain VMCH2 showed the maximum zone of size 13 mm and in others, the zone size varied from 5 to 8 mm. A total of 58 actinomycetes were isolated from various habitats of Lucknow, India by Kuddus and Ahmad (2013). Isolates were then screened for their ability to produce chitinase enzyme. Based on the results, six isolates showing zones of clearance above 0.2 cm were selected for further studies. Similar work has been reported by Thirumurugan et al. (2015) and Wang et al.



(a) Isolate 130 culture showing zone of clearance on Chitin medium containing colloidal chitin

(b) Isolate 194 culture showing zone of clearance on Chitin medium containing colloidal chitin

(c) Isolate 184 culture showing zone of clearance on Chitin medium containing colloidal chitin

(d) NRRLB 16746 (Positive control) culture showing zone of clearance on Chitin medium containing colloidal chitin

(e) NRRLB 24916 culture showing zone of clearance on Chitin medium containing colloidal chitin

Figure 2. Plates showing zone of clearance of isolates due to production of chitinase enzyme.

Table 4. Chitinase enzyme activity and protein content in crude culture extract.

Culture	Absorbance at 585 nm	Concentration of NAGA (mg)	Protein content (mg/ml)	Protein content after equalization (mg/ml)	Enzyme activity (IU/ml)
Isolate 130	0.702	0.812	0.71	0.39	14.12
NRRLB 16746 (Positive control)	0.680	0.718	0.70	0.38	12.81
Isolate 194	0.542	0.598	0.55	0.35	11.6
Isolate 184	0.482	0.401	0.52	0.34	10.45
NRRLB 24916	0.29	0.212	0.33	0.33	7.16

(2015).

Secondary screening for chitinase activity

For quantitative analyses of chitinase activity in crude extracts, isolate 130, 184, 194 and *Streptomyces mexicanus* (NRRLB 24916) were selected. *Streptomyces albidoflavus* (NRRLB 16746) was taken as positive control for chitinase activity. The results are shown in Table 4.

The results obtained during secondary screening were compared to studies reported in literature. Chitinase activity in *Streptomyces* sp. strain A was estimated by

Table 5. Optimization of fermentation conditions (pH, temperature and substrate concentration) for highest chitinase producer (in crude), isolate 130 utilizing colloidal chitin as a substrate.

Cultures/ collection site	pH	Enzyme activity (*IU/ml)	Temperature (°C)	Enzyme activity (*IU/ml)	Substrate concentration (%)	Enzyme activity (*IU/ml)
Isolate 130 (Chemical Plant, Faridabad)	6.5	2.00	2	1.20	0.25	3.12
	7.0	5..89	25	16.14	0.5	14.22
	7.5	7.56	3	17.56	1	17.68
	8.0	16.28	35	17.05	1.5	17.12
	8.5	17.12	4	2.12	2	10.22
	9.0	0.9	45	0.16	2.5	4.01

Deepthi et al. (2012). The culture was inoculated in 50 ml of colloidal chitin broth, incubated for seven days at 30°C and centrifuged to obtain cell free extract. Strain A showed 1.356 µmol/ml/min of enzyme activity, 0.225 mg/ml protein content in crude. Kumaran et al. (2012) estimated enzyme activity in *Streptomyces* sp. CDB20. The colloidal chitin medium was inoculated with culture spores and incubated for 10 days at 28°C. Crude supernatant was obtained after centrifugation and specific activity was measured by using N-acetyl glucosamine as standard and protein content was measured by Bradford method. Activity shown by CDB20 was found to be 1.22 IU/ml.

Optimization of fermentation parameters (pH, temperature and substrate concentration) for enzyme production in crude extract

Based on the results of primary screening, isolate 130 was selected for secondary or quantitative analyses by submerged fermentation process at a range of temperature, pH and substrate concentration conditions to determine maximum enzyme activity. Isolate 130 showed maximum chitinase activity, 17.12 IU/ml at pH range of 8.0 to 8.5, 17.56 IU/ml activity at temperature range of 25 to 35°C and 17.68 IU/ml at substrate concentration range of 1 to 1.5% (Table 5 and Figure 3A to C).

As reported previously, Santhi (2016) performed optimization of fermentation conditions in crude extract for improving chitinase activity of *S. albus* FS2. The result showed the following: Maximum activity was observed on 5^{th} day of incubation (80 IU/ml), at temperature 37°C (82 IU/ml) and pH 8 (90 IU/ml). Similar results have been reported in *Streptomyces* sp. by Singh et al. (2008) and Subramaniam et al. (2012). The values for enzyme activity and protein content mentioned in aforementioned references was first multiplied by the total volume of the sample and then subsequently used for calculating specific activity. However, in the present study, activity and protein content values “per millimeter” have been reported.

Statistical analyses of enzymatic activity using SPSS software

Statistical analyses of fermentation conditions (pH, temperature and substrate concentration) using one way ANOVA showed that there is a significant effect of pH, temperature and substrate concentration on the enzyme activity shown by isolate 130. The values obtained were $F(5,12) = 66581.475$, $p = 0.000$ for pH, $F(5,12) = 137621.778$, $p = 0.000$ for temperature and $F(5,12) = 111638.172$, $p = 0.000$ for substrate concentration. This was also proved by Post Hoc test (Turkey HSD) analyses which demonstrated that there lies a statistically significant difference in the activity observed at different pH and temperature values. This means that with the increasing pH, temperature and substrate concentration, activity initially increases, attains a maximum level then gradually decreases to produce a bell shaped curve.

In a similar study, Gherbawy et al. (2012) analyzed the data recorded for chitinase activity in case of 7 actinomycete isolates by two-way ANOVA by using ‘Proc Mixed”. The level of statistical significance was checked with $P<0.05/P<0.01$. However, the results showed statistically no significant differences ($P<0.05$ or $P<0.01$) in the data.

Apart from using ANOVA/Post Hoc test (Turkey HSD) analyses, other statistical methods such as Plackett-Burman and response surface methodology can also be used to optimize the medium components and improve chitinase production from strains (Meriem and Mahmoud, 2017).

Purification of enzyme and enzyme assay in purified products

Purification by ion exchange chromatography

Chitinase enzyme activity and protein content were determined in the partially purified protein samples using NAGA and BSA standard curves, respectively as already mentioned in the analyses of crude extracts. It was found that protein content also increased after purification. For chitinase enzyme activity comparison,

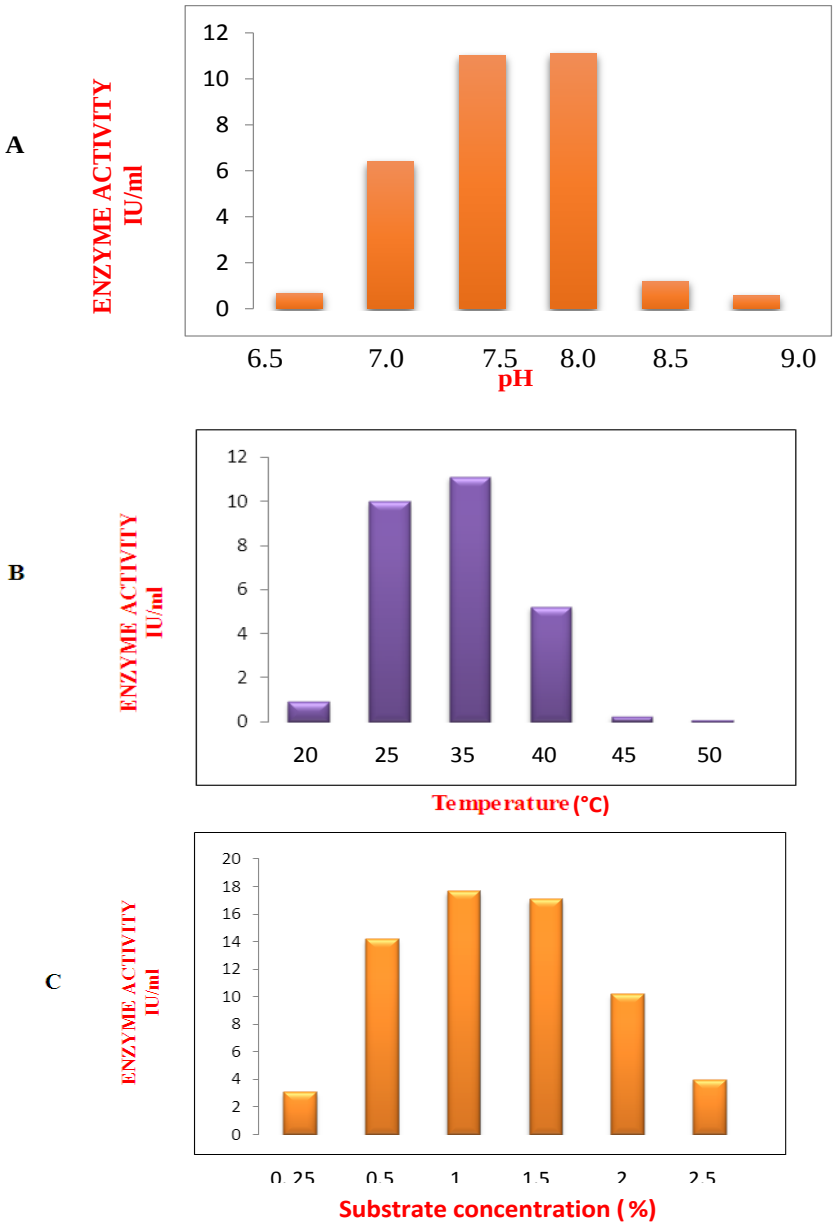


Figure 3. Comparison of fermentation conditions for highest chitinase producer (in crude at different pH (A), temperature (B) and substrate concentration (C).

protein content in all samples was equalized. Chitinase activity increased in culture extracts after purification as indicated by the higher values of released NAGA (Table 6 and Figure 4). Enzyme activity was found maximum in isolate 130, followed by NRRLB 16746, isolate 194, 184 and NRRLB 24916. Therefore, it can be concluded that isolate 130 is an efficient producer of extracellular chitinase enzyme in comparison to even the known chitinase producer like NRRLB 16746. The highest chitinase producing isolate 130 was selected for further purification by ion exchange column chromatography. Enzyme activity in purified fraction was found to be 32.12 IU/ml/min.

The results obtained after purification were compared with data reported in literature. Narayana and Vijayalakshmi (2009) performed a single-step purification of chitinase from *Streptomyces* sp. ANU 6277. The crude culture supernatant was 80% saturated with ammonium sulphate, dialyzed and then concentrated. The sample was loaded on Sephadex G-100 column. Activity and protein content in purified fraction was examined. Molecular size of purified fraction was estimated by SDS-PAGE. Total activity (U), total protein content (mg) and specific activity (U/mg) recorded in ammonium sulphate saturated sample was 3120 U, 118 mg and 26.4 U/mg, whereas in case of Sephadex G-100 purified sample it

Table 6. Protein content and chitinase enzyme activity in partially purified partially purified samples.

Culture	Protein content (mg/ml)	Protein content after equalization (mg/ml)	Enzyme activity (IU/ml) in partially purified	Enzyme activity as previously observed in crude culture extracts (IU/ml)	Enzyme activity in extract purified by ion exchange chromatography (IU/ml)
Isolate 130	1.071	0.59	23.10	14.12	32.12
NRRLB 16746 (control)	0.930	0.59	19.08	12.81	-
Isolate 194	0.813	0.569	13.19	11.6	-
Isolate 184	0.740	0.560	12.0	10.45	-
NRRLB 24916	0.572	0.553	12.1	7.16	-

*The highest chitinase producing isolate 130 was selected for further purification by ion exchange column chromatography.

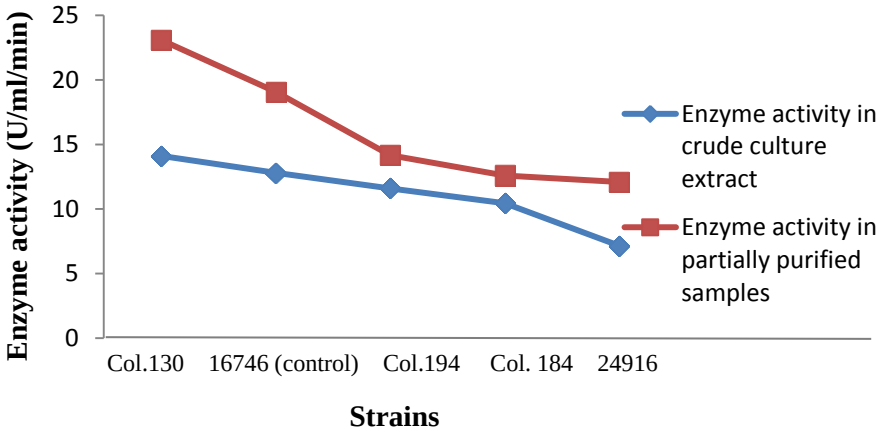


Figure 4. Comparison of chitinase enzyme activity of crude culture extracts and partially purified samples.

was found to be 1649 U, 27.5 mg and 59.9 U/mg. A single protein band of size approximately 45 kDa was obtained.

Mander et al. (2016) purified chitinase from *Streptomyces anulatus* CS242. The crude sample was precipitated by ammonium sulfate followed by dialysis and concentration. The resultant sample was purified using gel permeation chromatography with Sepharose CL-6B column. Protein content and chitinase activity was assayed in each fraction by Bradford and DNS method respectively. Total activity (U), total protein content (mg) and specific activity (U/mg) recorded in ammonium sulphate saturated sample was 5300659 U, 51.24 mg and 10356 U/mg whereas in case of Sepharose CL-6B purified sample it was found to be 270102 U, 10 mg and 27010 U/mg.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Partially purified samples (crude cell extract, dialyzed and concentrated) of isolate 130 were run on denaturing SDS-PAGE for observing the protein profile. Multiple bands of different sizes were observed in all crude extracts. However, number of bands started decreasing when the samples were dialyzed and concentrated. This showed the extent of purification in each case (Figure 5a). Purified fraction was also run on gel and showed presence of one band of approximately 65 to 70 kDa corresponding to purified chitinase enzyme (Figure 5b). The purified band from the gel was eluted and processed for further studies.

Molecular sizes of bacterial chitinases reported in literature were 28, 35 and 45 kDa from *Streptomyces* sp. NK 1057, 43 and 45 kDa from *S. albovinaceus* S-22(5), 49 kDa from *S. griseus* HUT 6037, 20 to 70 kDa, 38 kDa from *S. anulatus* CS242 (El-Sayed et al., 2000; Nawani and Kapadnis, 2004; Bhattacharya et al., 2007; Narayana and Vijayalakshmi, 2009; Mander et al., 2016).

Analysis of kinetic parameters

The K_m and V_{max} values of the purified fractions for isolate 130 were found to be 2.11 µm/ml and 53.11 mg/ml respectively (Figure 6). This shows that the enzyme have high affinity for the substrate and moderate turnover

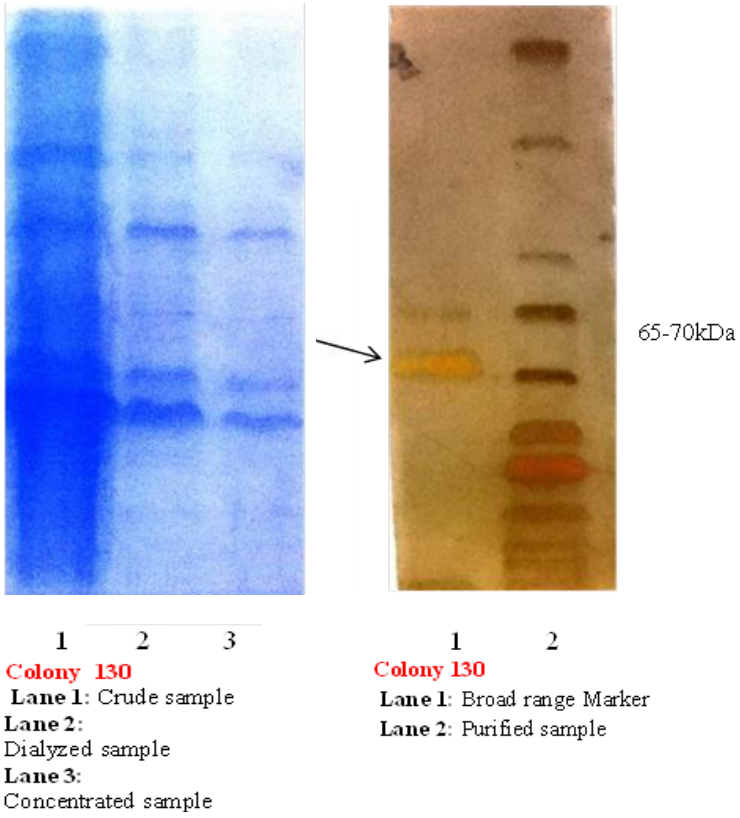


Figure 5. (a) SDS-PAGE profile of partially purified samples of isolate 130; (b) SDS-PAGE profile of isolate 130 purified fractions.

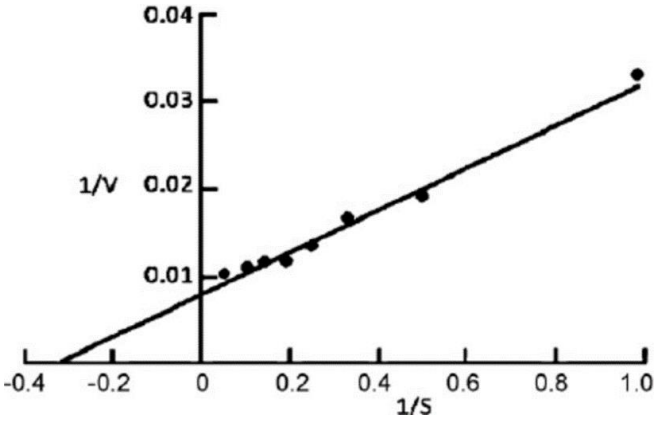


Figure 6. Lineweaer-Burk plot for isolate 130.

number.

The results obtained were compared with data reported in literature. Karthik et al. (2015) determined Km (6.74 mg/ml) and Vmax (61.3 U/mg) of purified chitinases produced by a *Streptomyces* sp. using colloidal chitin. Rabeeth et al. (2011) reported that the purified chitinase produced by *Streptomyces griseus* exhibited Km and Vmax values of 400 mg and 180 IU mL^{-1} respectively for colloidal chitin.

Characterization of enzymes by molecular approach

Protein identification by mass spectrometry

Sequences obtained after MS/MS for chitinase were assembled using MASCOT. ESI-MS spectra of isolate 130 are shown in Figure 7. Total assembled amino acid sequence obtained for isolate 130 had a length of 453 amino acids. The assembled sequences were then used for further analyses.

Analysis of structure and catalytic sites

For identifying the type of protein in isolate 130, the assembled amino acid query sequence was searched using pBLAST against Protein data base (PDB). The target sequence of isolate 130 showed high identity (70%) with chtA of family 18 from Streptomyces plicatus (PDB id: 1hp4). Multiple sequence alignment of N-terminal sequence of isolate 130 was done with sequences of known chitinase producing Streptomyces sp. using CLUSTALW software (Figure 8).

For constructing the structure for the enzymes, N-terminal sequences of isolate 130 were submitted in SWISS-MODEL. The software search the database for similarity with the query structure. The best suited

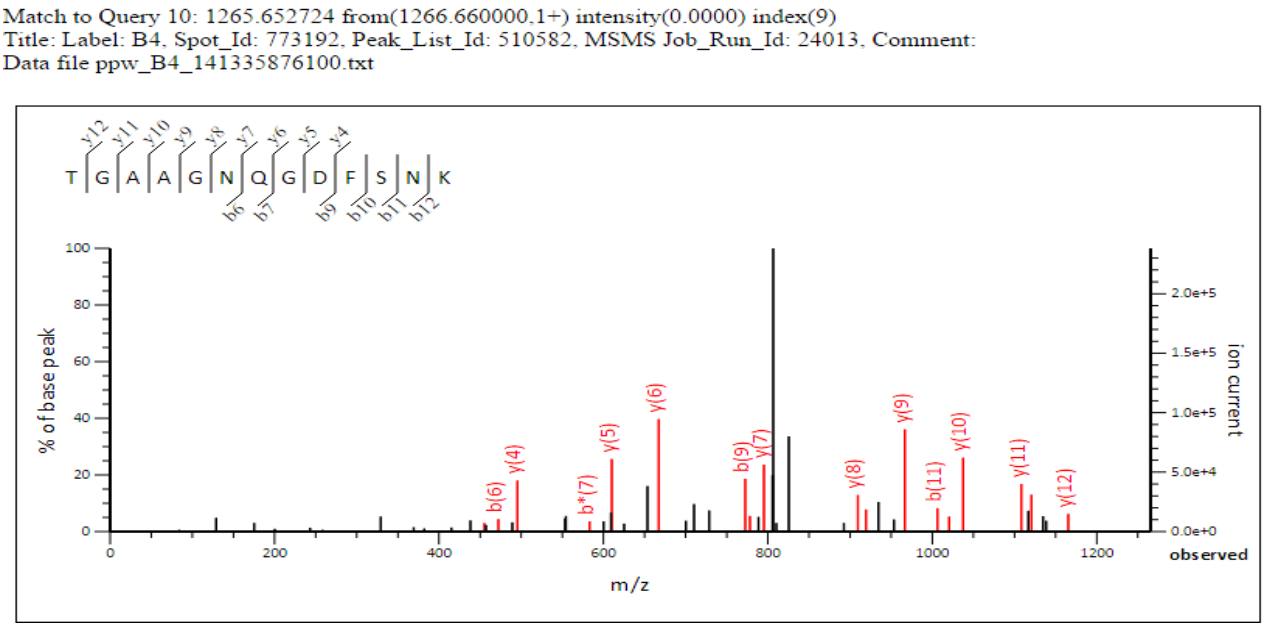


Figure 7. ESI-MS spectra for isolate 130.

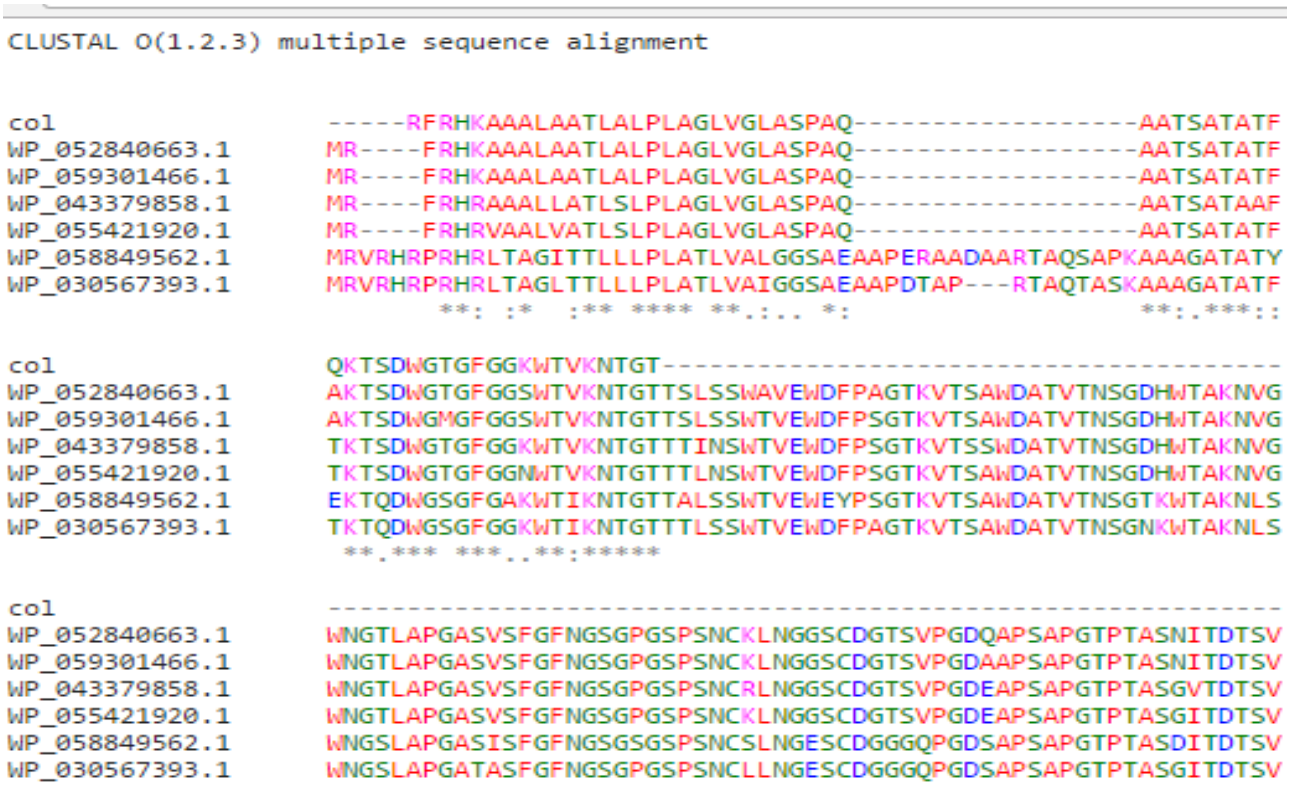


Figure 8. Multiple sequence alignment of target isolate 130 and template *Streptomyces plicatus (*PDB ID: 1hp4). The important residues from active site point of view are highlighted with red and hydrophobic residues with blue.

structure is chosen by the SWISS-MODEL and based on the sequence of that model a tentative three dimensional structure was made for the submitted sequence (Figures 9 and 10). The 453 amino acid residues of isolate 130

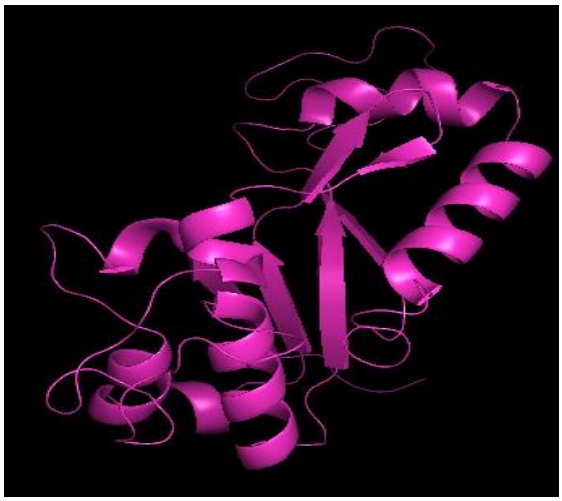


Figure 9. Modelled structure of isolate 130 using SWISS-MODEL.

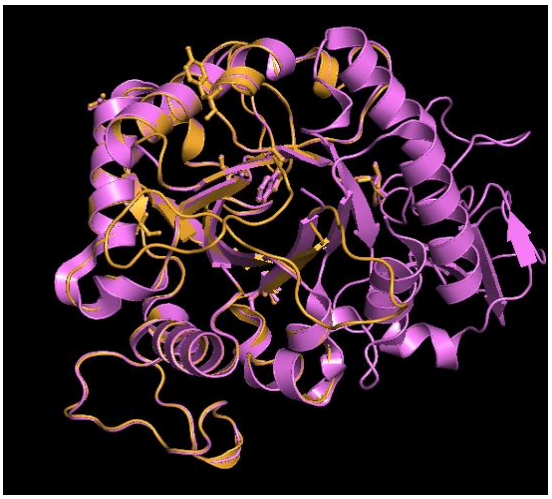


Figure 10. Superimposed modeled structure of isolate 130 by MODELLER (violet: 1hp4; orange: isolate 130).

were folded into a domain (β-jelly roll) structure comprising 3 antiparallel α-helices and five parallel β-sheets, like other enzymes belonging to the glycoside hydrolase family 18 (Watanabe et al., 1999, Hamid et al., 2013, Yan and Fong, 2015).

Ramachandran plot revealed that total residues in allowed region and additional allowed region were 89.8 and 9.5% respectively (Figure 11). The 453 amino acid residues of isolate 130 were folded into a domain of ((α/β)8 barrel) structure comprising of 3 antiparallel α-helices and five parallel β-sheets, similar to enzymes of the glycoside hydrolase family 18. Verify 3D comparison results for isolate 130 showed that in a 3D/1D profile, 88.75% of the residues had an average 3D-1D score >=0.2. The obtained data was also compared against protein database (PDB) using DaliLiteV3.1 server. In case of isolate 130, RMSD and Z score values for the top 5 matches were in the range of 0.7-0.9 and 24.9-28.3 respectively. This data further confirmed and validated the modeled structure obtained for the isolate.

Information about the active site was obtained through superimposing 3-D model structure of the target enzyme with that of template protein of chitinase from S. plicatus. This provided accuracy of homology between the structures, and also helped in positioning the conserved active site residues. Information related to active site of the template structure was obtained from Catalytic Site Atlas (CSA) data base of European Bioinformatics Institute. Overlapping of isolate 130 chitinase with the template placed the amino acid residues Aspartic acid (D) at position 477, Glutamic acid (E) at position 368, Tyrosine (Y) at positions 448, 475, 490, 524 and Arginine (R) at position 220. Combination of these amino acids constitutes the active site of the enzyme. Out of these, Glutamic acid (E) at position 368 and Tyrosine (Y) at position 524 were found to be shared by both isolate 130 and *S. plicatus* as shown in Figure 12.

Circular dichroism for protein secondary structure analysis

The far-UV CD spectrum of isolate 130 exhibited a pronounced maximum and minimum at 195 and 222 nm, respectively, which are characteristics of β-sheet and α-helix structures in aqueous solution, respectively (Figure 13). Thus isolate 130 chitinase is an autonomous structural protein that contains both α-helix and β-sheet secondary structures as predicted from homology modelling.

Results obtained in this study were compared with literature review. Mander et al. (2016) determined the N-terminal amino acid sequence of the purified enzyme of *S. anulatus* CS242 by the Edman degradation method. Sequence obtained was APGAPGTGAL. This was then searched against NCBI-BLAST database and it was found that chitinase from *S. anulatus* showed high degree of sequence similarity (80 %) with chitinase A1 from *Stigmatella aurantiaca* DW4/ 3-1, followed by enzymes from *Amycolatopsis mediterranei* S699 (70 %), *Streptomyces sp*. Mg1 (70%) and *Streptomyces* sp. AA4 (40%). Ubhayasekera and Karlsso (2012) performed homology modelling to determine the structure of chitinase producing *Streptomyces* sp. Mg1. Similar chitinase catalytic module structures were obtained from the Protein Data Bank (PDB), then superimposed and compared with the program O. Multiple sequence alignments were used to identify the best pair-wise alignment of the *Streptomyces sp.* Mg1 enzyme with that of *Lactococcus lactis* subsp. *lactis*. This pair-wise alignment was the basis of creating a homology model,

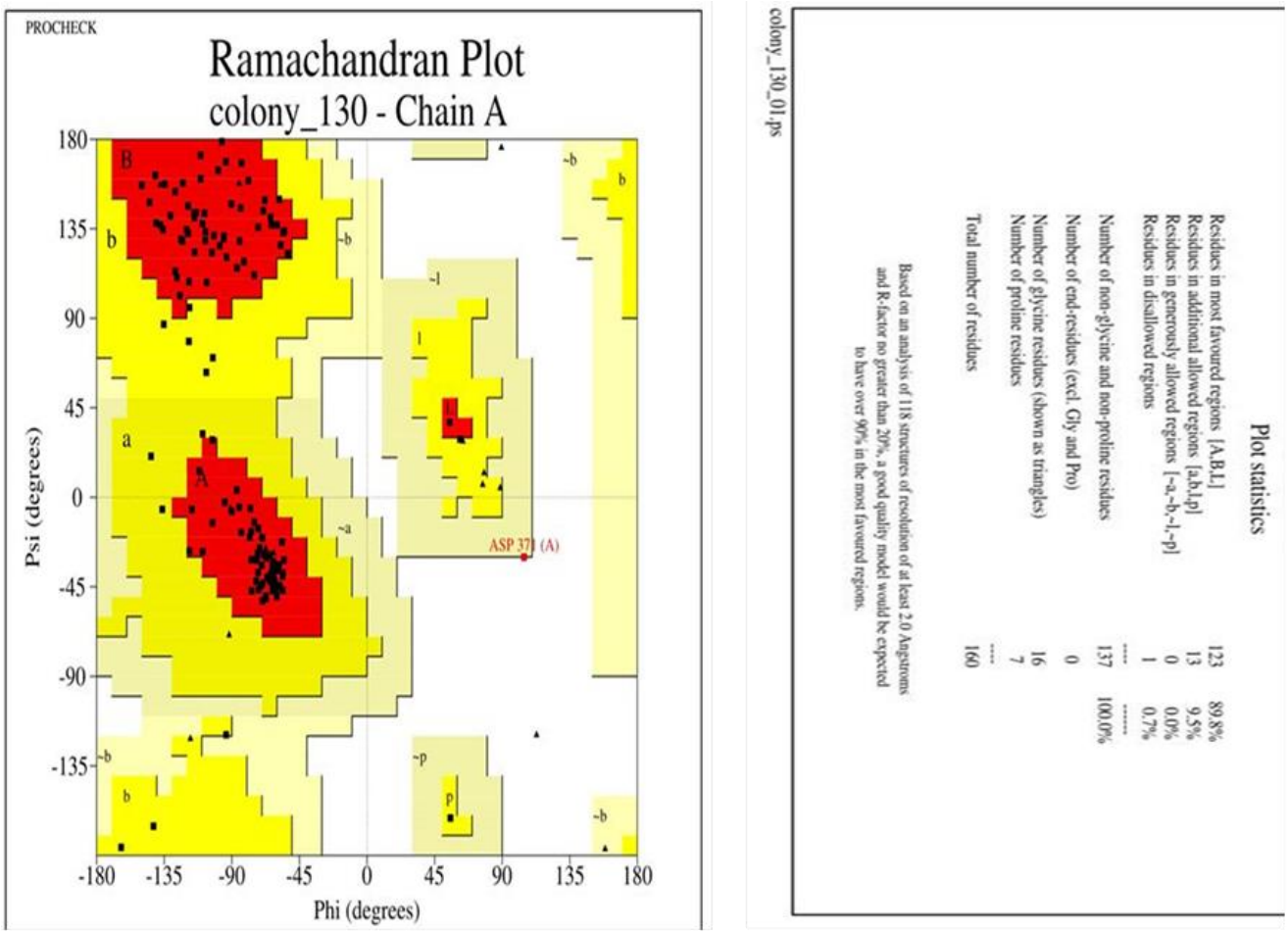


Figure 11. Ramachandran plot of modelled isolate 130 obtained by PROCHECK validation package.

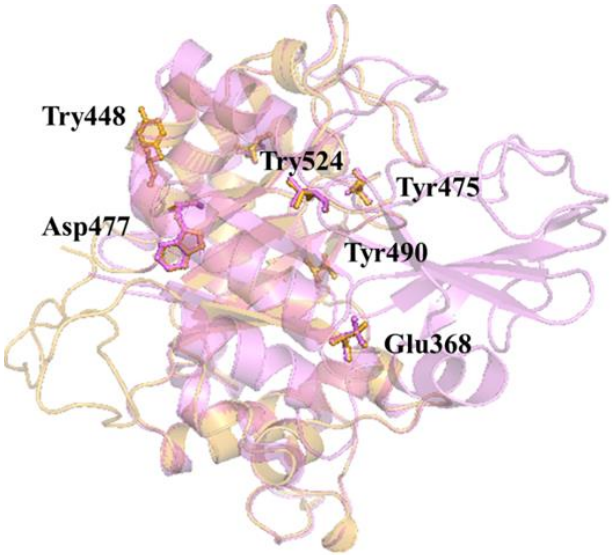


Figure 12. Superimposition of active site residues of modelled chitinase and template 1hp4. (Violet and orange color ribbons represents modelled and template proteins, respectively).

with PDB entry [PDB:3IAN] (*L. lactis* subsp. *lactis*) as the template in the program SOD. The model was adjusted in O, using rotamers that would improve packing in the interior of the protein. Homology modelling revealed that *Streptomyces* sp. Mg1 chitinase had a TIM barrel fold with six insertions and three deletions compared to the chitinase structure from *L. lactis* subsp. *Lacti.*

Role of extracellular enzymes in bioremediation of wastes

Primary and secondary screening of isolates for degradation of wastes

Results obtained during screening procedure showed that isolate 130 efficiently degraded crustacean (prawns, shrimp and crab) shells, with a zone of hydrolysis of 8 mm (Figure 14). The extent of degradation of commercial colloidal chitin by isolate 130 (zone of hydrolysis observed was 16 mm) versus its potential to degrade shell wastes has been shown in Figure 15.

For quantitative analyses of enzymatic activity, the

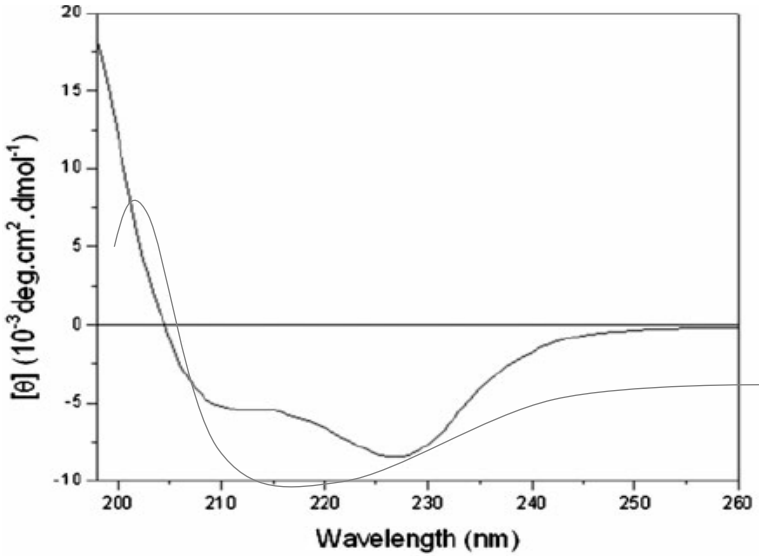
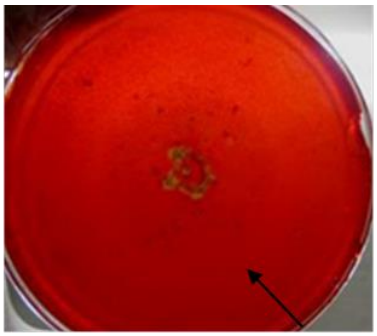


Figure 13. Far-UV CD spectra of isolate 130.



Isolate 130 (control) on CM + crustacean (prawns, shrimp and crab) shells (congo red staining)

Figure 14. Primary screening results for isolate 130.

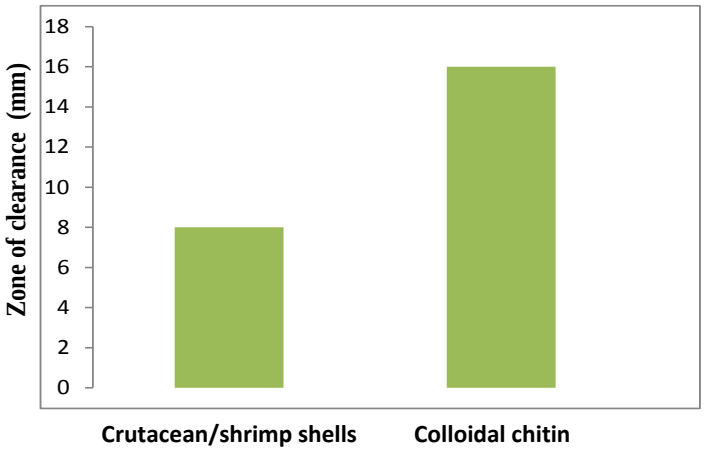


Figure 15. The extent of degradation of commercial colloidal chitin versus degradation of biodegradable waste.

production broth supplemented with 0.4% waste was inoculated with culture. Enzyme activity was measured under the solid state fermentation by using pDMAB method as mentioned in materials and methods. Activity observed was 9.66 IU/ml. As per literature, Brzezinska et al. (2014) reported efficient degradation (38.2%) of shrimp shells by *Streptomyces rimosus.* Similarly Hoang et al. (2011) reported the decomposition of shrimp shells by *Streptomyces* sp. TH-11 between 7 to 16 days. Similar results have been reported by Rabeeth et al. (2011).

Apart from chitin, there are many other derivatives which have large number of applications in industries. For example, chitosan, which is made by treating the chitin present in crustacean/shrimp shells with an alkaline substance. It can be used as biopesticide in agriculture, fining agent in winemaking, antibacterial agent in medicine and flocculent coagulant in removal of toxic metals etc. (Bouhenna et al., 2015, Ferhat et al., 2016).

For future studies, bacterial cultures with potential to degrade wastes can be converted into powdered form by lyophilization and can be packed in containers along with nutrient supplements. The sample can be dissolved in water to prepare a formulation and sprayed on to the waste materials for degradation. However, optimization of environmental parameters (pH, temperature and nutrients) is required to allow microbial growth and speed up the process of metabolism. Hence initially the *ex-situ* degradation of the waste samples can be done under controlled conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Improvement of decontamination and isolation protocols for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from raw milk samples

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Most protocols regarding sample decontamination for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolation are based on the MAP detection from feces and not milk. The choice of the best decontamination protocol is crucial to a successful MAP isolation. In this study, 36 combinations of variables for sample decontamination and MAP isolation from raw milk presented in the literature were carried out on milk samples artificially contaminated which were then inoculated into tubes with three different culture media: Herrold egg yolk medium (HEYM) prepared with fresh egg yolk, HEYM prepared with commercial egg yolk and Lowenstein-Jensen medium (LJ). Each treatment was performed in triplicate for each medium, with a total of 324 observations. The protocol combination which provided higher MAP growth and lower nonspecific contamination in a shorter period of time was considered improved. In this study, the protocol involving 0.75% HPC at room temperature for 24 h, using centrifuge at 2500 × *g* for 15 min and addition of antimicrobial solution immediately before inoculation into tubes with HEYM prepared with fresh egg yolk provided the greatest MAP isolation from raw milk samples.

Key words: *Mycobacterium avium* subsp. *paratuberculosis*, milk, decontamination protocols.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis, a chronic granulomatous enteritis that affects all ruminants and has been proposed as one of the etiologic agents of Crohn's disease, a chronic granulomatous enteritis seen in humans. The transmission vehicle could be milk and dairy products (Abubakar et al., 2008; Atreya et al., 2014; Liverani et al., 2014). Despite the ban on the marketing of

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raw milk in Brazil since the 1950s (BRASIL, 1950), it is estimated that about 20 to 30% of bovine milk production in Brazil is marketed without sanitary inspection: the sale of these products is held freely in several cities (Abrahão et al., 2005, Motta et al., 2015), and this is a significant public health problem.

Animals with paratuberculosis excrete MAP in feces and, in smaller quantities, in milk (Sweeney et al., 1992). Most protocols regarding sample decontamination for MAP isolation are based on MAP detection in feces and not in milk (Stabel, 1997, Whitlock et al., 2000, Bradner et al., 2013). Due to the characteristics of each type of sample, different protocols must be followed. Therefore, it is necessary to develop methods accordingly.

MAP isolation also depends on chemical decontamination to inactivate other microorganisms in the sample that could inhibit the growth of MAP, since this presents a very slow rate of growth (Collins, 2003, Bradner et al., 2013). Chemical decontamination, however, is known to affect also the viability of MAP and therefore increases the likelihood of a false-negative culture result (Grant and Rowe, 2004). In addition, the existing culture protocols take from 12 to 18 weeks to isolate a suspect colony (Grant et al., 2001). Thus, a balance between an efficient inactivation of undesirable microorganisms and low environment toxicity for MAP is needed. The choice of the best decontamination protocol is crucial to a successful isolation of this potential zoonotic organism.

This study compared protocol combinations for sample decontamination and MAP isolation from raw milk, aiming at a protocol with earlier isolation, less contamination and facility of application.

MATERIALS AND METHODS

MAP K10 strain

A MAP K10 strain certified by genetic sequencing was grown in Middlebrook 7H9 supplemented with OADC. After that, 100 µL of the prepared suspension at a concentration of 10^6 CFU/mL was inoculated into 40 mL raw milk aliquots, collected from a bulk tank from a historically paratuberculosis-free farm, which also tested negative for MAP presence by IS*900*-PCR using the primers BN1 (5' GTT ATT AAC GAC GCC CAG C 3') and BN2 (5' ACG ATG CTG TGT TGG GCG TTA G 3') accordingly Sivakumar et al. (2005).

Combinations of variables

A total of 36 combinations of variables for sample preparation for MAP isolation presented in the literature (Collins et al., 1993, Grant et al., 1996, Dundee et al., 2001, Pillai and Jayarao, 2002, Stabel et al., 2002, Bradner et al., 2013) were carried out on milk samples artificially contaminated which were then inoculated onto slant agar in tubes with three different culture media: Herrold Egg Yolk Medium (HEYM) prepared with fresh egg yolk, HEYM prepared with commercial egg yolk and Lowenstein-Jensen medium (LJ) (Himedia, Mumbai, India).

Two binomials time-speed of centrifugation were compared: 3100 × *g* for 30' and 2500 × *g* for 15'; two concentrations of hexadecylpyridinium chloride (HPC) (Sigma, Mumbai, India): 0.75% and 0.9%; three times and two contact temperatures with HPC: 2, 5 and 24 h and room temperature and 37ºC, respectively; and two times of contact with an antimicrobial solution (nalidixic acid - 50 mg/L, vancomycin - 50 mg/L and amphotericin B - 150 mg/L), used at the end of the decontamination: immediately (mixing and direct inoculation) and 2 h (Table 1).

HEYM was prepared according Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, from World Organisation for Animal Health (OIE, 2014). Briefly, for 1 L of medium we used 9 g of peptone, 4.5 g of sodium chloride, 2.7 g of beef extract, 27 mL of glycerol, 4.1 g of sodium pyruvate, 15.3 g of agar; 2 mg of mycobactin, 870 mL of distilled water; 120 mL of egg yolks and 5.1 mL of a 2% aqueous solution of malachite green.

Statistical analysis

Each treatment was performed in triplicate for each medium, with a total of 324 observations. Data were analyzed by ANOVA and discriminated means were compared by F test and Scott-Knott test at 5% probability. Observation of bacterial growth was made considering score 5 for optimum growth, score 3 for good growth, score 1 for no growth and score 0 for contamination.

RESULTS AND DISCUSSION

After observation of bacterial growth on tubes with HEYM prepared with fresh egg yolk, 25 (23.1%) showed score 5; 16 (14.8%) showed score 3; 29 (26.9%) showed score 1 and 38 (35.2%) showed score 0. For tubes with HEYM prepared with commercial egg yolk, none showed score 5; 10 (9.3%) showed score 3; 69 (63.9%) showed score 1 and 29 (26.9%) showed score 0. For tubes with Lowenstein-Jensen medium, 3 (2.8%) showed score 5; 2 (1.9%) showed score 3; 7 (6.5%) showed score 1 and 96 (88.9%) showed score 0.

These results showed significant differences considering the culture media and 36 treatments (Table 2). Comparing the different culture media used, HEYM with fresh egg yolk was significantly better than HEYM with commercial egg yolk and LJ (Table 3).

There are no studies showing differences between the use of a fresh egg yolk emulsion or commercial egg yolks in the composition of HEYM. However, these differences can be explained by the possible use of some kind of preservative in the manufacture of commercial emulsions of egg yolks which may influence MAP growth. Unlike some studies (Juste et al., 1991, Florou et al., 2009) where there have been no reported differences between HEYM and LJ, in this study, 88% of tubes containing LJ were discarded due to contamination. However, production of LJ is more difficult compared to HEYM and one objective of this study was the ease of handling.

Comparing the 36 treatments used for *M. avium* subspecies *paratuberculosis* (MAP) isolation, the protocols followed by letter 'a' were significantly better than those followed by letter 'b' (Table 4).

Similar to some studies (Dundee et al., 2001, Gao et al., 2005) which have shown that treatment of milk with

Table 1. Protocol combinations for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolation carried out on artificially contaminated milk samples.

Treatment	Time-speed of centrifuge	HPC[1] concentration (%)	Time of contact with HPC (h)	Temperature of contact with HPC	Time of contact with antimicrobial solution[2]
1	3100 × *g* / 15'	0.75	2	Room	immed[3]
2	3100 × *g* / 15'	0.75	2	Room	2h
3	3100 × *g* / 15'	0.75	2	37°C	2h
4	3100 × *g* / 15'	0.75	5	Room	immed
5	3100 × *g* / 15'	0.75	5	Room	2h
6	3100 × *g* / 15'	0.75	5	37°C	2h
7	3100 × *g* / 15'	0.75	24	Room	immed
8	3100 × *g* / 15'	0.75	24	Room	2h
9	3100 × *g* / 15'	0.75	24	37°C	2h
10	3100 × *g* / 15'	0.9	2	Room	immed
11	3100 × *g* / 15'	0.9	2	Room	2h
12	3100 × *g* / 15'	0.9	2	37°C	2h
13	3100 × *g* / 15'	0.9	5	Room	immed
14	3100 × *g* / 15'	0.9	5	Room	2h
15	3100 × *g* / 15'	0.9	5	37°C	2h
16	3100 × *g* / 15'	0.9	24	Room	immed
17	3100 × *g* / 15'	0.9	24	Room	2h
18	3100 × *g* / 15'	0.9	24	37°C	2h
19	2500 × *g* / 15'	0.75	2	Room	immed
20	2500 × *g* / 15'	0.75	2	Room	2h
21	2500 × *g* / 15'	0.75	2	37°C	2h
22	2500 × *g* / 15'	0.75	5	Room	immed
23	2500 × *g* / 15'	0.75	5	Room	2h
24	2500 × *g* / 15'	0.75	5	37°C	2h
25	2500 × *g* / 15'	0.75	24	Room	immed
26	2500 × *g* / 15'	0.75	24	Room	2h
27	2500 × *g* / 15'	0.75	24	37°C	2h
28	2500 × *g* / 15'	0.9	2	Room	immed
29	2500 × *g* / 15'	0.9	2	Room	2h
30	2500 × *g* / 15'	0.9	2	37°C	2h
31	2500 × *g* / 15'	0.9	5	Room	immed
32	2500 × *g* / 15'	0.9	5	Room	2h
33	2500 × *g* / 15'	0.9	5	37°C	2h
34	2500 × *g* / 15'	0.9	24	Room	immed
35	2500 × *g* / 15'	0.9	24	Room	2h
36	2500 × *g* / 15'	0.9	24	37°C	2h

Each combination was inoculated into tubes with Herrold egg yolk medium (HEYM) prepared with fresh egg yolk, HEYM prepared with commercial egg yolk and Lowenstein-Jensen medium (LJ).[1]HPC = hexadecylpyridinium chloride; [2]antimicrobial solution = nalidixic acid - 50 mg/L, vancomycin - 50 mg/L and amphotericin B - 150 mg/L, [3]immed = immediately (mixing and direct inoculation).

Table 2. ANOVA for comparing means between variables 'treatment' and 'culture media'.

Source of variation	DF	Mean square
Treatment	35	0.43**
Culture media	2	20.52**
Treatment x Culture Media	70	0.23[ns]

** Significant differences at 1% probability; [ns] No significant differences.

Table 3. Mean comparison among three different culture media used for *M. avium* subspecies *paratuberculosis* (MAP) isolation carried out on artificially contaminated milk samples.

Culture media	Mean
HEYM prepared with fresh egg yolk	1.67[a]
HEYM prepared with commercial egg yolk	1.15[b]
Lowenstein-Jensen medium	0.81[c]

Means followed by the same letters does not differ statistically by Scott-Knott test at 5% probability.

Table 4. Mean comparison among 36 treatment used for *M. avium* subspecies *paratuberculosis* (MAP) isolation carried out on artificially contaminated milk samples.

Treatment	Mean	Treatment	Mean
1	1.06[b]	19	1.01[b]
2	1.13[b]	20	1.02[b]
3	1.37[a]	21	1.07[b]
4	0.97[b]	22	0.89[b]
5	1.35[a]	23	1.31[a]
6	1.3[a]	24	1.07[b]
7	1.61[a]	25	1.43[a]
8	1.61[a]	26	1.5[a]
9	1.19[b]	27	1.37[a]
10	1.19[b]	28	0.84[b]
11	1.15[b]	29	0.89[b]
12	1.14[b]	30	1.18[b]
13	1.32[a]	31	0.77[b]
14	1.28[a]	32	1[b]
15	1.27[a]	33	1.24[a]
16	1.12[b]	34	1.2[b]
17	1.19[b]	35	1.62[a]
18	1.56[a]	36	1.3[a]

Means followed by the same letters does not differ statistically by Scott-Knott test at 5% probability.

0.75% HPC is better for the detection of MAP, in this study 0.75% HPC was used in the improved protocol. Some studies have used other products for chemical decontamination, such as BHI with HPC and CB-18^{TM} (Dundee et al., 2001; Ozbek et al., 2003; Ruzante et al., 2006). However, this study aimed at a high isolation rate of MAP and ease of application, and considering that these other reagents are more costly compared to HPC and that more work is necessary for the implementation of these protocols, this study used only HPC. The other agents would be greatly disadvantageous if a large number of samples needed to be tested. Meanwhile, studies carried out by Dundee et al. (2001) indicated that treatment with HPC for 5 h was more effective, while in this study HPC for 24 h was used in the improved protocol.

Although in this study just K10 strain was used for comparing protocol combinations for sample decontamination, it is important to highlight that the types of culture media could determine differences in the growth of MAP strains (Cernicchiaro et al., 2008).

Considering that animals with paratuberculosis excrete MAP in small quantities in milk (Sweeney et al., 1992) and that a significant proportion of MAP cells was observed to be present in the initial sample of milk were not recovered after decontamination, regardless of the method used, there is a consensus that decontamination methods may also affect MAP cells, resulting in false negatives (Reddacliff et al., 2003). These researchers have found that, during decontamination, the number of microorganisms is greatly reduced as well as in subsequent removal of aliquots for inoculation into media. This increases the necessity of using other diagnostic methods, for example molecular tools, as complementary instruments in MAP detection, although isolation is considered the gold standard.

In this study, it was considered that a protocol involving 0.75% HPC at room temperature for 24 h, using a centrifuge at 2500 × *g* for 15 min and an antimicrobial solution immediately before inoculation into tubes with HEYM prepared with fresh egg yolk provided the optimal MAP isolation from raw milk samples. This protocol was also less laborious, shows an ideal quality for the simultaneous processing of large quantities of raw milk samples, although the protocol was somewhat time consumed requiring 24 h.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Biological control of potential antagonistic bacteria isolates to restrict *Magnaporthe grisea* infection on rice

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Rice blast caused by *Magnaporthe grisea*, frequently affects rice in the world. This research is intended to screen biological control agents for controlling *M. grisea*, referencing the study biological control agents testing approaches, since biological control is an environmentally friendly plant disease controlling approach. 710 bacterial isolates were discovered from rice tissues, of which hopeful biological control scores were discovered referencing their abilities in antagonism inhibition and secreting extracellular hydrolytic enzyme. Biological control discovery against *M. grisea* were experimented on 35 bacterial strains with hopeful biological control characteristic examining through amplified ribosomal DNA restriction analysis (ARDRA) and BOX analysis on isolates with high assessment scores. Five biological control agents (BCAs) with protection efficacy of more than 40% in greenhouse and field experiment were discovered. *Pantoea ananatis* HS-8 and *Bacillus cereus* DL-7 performed well in greenhouse experiment, and field test respectively. In general, correlation coefficient is 0.95 between assessment scores of 35 experimented BCAs and correlation coefficient between antagonism test and biological control efficacy show 0.72 against *M. grisea*. Biological control efficacies results in greenhouse and field experiments showed positive correlation with assessment scores, proposing that the BCAs evaluating and screening method set-up is reference for screening BCAs for controlling *M. grisea.*

Key words: Biological control agents (BCAs), biological control efficacy, extracellular metabolites, *Magnaporthy grisea.*

INTRODUCTION

Magnaporthy grisea is one of the most important diseases of the many diseases that attack rice. Failures of entire rice crops have resulted directly from rice blast epidemics. All of the plant disease management strategies and techniques that have been generated have been brought to bear against rice blast, but often with limited success. Rice blast has never been eliminated from a region in which rice is grown, and a single change

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in the way rice is grown or the way resistance genes are deployed can result in significant disease increase even after years of successful management. This disease is a model that demonstrates the seriousness, elusiveness, and longevity of some plant diseases. Rice blast has been widely studied throughout the world. Many investigators have considered it to be a model disease for the study of genetics, epidemiology, molecular pathology of host parasite interactions and biology (Xiao et al., 2015), which helped discover new biological control agents, for agriculture crop protection (Tokpah et al., 2016).

Biological control agents (BCAs) antagonistic to plant pathogens are a sustainable strategy for plant protection (Chen et al., 2013). Successful biological control based on plant associated antagonists not only requires a better knowledge of the complex regulation of disease suppression by antagonists in response to biotic and abiotic factors, but also requires a knowledge of the dynamics and composition of plant-associated bacterial communities and what triggers plant colonization (Cretoiu et al., 2013). However, research of screening efficient biological control agents could be used to limit the fungal pathogen (blast). The study intend to discover bacterial strains with potential biological control characteristic from different rice parts, through efficient and useful BCAs experimenting strategy based on activities of extracellular hydrolytic enzymes, antagonism inhibition ability and phylogenetic analysis and BOX fragment sequencing. The practical results of greenhouse and field experiments are conducted for controlling *M. grisea* and the correlation is analyze between antagonism and biological control efficacies, assessment score, and biological control efficacies. Finally, conidia germination and appressorium penetration on the rice leaf, which led to the control of *M. grisea* in actual production (Qi et al., 2012).

MATERIALS AND METHODS

Plants and bacteria culture

M. grisea strain *Guy11* was used in this study. Conidiation strain blocks were maintained on strew decoction and corn (SDC) media at 28°C for 7 days in the dark followed by 3 days of continuous illumination under fluorescent light (Shlipak et al., 2013). Biological control isolates were cultured on Luria-Bertani (LB) agar at 28°C for 1 to 3 days. Rice cultivar, particular Nanjing 47, used in this experiment is widely planted by framers. Plastic pots (30 cm bottom diameter and 35 cm height) were filled with soil rich in humus, which had been sterilized at 121°C for 1 h three times individually on three simultaneous days, were sown with rice seeds in greenhouse experiment. An insect-free greenhouse preserved at 20 to 35°C with relative humidity of 70% and a 12 h/12 h day/night photoperiod (600-µmol photons/m^2/s of light supplied during the daytime) was where plants were grown.

Separation of rice habitant isolates

Rice samples, collected from farmers' fields in Taicang, Jiangsu Province in China were separated from surface and interior of stems, endorhiza, rhizosphere, endosphere and phyllosphere individually for rice habitant isolates and bacterial isolates. Surface and interior of its stem, or its root system, three grams fresh weight (FW) of soil, roots, stems or leaves were placed into a sterilized Erlenmeyer flask and suspended in 27 mL of a sterile 0.85% NaCl solution while screening of bacteria from rice rhizosphere. The suspension was incubated at 25°C with shaking at 180 rpm for 30 min and then settled for 5 min; the resulting supernatant was serially diluted, plated on R_2A medium plate (for soil samples) or LB agar (for tissue samples), and incubated at 28°C for 72 h to obtain cultures (form, color and texture) containing 50 to 300 CFU. Colonies with different morphologies from each microenvironment were transferred to LB agar, purified, and then stored at -70°C in LB broth containing 40% glycerol referenced by Balsanelli et al. (2016). Three gram (FW) of sampled (leaf, stem and root), was first soaked in 1% sodium hypochlorite (NaClO) for 5 min and then in 70% ethanol for 2 min assumed to be surface-sterilized, rinsed three times with sterile water, and finally imprinted on R_2A agar plates to screen endophytic bacteria and check sterility. Sample of 3.0 g sterile, was placed in a sterilized mortar including 27 mL of sterile 0.85% NaCl solution and homogenized with a sterilized pestle. Later 10^{-1}, 10^{-2} and 10^{-3} aliquots were taken from 0.1 ml extracts of each sample and smeared on R_2A plates, and incubated at 28°C for 72 h referenced by (Tokpah et al., 2016).

Screen for antagonism towards *M. grisea*

Hundred microliters of the supematants were diluted to obtain aliquots of 10^{-4} to 10^{-6} ml, which were smeared on WA medium. A WA plate medium was inoculated with *M. grisea* hyphae block in the center, and with a candidate antagonistic strain, which one bacterial colony was picked with sterile toothpick, in 3 cm away from the block, and incubated at 28°C for 48 to 72 h. Activity of *in vitro* antagonistic was graded with 0, 1, 2 or 3 based on the diameter (in cm) of the semicircular hyaline zones after 48 to 72 h: Grade 0, no antagonism; grade 1, (1-5 cm); grade 2, (5.1-10 cm); grade 3, >10 cm described by Tokpah et al. (2016).

Activities of extracellular hydrolytic enzymes and siderophores evaluation

In vitro activities of their extracellular hydrolytic enzymes (cellulase, chitinase, glucanase, protease and siderophores), which were indicated by distinct semi-circular hyaline zones around bacterial colonies on specific agar media were examined for bacterial strains. Activity of cellulase was discovered as described by Marjamaa et al. (2013), chitinase activity was tested in minimal medium (Cretoiu et al., 2013), and glucanase activity was examined referencing to Tomkins et al. (2013). Skim milk agar (50 mL of sterilized skim milk mixed at 55°C with 50 mL of 1/5 WA medium containing 2% agar) was used for the detection of protease activity, which was indicated by casein degradation (Durante et al., 2013). Determined expression of siderophore's was done as previously referenced (Tokpah et al., 2016).

Evaluation of possible biological control agents for their biological control ability

A biological control evaluation method was discovered to assess each BCA with different importance referencing to their antagonistic activity and enzyme producing activity (Tokpah et al., 2016). The *in vitro* antagonistic reaction was graded with 0, 1, 2 and 3 based on the diameter (cm) of the transparent-circular zones: No antagonism (0 score); 1-5 cm (1 score); 5.1-10 cm (2 scores); > 10

cm (3 scores). The ability of strains to produce cellulose, chitinase and glucanase were reference the same way. For scoring protease and siderophore production, each value was halved, as protease might play better role in biological control of nematode and siderophores is better in biocontrol of bacterial pathogens, rather than controlling fungal pathogen (Siddiqui et al., 2005). In addition, grade test is the same as accounted in antagonism method and 12-referenced highest assessment score of each bacterial isolate.

Identification of bacterial isolates, by phylogenetic analysis and BOX fragment sequencing

For ARDRA analysis, 89 bacteria DNA was prepared using the Mini BEST Bacterial Genomic DNA Extraction kit Takara Bio Inc. The partial nucleotide sequence of the amplified 16S rDNA was determined using the following primers: L1494-1514 (reverse) 5'-CTA CG (or A) G TA CCT TGT TAC GAC-4' in an automated DNA sequencer (Durante et al., 2013). Amplification was performed with a Pettier Thermal Cycler PTC-200 (Bio-Rad, Watertown, MA, USA) using an initial denaturation step at 94°C for 5 min, and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR products (10 µl) were digested for 2.5 h using the restriction enzymes AluI and MspI. The restriction fragments were separated on a mix gel (1.5% agarose + 2.25% Synergel) running in 1.0 × TBE buffer at 80 V for approximately 5 h, and then stained with ethidium bromide, and photographed under UV transillumination. The experiment was repeated three times to examine the reproducibility of the results.

35 bacterial DNA was prepared using the Mini BEST Bacterial Genomic DNA Extraction kit. BOX-PCR was carried out as described by Rademaker and De Bruijn (1997) using the BOX A1R primer 5'-CTA CGG CAA GGC GAC GCT GAC G-4'. Amplification was performed with a Peltier Thermal Cycler PTC-200 (Biozym Diagnostic, Hessisch Oldendorf, Germany) using an initial denaturation step at 95°C for 6 min, and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 65°C for 8 min, followed by a final extension at 65°C for 16 min. A 5 µl aliquot of amplified PCR products were separated by gel electrophoresis on mixed gel (0.5% agarose + 0.75% Synergel) in 1.0 × TBE buffer at 120 V for 6 h, stained with ethidium bromide, and photographed under UV transillumination (Bio-Rad). The reproducibility of the results was verified in three independent experiments.

Greenhouse experiment

Thirty-five bacterial strains were grown in LB individually at 28°C with 280 r/min for 24 h carefully shaking in greenhouse experiment. Then, bacterial cells were pelleted by centrifugation, washed and suspended in a sterile 0.85% NaCl solution, and well-adjusted to 5 × 10^5 CFU/mL with water for use. Thirty-five bacterial treatments and 2 control treatments were investigated for their biological control efficacies in greenhouse experiments with 24 rice plants per replicate.

Mycelia plugs were grow on PDA medium at 25°C for three days and then inoculated on SDC medium, incubated at 28°C for 3 days, and transferred to a dark chamber for 5 days. The fungus conidia were harvested using 15 ml sterile distilled water containing 0.5% gelatin and used to inoculate the plants. The suspension was filtered through four layers of sterile cheesecloth, and kept in a flask 4°C. Conidia concentration was measured using a hemocytometer and adjusted to 10^5 conidia/ml, before spraying. Inoculation was done in the evening by spraying the *M. grisea* spore suspension (at 15 ml/per replicate). After inoculation, the plants were well kept in the dark chamber and covered with black plastic sheets for 24 h, in order to stimulate infection. Thereafter, the plants were exposed simultaneously to 12 h of light and 12 h of dark for up to three to seven days. At 21 days after inoculation, recorded disease grade were reference and statistically assessed. Relative disease severity and biological control efficacy were calculated with the referencing formula. Rice plant were scored for the disease severity (DS) using a scale of (0-5) as described: 0 indicate no symptoms; 1 indicate typical blast lesions with elliptical shapes measuring 1 to 2 cm long and usually confined to the area of the two main veins and infecting < 2% of the total leaf area; 2 indicate typical blast lesions infecting 10 to 25% of the leaf area; 3 indicate typical blast lesions infecting 26 to 50% of the leaf area; 4 indicate typical blast lesions infecting 51 to 75% of the leaf area, and 5 indicate all leaves that are dead respectively (Harish et al., 2008).

Biological control efficacy and relative disease severity were calculated as follows:

Relative disease severity (%) = [∑ (The number of diseased plants in each grade × the number of grade) / (Total number of plants investigated × the highest disease grade)] × 100%

Biological control efficacy (%) = [(Relative disease severity of Control 1-Disease severity with bacterial treatment) / Disease severity of Control 1] × 100

Examination of Conidia germination, and appressorium penetration on the rice leaf

Bacteria strains were cultured on LB medium plates for spores germination while *Guy11* strains were maintained on straw decoction and corn (SDC) medium at 28°C for a week in the dark followed by 3 days of continuous illumination under fluorescent light. Conidial germination and appressorium formation were measured on a hydrophobic surface (Qi et al., 2012). Appressorium formation rate was counted according to preceding study (Zhang et al., 2011). More than 100 appressoria were observed for each replicate and the experiments were repeated three times. The conidia germination, and appressoria penetration on the rice leaf was observed using an epifluorescenc microscope (Shlipak et al., 2013).

Field test

The field tests were on private land located in Taicang, Jiangsu province, China, referencing GPS coordinate, as N 32.420364, E 121.398103 and the field studies did not involve protected or endangered species. Six biological control agent's treatments were set in the field trials (according to the assessment criteria) and water treatment as mock control, each treatment comprising of 3 replicates and 24 rice plants per replicate. Each treatment includes 1 m^2 normal growth rice field. BCAs suspensions were prepared as reference in greenhouse experiment. Rice ears were deal with by spraying 1 L 5 × 10^5 CFU/mL of BCAs suspension per treatment when rice field come to vegetation stage. After 5 days of BCAs treatment, 1 L 1 × 10^5 CFU/mL *Guy11* spore suspension was sprayed on each treatment and black plastic sheets were used to keep moisture around individual ear. The disease grade was statistically assessed and reference in one week after pathogen inoculation. Relative disease severity and biological control efficacy and relative disease severity were calculated as above according to Harish et al. (2008).

Statistical analysis

Clustering analysis was performed using the unweighted pair grouping method based on arithmetic averages (UPGMA) in order

to determine the population structure of the isolates. After deleting the isolates with same potential biological control characteristics and BOX fingerprint the selected bacterial strains were examine by sequencing their 16s rRNA gene, and the sequences were compared, using the basic local alignment search tool (BLAST), with the reference sequences in the Nucleotide Sequence Database of NCBI (National Center for Biotechnology Information).

Biological control efficacy and relative disease severity were subject to two ways analysis of variance (ANOVA) referencing the statistical software Data Processing System (DPS version 7.05) to determine the differences among the treatments. The means of treatments showing significant differences were separated at the 5% level of significance using the Fisher's least significant difference (LSD) test in order to determine the best treatment. Microsoft Excel 2010 (Microsoft Corporation) was used to calculate the conventional correlation coefficients of the biological control efficacy of isolates with their assessed biological control potential assessments basing on production of siderophores antagonism and extracellular hydrolytic enzymes *in vitro*, antagonism. In addition, Microsoft Excel 2010 Microsoft Corporation was used to calculate the coefficients of, conidia germination, and appressoria penetration on the rice leaf and was observed using an epifluorescenc microscope (Shlipak et al., 2013).

RESULTS

Examining isolates with potential biocontrol efficacy against *M. grisea* disease

Rice samples were gathered from farmers filed in Taicang of which bacterial population density was discovered and referenced in (Table 1). Seven hundred and ten bacterial strains were isolated from rice tissue, comprising root, surface and interior of stems, endorhiza, phyllosphere, soil, and rhizosphere. Generally, the amount of bacterial isolates from surface of plant tissues was higher than those from interior (Table 1). Activities of extracellular hydrolytic enzymes (cellulase, chitinase, glucanase, protease and siderophores) were measured, and the bacterial isolates and as well as antagonistic ability were evaluated with reference method (Table 1).

ARDRA and BOX analysis of potential biological control efficacy strains

Eighty-nine isolates with more than 2 in the evaluating score were chosen out of 710 bacterial isolates, and subjected to ARDRA/BOX fingerprint analysis to avoid redundancy in further analysis. The isolates were separated to 10 clusters on 65% similarity (Figure 1A), of the 10 clusters with 1 isolate accounted for cluster 1, 2, 7, 9, 10 and 2 clusters with at least 2 isolates cluster 6, 8, while, others clusters with 4 strains, 29 strains and 47 isolates accounted for 3, 4 and 5 (Figure 1A). Thirty-five bacterial strains with highest evaluating score were taken from each cluster; BOX-PCR experiment was taken to rearrange them into 6 clusters with very important variability (Figure 1B).16S rRNA gene fragments were amplified from genome of these 35 bacterial strains and identification of physiological and biochemical shows that HS-8 and DL-7 are *Pantoea ananatis* and *Bacillus cereus* strains, and other stains were identified using similar method (Table 2).

Biological control efficacy experiment against *M. grisea* under greenhouse state

Greenhouse test were carried out with 35 selected bacterial strains and 6 isolates with apparent biological control efficacy (from 50%, up Table 2) which approved HS-8, DL-7, DS-26, HL-22, HR-12 and DR-42. Two strains (HR-12, and DR-42) were isolated from rhizosphere, two (DL-7, and HL-22) from phyllosphere, and two (HS-8, and DS-26) from stem sample of rice plant.

Conidia germination and Appressorium penetration on rice leaf treated with (HS-8 and DL-7)

Examination of conidia germination and appressorium formation, found that there were 70% increased rate of conidia germination at 8 h and 20% decreased rate in appressorium formation at 24 h among isolates referencing significant difference at ($P<0.05$) in the rate of conidia germination and appressorium formation between isolates (HS-8 and DL-7) to control (Figure 2A and B).

Correlation assessment between assessment scores and biological control efficacy

Six isolates were received with significant biological control efficacy (more than 50%) in greenhouse experiment and two isolates with significant biological control efficacy in field experiment. Pearson correlation assessment was use to examine the association between their biological control efficacy and the assessment scores based on those statistics (Table 2). The correlation coefficient between antagonism test and greenhouse test results is 0.72 (Figure 3A), correlation coefficient between assessment scores and greenhouse test results is 0.95 (Figure 3B). However, *P. ananatis* HS-8 and *B. cereus* DL-7 showed significant results in greenhouse and field experiments. HS-8 and DL-7 evidently decrease *M. grisea* severity in greenhouse and field experiments (Tables 2 and 3).

DISCUSSION

With the alarming news of rice blast disease, and the aggravation of global warming on the rice crop, *M. grisea* has caused serious loss not only in China but also in other parts of the world (Graham et al., 2013).

Biological control agents were separated in different

Table 1. Antagonistic activities of metabolic enzymes on isolates screened from rice tissue.

Strain habitat	Crop growth stage[A]	Bacteria concentration (CFU/g)[B]	Number of bacterial strains	Isolates with antagonism activity and metabolite enzyme activity[C]					
				Antagonism test	Chitinases	Cellulases	Proteases	Glucanase	Siderophores
Surface stem	Tiller stage	6.4×10^{6}	59	25	5	8	4	4	4
	Booting stage	6.6×10^{6}	80	42	4	15	7	12	4
	Ripe stage	6.2×10^{5}	31	15	1	6	2	3	3
Interior stem	Tiller stage	6.3×10^{4}	22	9	2	5	1	1	0
	Booting stage	6.7×10^{5}	36	15	2	4	4	2	3
	Ripe stage	5.5×10^{6}	28	8	0	3	1	2	2
Endorhiza	Tiller stage	3.9×10^{3}	30	6	0	2	0	2	2
	Booting stage	3.8×10^{4}	43	13	1	5	3	2	2
	Ripe stage	6.9×10^{7}	21	4	1	1	0	2	0
Endosphere	Tiller stage	6.5×10^{3}	18	10	1	6	1	2	0
	Booting stage	3.3×10^{8}	12	7	0	3	1	1	2
	Ripe stage	4.3×10^{5}	8	4	1	0	1	0	2
Phyllosphere	Tiller stage	5.6×10^{6}	66	12	0	8	1	2	1
	Booting stage	7.5×10^{6}	80	21	6	10	0	2	3
	Ripe stage	6.4×10^{6}	28	6	0	2	0	1	3
Rhizophere	Tiller stage	4.9×10^{5}	39	16	3	8	0	3	2
	Booting stage	6.7×10^{5}	41	19	4	8	2	3	2
	Ripe stage	4.2×10^{6}	25	5	0	1	1	2	1
Soil	Tiller stage	3.2×10^{6}	11	5	0	3	0	1	1
	Booting stage	4.3×10^{5}	19	8	2	2	1	3	0
	Ripe stage	5.5×10^{6}	13	6	2	1	1	2	0

(A) BCAs screened from two-growth period of rice, tillering and heading periods. (B) Bacteria concentration represents the total bacteria concentration screened in tissue of rice samples. (C) *M. grisea* strain *Guy11* use for antagonistic experiment. Isolates which have a noticeable halo on WA medium confront cultured with fungal pathogen were called antagonist. The width of the clear semicircular halo surrounding the bacterial streak was measured after incubation.

positions (rhizosphere, surface and interior of stems, endorhiza, phyllosphere, soil, and root) from non-infected/infected rice in this experiment. Bacterial strains with antagonist activities were examined from rice *vivo* tissue (Table 1); demonstrating *vivo* plant habitats might include more bacteria cultural antagonism to plant pathogen as proposed (Villarroya et al., 2016). It might also be related with adaption in the microbial communities in infested fields (Bulgarelli et al., 2013).

To discover acceptable origin of potential BCAs is to obtain successful biological control and assess the vivo and *in vitro* of the crop. Previous, research has mainly focused on isolating nitrogen-fixing bacteria (Balsanelli et al., 2016) and plant growth-promoting bacteria (Corcione et al., 2013).

On the contrary, the study focus is on biological

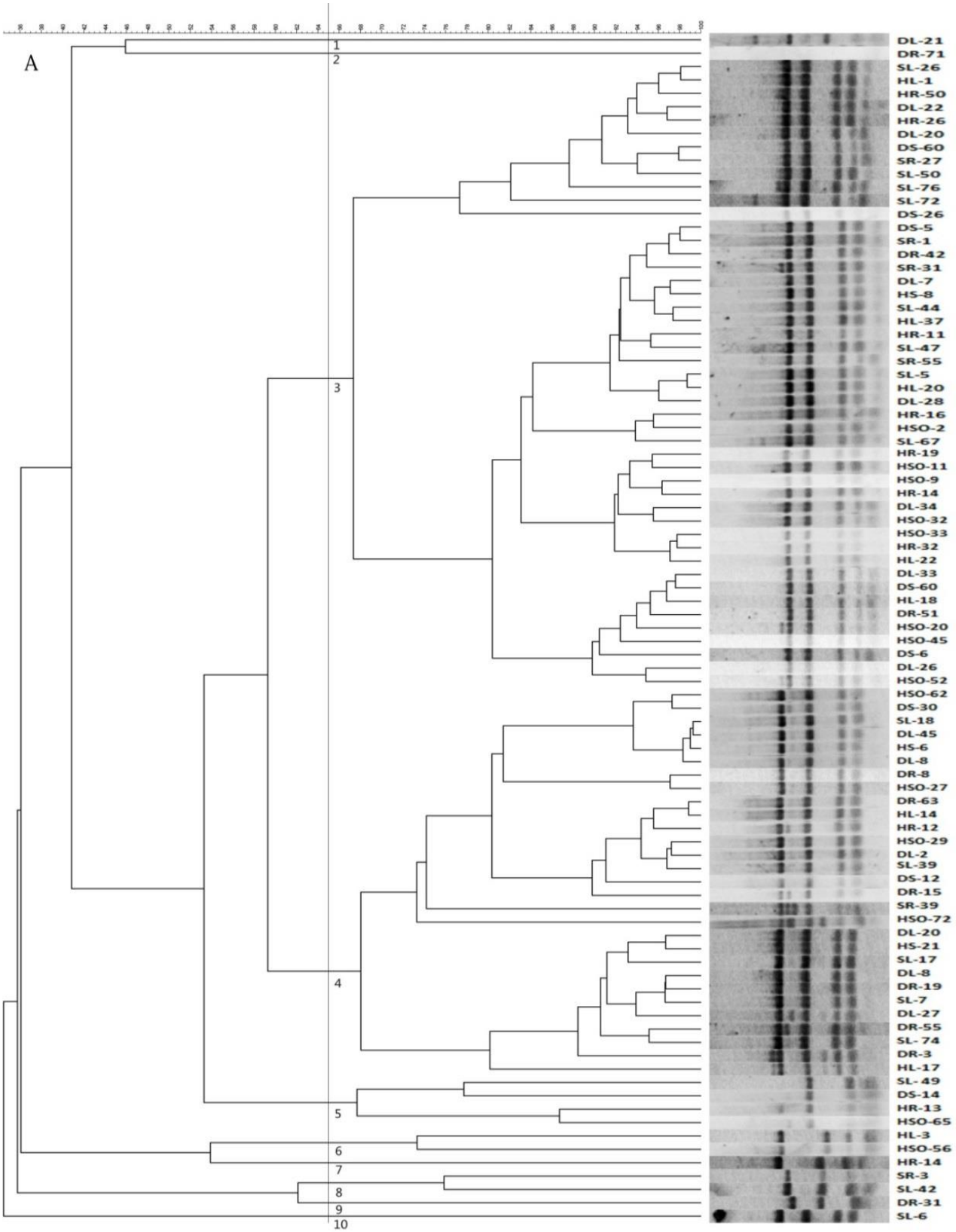

Figure 1. Analysis of the fingerprint (A) ARDRA and (B) BOX. The dendrogram was constructed using GelCompar®II version 4.5 (Applied MathsBVBA). The analysis was performed using Pearson correlation applied to the densitometric curves reference by Rademaker and De Bruijn (1997), followed by clustering analysis using the unweighted pair-rouping method based on arithmetic averages (UPGMA).

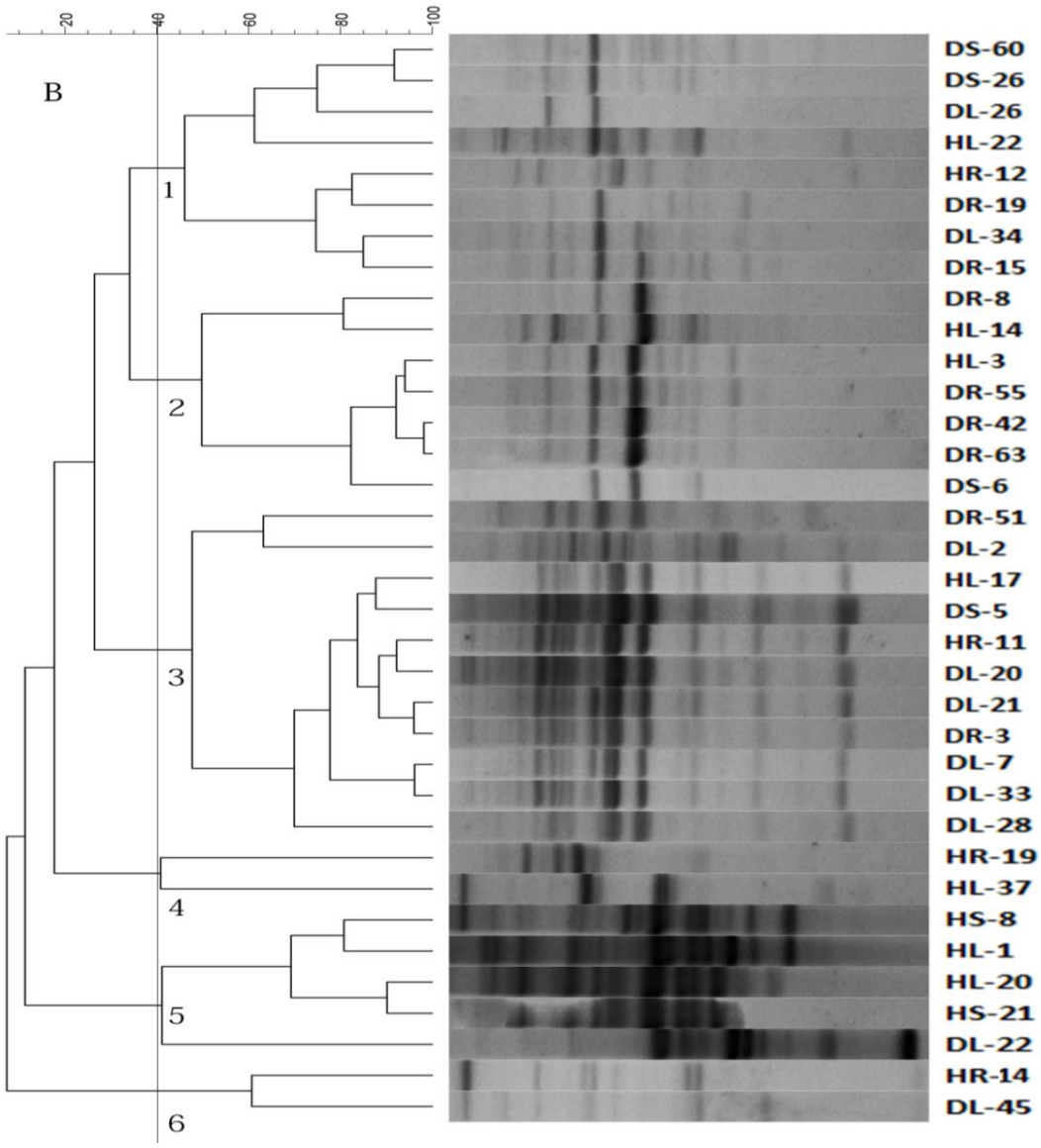

Figure 1. Contd.

control isolates to restrict *M. grisea* infection on rice (Figure 4A and B) which, suggest that antagonistic activity against *M. grisea* and activities of hydrolytic enzymes, were in essential to the biological control agents. Thus, recommending the study assessment strategy for testing biological control agents in similar works.

ARDRA and BOX classifies assessment strategy and BCAs helped avoid using biological control agents of same species in greenhouse and field test and receive different genetic background, which reduce workload in follow-up test. In other experiments, classifying assessment strategy based on genetic background was not included (Er et al., 2016), which resulted in duplication of same species, which is time-consuming.

Biological control success may depend on suitable formulations as well as survival of the microbial agents. Bacteria as biological control agents have advantages over fungal when applied as a preventive application to suppress the disease. In line with this study, several strains of bacteria isolated from rice plant were previously evaluated for their antagonistic ability against *M. grisea* (Tokpah et al., 2016). Six BCAs were acquired with more than 50% which displayed preferable biological control ability (Table 2).

Moreover, those six BCAs with more than 50% showed significant correlation in greenhouse with strains' assessment score but some disparity of acting in field experiment against *M. grisea* (DR-42 and HR-12), showing that similar BCA isolates might give different

Table 2. Recognition and assessments of 35 bacterial isolates disease severity and biological control efficacy in controlling *M. grisea* caused by *Guy11.*

Strains	Identify results	Similarity (%)	Atangonim value	Proteae value	Cellulse value	Chitinse value	Glucanse value	Siderophores value	Scores	Disease severity (%)	Biological control efficacy (%)
HS-8	*Pantoea* sp.	80	3	1	3	1	2	2	12	16.67±5.56^{qr}	86.50
DL-7	*Bacillus cereus*	98	2	1	1	2	2	2	10	18.52±3.21^{qr}	84.00
DS-26	*B. cereus.*	99	2	1	3	0	1	1	7	33.21±4.97^{op}	77.00
HL-22	*Bacillus.* sp.	99	2	0	1	1	1	1	6	35.19±8.49^{nop}	71.50
HR-12	*Enterobacter* sp.	98	2	1	2	0	1	0	6	36.11±2.78^{mnop}	70.25
DR-19	*B. cereus*	99	1	0	1	1	0	0	3	45.19±7.14^{ghijklmn}	32.00
DL-34	*B. cereus*	99	2	0	1	0	0	1	3	49.81±3.16^{fghijkl}	32.75
DR-15	*B. cereus*	99	0	0	0	0	0	0	2	53.7±6.42^{defghij}	27.50
DR-8	*Bacillus subtilis*	99	1	0	1	0	0	0	2	54.32±13.4^{defghi}	26.67
HL-14	*B. cereus*	99	1	0	0	0	1	0	2	53.02±9.92^{efghijk}	28.42
HL-3	*B. subtilis*	99	2	0	0	0	0	0	2	54.07±6.42^{defghi}	27.00
DR-55	*B. subtilis*	99	2	0	0	0	0	0	2	46.11±10.2^{ghijklmn}	24.17
DR-42	*B. pumilus*	92	2	0	0	1	2	0	5	44.07±5.01^{hijklmno}	54.50
DR-63	*Enterobacter* sp.	99	1	0	0	0	0	0	2	47.04±0.32^{ghijklm}	28.50
DS-6	*B. subtilis*	99	2	0	0	0	1	1	3	50.99±7.86^{fghijkl}	31.17
DR-51	*B. subtilis*	99	1	0	1	0	1	0	3	41.67±7.35^{klmno}	33.75
DL-2	*B. pumilus*	85	1	0	1	1	0	0	3	43.09±4.69^{ijklmno}	31.83
HL-17	*B. cereus*	96	1	0	1	0	1	0	3	41.23±2.04^{lmnop}	31.33
DS-5	*B. cereus*	99	0	2	0	0	0	0	2	58.77±7.46^{cdef}	20.67
HR-11	*B. cereus*	99	1	1	0	1	0	0	3	50.68±3.26^{fghijkl}	31.58
DL-20	*B. subtilis*	99	1	0	0	1	0	0	2	54.94±2.47^{defgh}	25.83
DL-21	*B. subtilis*	99	1	0	0	1	0	0	2	52.96±9.71^{efghijk}	28.50
DR-3	*B. subtilis*	99	0	0	1	0	0	0	1	64.81±8.49^{cdef}	12.50
DL-26	*B. cereus*	99	1	0	0	0	0	1	2	56.67±4.01^{cdefg}	23.50
DL-33	*B. cereus*	99	1	0	0	0	1	0	2	54.32±13.4^{defghi}	26.67
DL-28	*Bacillus* sp.	99	1	0	0	0	1	1	3	51.23±4.66^{fghijkl}	30.83
HR-19	*B. cereus*	99	0	0	1	0	0	0	1	63.7±7.56b^{cde}	17.50
HL-37	*B. idriesis*	99	0	0	0	1	0	0	1	65.12±6.17b^{cd}	12.08
DS-60	*B. cereus*	99	0	0	1	0	1	0	2	56.51±2.2^{cdefg}	23.71
HL-1	*Pantoea* sp.	99	0	1	0	0	0	0	1	53.02±9.92^{efghijk}	19.25
HL-20	*Pantoea* sp.	97	0	0	1	1	1	0	3	41.13 ± 2.47^{mn}	30.46
HS-21	*B. cereus*	94	1	0	0	0	0	0	1	60.66 ± 1.43b	13.43
DL-22	*Acinetobacter* sp.	99	0	0	0	1	0	1	2	60.89 ± 3.49b^{c}	29.83

Table 2. Contd.

HR14	*B. cereus*	99	1	1	0	1	0	0	3	31.12 ± 3.35^{q}	35.51
DL-45	*B. cereus*	99	0	0	0	0	1	0	1	58.56 ± 3.52^{cde}	16.58

(A) Genbank library closest isolate, using nucleotide blasting of the 16S rDNA sequence in NCBI (National Center of Biotechnology Information. USA). (B) The percentage means the similarity between our BCAs known bacterial isolates with references in NCBI. (C) The values are Mean ± Std. Deviation; followed by the same letter within a column are not significantly different as discovered by the LSD test ($P < 0.05$).

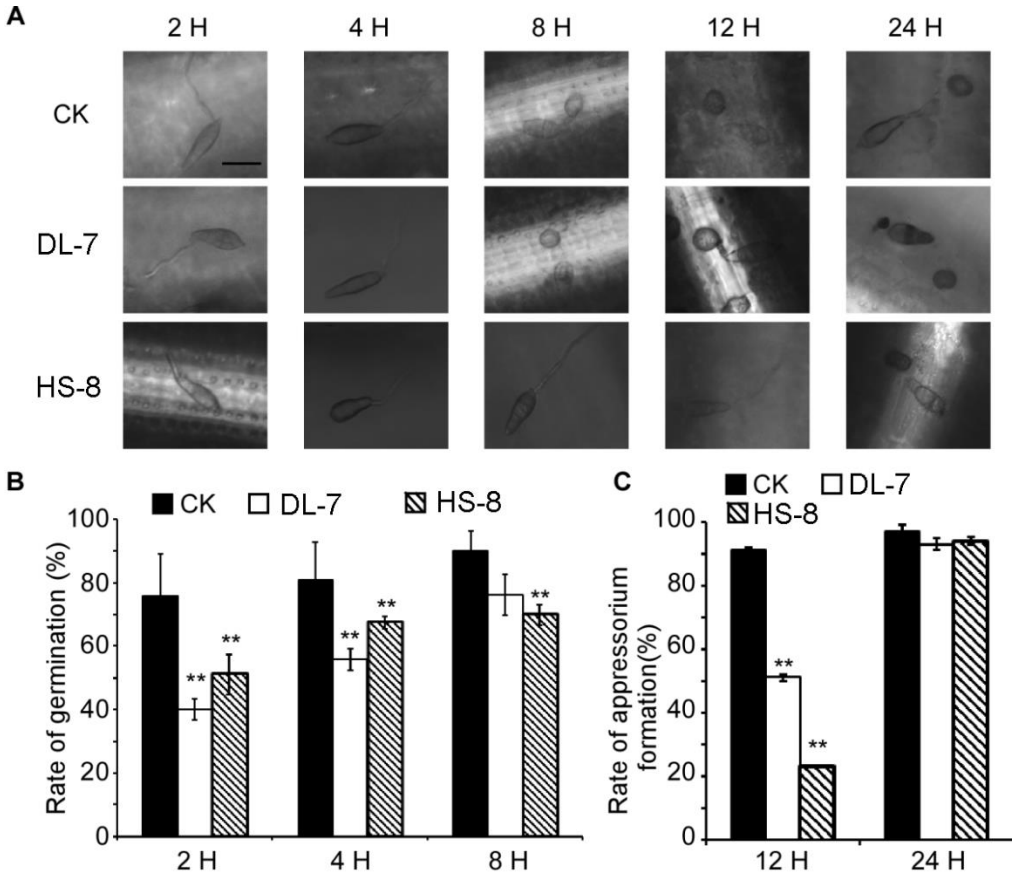

Figure 2. Infectious conidial germination and appressoria growth on rice leaves of strains (HS-8 and DL-7) and control. Analysis for each type of infectious hyphal shape and 100 infecting hyphae were coun1ted per replicate and the experiment repeated three times. (A) Appressoria transformant examined under an epifluorescence microscope and infectious growth was observed at different time points, post-inoculation (hpi). (B) Rice leaves from 14-day-old rice seedling inoculated with conidial suspension (1 ×10^5 spores/ml). (c) Appressorium penetration on the rice leaf.

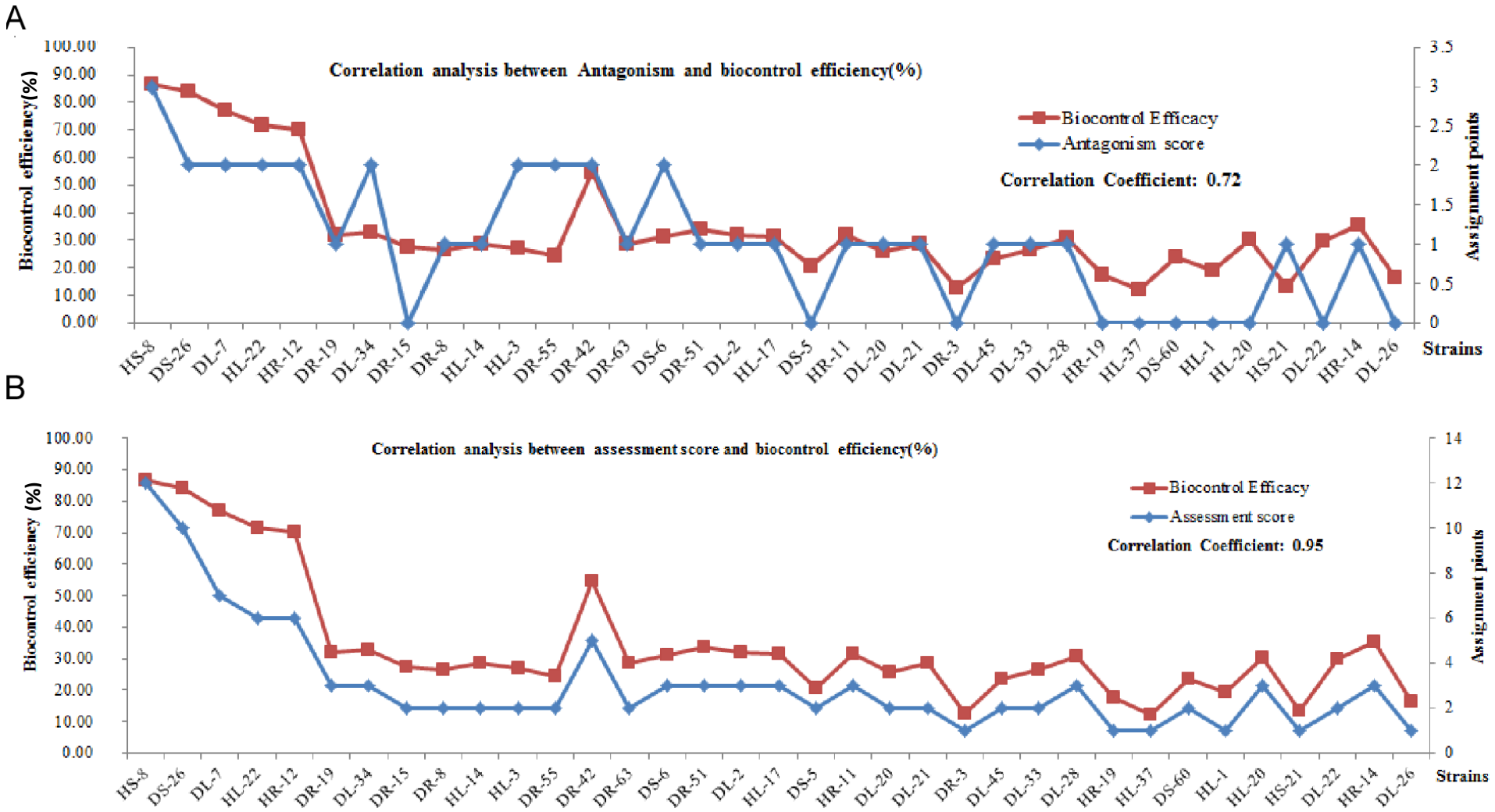

Figure 3. (A) Correlation analysis between antagonism and biological control efficacy to *M. grisea* disease is coefficient 0.72. (B) Correlation analysis between assessment and biological control efficacy to *M. grisea* disease in greenhouse test; Correlation analysis and the coefficient is 0.95. (C) The dark red square rhombus lines representing bacterial biological control efficacy uses the ordinate on the left, while the blue diamond rhombus lines representing the assessment of antagonism and hydrolytic enzymes activities on the right ordinate.

biological control characteristic against *M. grisea* in the same environment. Biological control efficacy was higher in greenhouse and lower in field test (Tables 2 and 3), perhaps resulting to different environmental sites, and temperature (Zaida et al., 2014), with further consequences

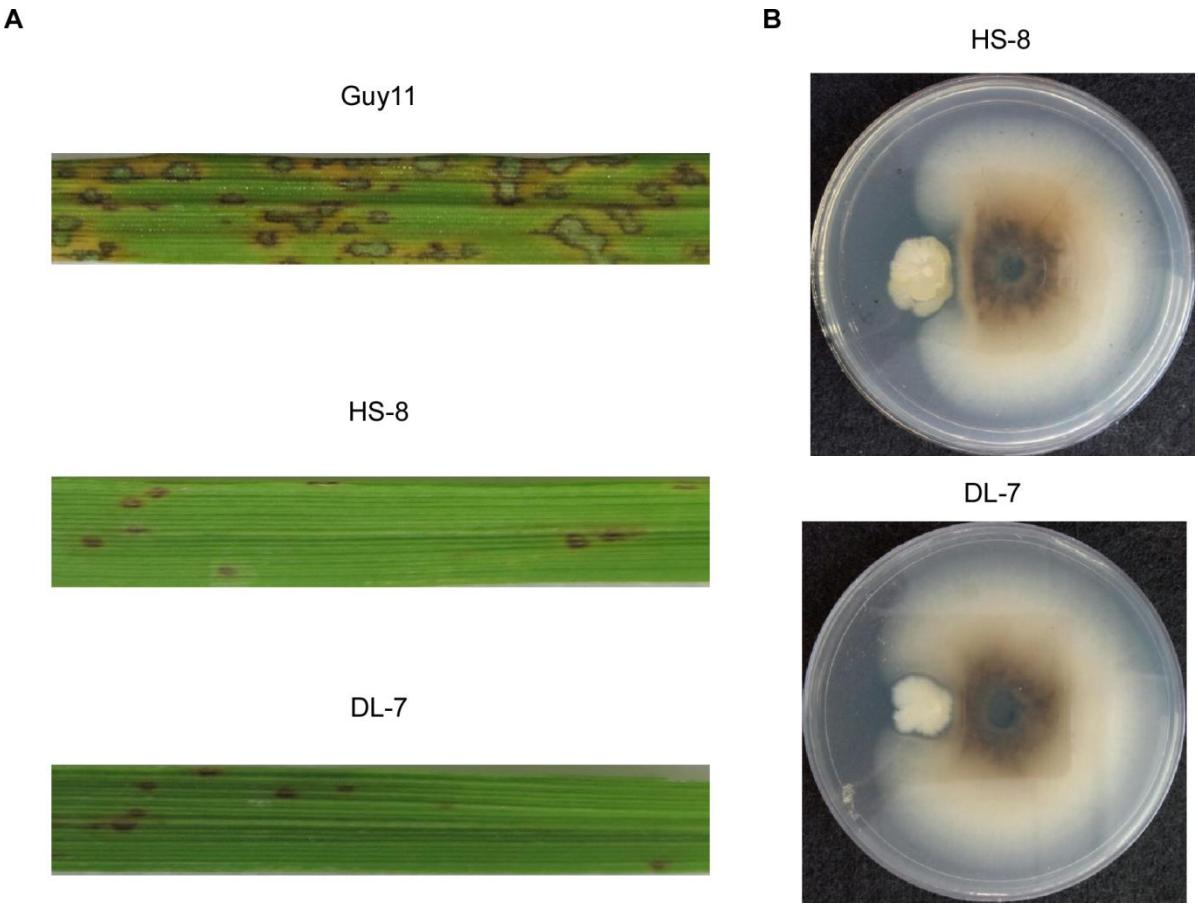

Figure 4. (A) Pathogenicity test on rice leaf plants and conidia suspension (5×10^5 spores/ml) sprayed on rice leaves. Diseased leaf was photographed at 5 day after inoculation. (B) Biological control strains inoculated on WA medium and cultured at 28°C for 7 days.

in different of colonizing abilities. On the other hand, few other characters of bacterial isolates, such as motility and nutrient competing ability, are possibly altered by colonizing condition as well (LeRoux et al., 2015). For separating successful biological control bacteria in different environment, climates and soil types were also considered. Like most other pathogens, conidia of *M. grisea* play a central role in the disease cycle. The infection process is initiated with attachment and conidia germination on the plant surface and appressoria formation from the end of the germ tubes (Figure 2A and B) (Turrà et al., 2015). In this study, more experiment that will focus on the mechanisms of the biological control agents was discovered.

With reference to the authors knowledge, this research is the newest to discover collections of an effective bacteria's, namely *P. ananatis* spp. strain (HS-8) and *B. cereus spp*. strain (DL-7) as BCAs against *M. grisea* based on the study assessment strategy (Tokpah et al., 2016), which showed good feasibility resulting in high correlation coefficient (Figure 3A and B). *P. ananatis* HS-8 and *B. cereus* DL-7 show obvious biological control capacity against *M. grisea* in greenhouse and field experiments while, other isolates with good biological control efficacy in greenhouse (DR-42 and HR-12) did not do well in field experiment (Tables 2 and 3). It can be hypothesize that strains HS-8 and DL-7 may ascribe to better adaptability in various environment as in different colonizing sites even one rice plant might face complete disparate environmental condition. Focusing will be on the mechanism of strains HS-8 and DL-7 referencing BCAs against rice diseases.

Conclusions

In this work, greenhouse experiment results recorded significant correlation with isolates assessment score (0.95), and antagonism and biological control efficacy of greenhouse experiment record 0.72 referencing the study assessment method for choosing BCAs, is also acceptable for *M. grisea* disease. By the study assessment method, five potential BCAs was discovered, especially *P. ananatis* HS-8 and *B. cereus* DL-7 with

good biological control ability against *M. grisea* disease on rice.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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Full Length Research Paper

Prevalence and resistance profile of extended-spectrum β-lactamases-producing *Enterobacteriaceae* in Ouagadougou, Burkina Faso

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Increasing bacterial resistance to antimicrobial agents has become an issue of concern. A major problem of the treatment of infections caused by Enterobacteriaceae using antibiotics is the emergence of Extended-spectrum β-lactamases (ESBL)-producing Enterobacteriaceae. This study aims to determine the prevalence of ESBL-producing Enterobacteriaceae strains in Ouagadougou, Burkina Faso, and describe their resistance profile to other antibiotics commonly used in the infections treatment. 486 clinical strains of Enterobacteriaceae were obtained from patients attending three health centers in Ouagadougou (Burkina Faso) from November 2014 to October 2015. Biochemical identification was performed and antibiotics susceptibility test was performed using the disk diffusion method. Data was analyzed with the Excel and ANOVA one-way software GraphPad Prism version 5.01 software. Results revealed occurrence of *Escherichia coli* (60.9%, 194) predominated followed by *Klebsiella* spp. (22.4%, 109). Antibiotics susceptibility test revealed that 86.8% strains were resistant to amoxicillin, 81.3% to trimethoprim-sulfamethoxazole, 61.9% to ceftriaxone, 58.6% to cefotaxime and 58.4% to cefepime. It was observed that 99.8% were susceptible to imipenem while 16.6% were resistant to fosfomycin and 12.3% to amikacin. However, 38.5% (187/486) of the strains were ESBL-producing, 67.9% (127/187) of which came from Yalgado Ouedraogo University Hospital Center, 23.5% (44/187) from Charles De Gaulle Paediatric University Hospital Center and 8.6% (16/187) from Saint Camille Hospital. This study thus showed a high prevalence of Extended-Spectrum B-Lactamases producing Enterobacteriaceae strains in Ouagadougou (38.5%). It underlined the need for routine detection and systematic reporting of ESBL strains in different health facilities in Burkina Faso, so that measures could be taken to prevent their spread and treatment failures.

Key words: Enterobacteriaceae, Extended-Spectrum Beta-lactamases (ESBL), Burkina Faso.

INTRODUCTION

Bacterial resistance to antibiotics is on the raise worldwide in healthcare setting and in community which tend to be posing a lot of challenges to the effective treatment of infections. Resistance of pathogenic bacteria to β-lactam antibiotics, a group of antibiotic mostly used for the treatment of bacterial infections because of their broad antibacterial spectrum and excellent safety profile has taken a great threatening dimension with the emergence Extended-Spectrum B-Lactamase (ESBL) producing *Enterobacteriaceae* (Abdallah et al., 2015). The ESBLs first described in 1983 in Germany arose from a single nucleotide polymorphism in the bla_{SHV} genes that altered specificity to oxyimino-cephalosporins. Overtime there has been a wide spread of ESBLs with an ever evolving ability to hydrolyze penicillins, first, second and third generation cephalosporins and monobactams but not carbapenems (Lukac et al., 2015; Tekiner and Ozpinar, 2016). In Africa, there has been various reports of ESBL producing *Enterobacteriaceae* (ESBL-E) implicated in causing infections across all ages. Sangare et al., (2017) noted the very high and increasing frequency of ESBL-E in their report on the prevalence of ESBL-E in teaching hospitals in Mali. In a similar study Oduro-Mensah et al., (2016) reported an overall 37.96% of 137 *Enterobacteriaceae* clinical isolates exhibiting ESBL phenotype in Ghana with *Klebsiella* spp. and *Escherichia* coli taking a lead. To further substantiate this, Farra et al. (2016) identified the high rate of faecal carriage of ESBL-E in healthy children in Bangui Central African Republic which portend a high risk of continuous dissemination of multi-drug resistant pathogen with grave consequences to the general health of the public. However there is paucity of information and extended study of ESBL producing *Enterobacteriaceae* (ESBL-E) in Ouagadougou of which study conducted has been restricted to single health centers (Zeba et al., 2007; Métuor-Dabiré et al., 2014; Ouedraogo et al., 2016). Hence this study aimed to determine the prevalence of ESBL producing *Enterobacteriaceae* in three of the major health centers (Yalgado Ouedraogo Teaching Hospital (CHU-YO), Charles De Gaulle Paediatric Teaching Hospital (CHUP-CDG) and Saint Camille Hospital (HOSCO)) in Ouagadougou and to describe their resistance to antibiotics commonly used in the treatment of Gram negative bacterial infections.

MATERIALS AND METHODS

Study site

This cross sectional study was conducted between November 2014 to October 2016 to determine the prevalence and susceptibility of *Enterobacteriaceae* to β-lactams, aminoglycosides and quinolones in Ouagadougou. Three major health centers Yalgado Ouedraogo Teaching Hospital (CHU-YO), Charles De Gaulle Paediatric Teaching Hospital (CHUP-CDG) and Saint Camille Hospital (HOSCO) in Ouagadougou were chosen for the study because they received the highest number of patients and cases in the city. Ouagadougou is the capital city of Burkina Faso with a population of about 2 million people. CHU-YO is the largest medical institution located in Ouagadougou. Over 150,000 patients are annually attended to in these three health care facilities.

Sample collection, isolation and identification of bacteria

Four hundred and eighty-six samples were collected from 486 patients (Male: 246; Female: 240; Children < 15 years: 121) from the three study locations in the following order CHU-YO 312 patients, CHUP-CDG 94 patients and HOSCO 80 patients. Samples collected included 325 urine samples, 109 pus samples, 8 blood samples, 17 stool samples, 17 vaginal swab samples and 10 pleural fluid samples. Urine and stool samples were collected in sterile universal bottles, pus, pleural fluid and vaginal swab samples were collected with sterile swab sticks and blood samples were collected in EDTA bottles. Samples were immediately transported to the laboratory in a thermo-box container at 4°C after collection for processing. All samples were cultured on Eosin Methylene Blue (EMB) and Cystine Lactose Electrolyte Deficient (CLED) agar using standard microbiological procedure and incubated at 37°C for 24 h. Presumptive colonies were subcultured on nutrient agar to obtain pure colonies. Isolates were identified using Gram staining, biochemical testing and the API 20 E gallery (BioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Susceptibility to antimicrobial agents was determined by the Kirby Bauer disc diffusion method on Muller-Hinton agar as described by the Clinical and Laboratory Standard Institute (CLSI, 2005). The antibiotic discs were obtained from BioMérieux, France (BioMérieux, Marcy l'Etoile, France). Antibiotics used were: gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), amoxicillin (20 µg), amoxicillin + clavulanic acid (20 + 10 µg), cefepime (30 µg), cefotaxime (5 µg), ceftriaxone (30 µg), cefoxitin (30 µg), imipenem (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25 - 23.75 µg) and fosfomycin (200 µg). *E. coli* ATCC 25922 was used as control for susceptibility testing.

Detection of ESBL strains

The screening and phenotypic tests for ESBL strains were performed in line with CLSI guidelines on Muller-Hinton agar. In this test, a disc of amoxycillin+clavulanic acid (20 + 10 µg) was placed at the centre of the Petri dish already inoculated with the test strain while cefepime (30 µg), cefotaxime (5 µg) and ceftriaxone (30 µg) discs were placed at a distance of 20 - 25 mm (centre to centre) from the amoxycillin+ clavulanic acid disc on the same dish. Zones

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Table 1. Distribution of strains according their origin and the clinical samples.

Strain	Health Center n(%)				Clinical Samples n(%)						
	CHU-YO	CHUP-CDG	HOSCO	Total	Urine	Pus	Blood	VS	stools	PF	Total
E. coli	194	53	49	296	211	57	1	13	5	9	296 (60.9)
Enterobacter spp.	14	03	7	24	19	3	2	0	0	0	24 (4.9)
Citrobacter spp.	6	0	0	6	5	1	0	0	0	0	6 (1.2)
Klebsiella spp.	63	32	14	109	75	26	5	3	0	0	109 (22.5)
Proteus spp.	21	0	8	29	11	17	0	1	0	0	29 (6)
Providencia sp.	1	0	0	1	1	0	0	0	0	0	1 (0.2)
Salmonella spp.	8	5	1	14	0	4	0	0	9	1	14 (2.9)
Serratia spp.	1	1	1	03	3	0	0	0	0	0	3 (0.6)
Shigella boydii	1	0	0	01	0	0	0	0	1	0	1 (0,2)
Shigella flexxneri	3	0	0	03	0	0	0	0	3	0	3 (0,6)
Total	**312 (64.2)**	**94 (19.3)**	**80(16.5)**	486 (100)	**325(66.9)**	**108 (22.2)**	**08 (1.6)**	**17(3.5)**	**18 (3.7)**	**10 (2.1)**	486 (100)

VS = Vaginal swab, PF = Pleural fluid.

of inhibition between the third generation cephalosporin discs and amoxicillin+clavulanic acid were observed after 18-24 h incubation at 37°C. Extension of inhibition zone around one or more cephalosporin discs nearest to the amoxycillin+clavulanic acid, was considered ESBL positive (CLSI, 2005).

Statistical analysis

Data was analyzed using ANOVA one-way. Chi-square test ($\chi 2$) was used to establish statistically difference in proportions for categorical data and statistical significance was set as P values of < 0.05. The statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, Inc.).

RESULTS

Distribution of strains according to origin and clinical samples

Four hundred and eighty-six isolates were obtained from all three collection centers. Three hundred and twelve (64.2%) was isolated from CHU-YO, 94 (19.3%) from CHUP-CDG and 80 (16.5%) from HOSCO. Urine yielded 325 (66.9%) of Enterobacteriaceae isolates making it the highest while 108 (22.2%) was isolated from pus. Two bacterial species were predominant in the three collection sites. *E. coli* had an occurrence of 296 (60.9%) and *Klebsiella* spp. had an occurrence of 109 (22.5%) as shown in Table 1.

Antibiotic susceptibility testing

Isolates displayed a resistance rate of 86.8% to amoxicillin and 35.2% to amoxicillin + calvulanic acid. Resistance rate to ceftriaxone and cefotaxime were 61.9% (301) and 58.6 (285), respectively. Furthermore, it was revealed that 0.2% (1) of the isolate was resistant to imipenem. Resistance rate of isolates to aminoglycosides was 12.3% (60) to amikacin and 51.0% (248) to gentamicin. Quinolones resistance rate was 68.3% (332) to nalidixic acid and 61.1% (297) to ciprofloxacin.

ESBL-producing strains

Out of 486 isolates tested, 187 (38.5%) were ESBL-producing, 127 (67.9%) from CHU-YO, 44 (23.5%) from CHUP-CDG and 16 (8.6) from HOSCO (Table 3). Plate 1 shows double disc synergy and a key hole phenomenon that was exhibited by *Klebsiella pneumonia*, and Table 4 shows the ESBL species distribution according to the sample. Difference between the proportions of ESBL isolates from the 3 sites was statistically significant ($p < 0.0001$). Furthermore, the difference between the ESBL bacteria isolated was not statistically significant ($p=0.1260$) with respect to age. In addition, 81 (43.3%) ESBL-E isolates were obtained from patients on antibiotic treatment, of which 16.7% (31/187) of antibiotics used were β-lactams.

Resistance profile of ESBL isolates to other antibiotics

The rate of resistance of ESBL isolates to other antibiotics is shown in Table 5. Resistance rates to tobramycin, nalidixic acid and ciprofloxacin in this study was 81.3% (152), 89.8% (168) and 83.4% (154), respectively. The susceptibility test of ESBL-E to aminoglycosides resulted in 3 antibiotypic profiles: the wild-type susceptible to all aminoglycosides 28 (14.9%), those that had cross-resistance to kanamycin, tobramycin gentamycin 128 (68.4%) and those that were resistance to all amino-glycosides 15 (8.0%).

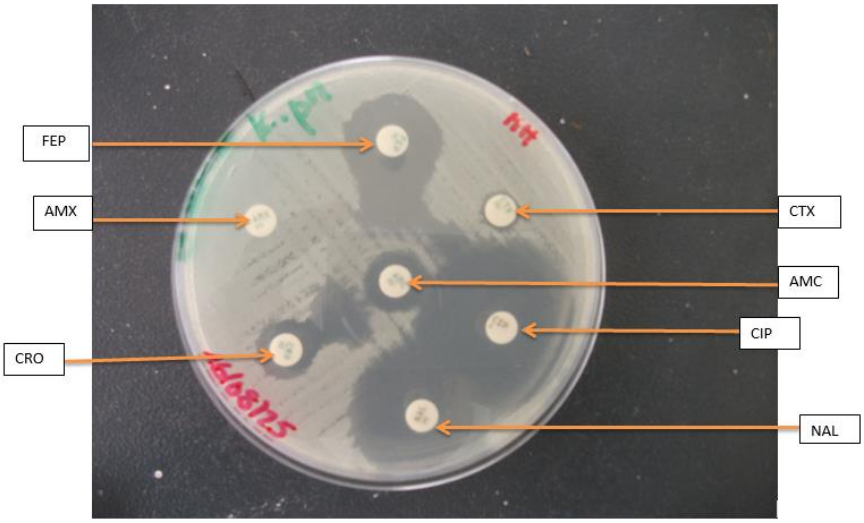

Plate 1. Representative image (*Klebsiella* spp.) of Double Disc synergy Test (DDS). Synergy between cefotaxime (CTX), ceftriaxone (CRO), cefepime (FEP) and amoxicillin + clavulanic acid (AMC) disc in center showing the keyhole phenomenon.

DISCUSSION

In this study, we determined the prevalence of ESBL producing Enterobacteriaceae (ESBL-E) and their resistance to antibiotics commonly used in the treatment of Gram negative bacterial infections in three major health care facilities in Ouagadougou. Enterobacteriaceae remains the major pathogens causing community-acquired and hospital-acquired infections including infections of the gastrointestinal tracts, urinary tract, sepsis, meningitis and medical device-associated infections (Mathlouthi et al., 2016). Urine, of all 6 clinical sample types analyzed gave the highest number of Enterobecteriaceae of which 50.1% were from Males. One hundred and eighty-seven (38.5%) of the 486 isolates obtained were ESBL producing with 21.0% from male. This is in line with the reports of Siraj et al. (2014) in Ethiopia and Ouedraogo et al. (2016) in Burkina Faso in which urine yielded a higher number of bacterial isolates. Hijazi et al. (2016) also reported a similar finding in Lebanon with male children having a higher colonization frequency (33.9%) of ESBL-E in contrast to their female counterparts that had a frequency of (15.9%). However this observation is a deviation from the normal trend of having more bacterial isolates from female urine samples since they were more at risk of acquiring urinary tract infection compared to their male counterparts (Ameri et al., 2014). *E. coli* and *Klebsiella* species has been identified as members of Entero-bacteriaceae that paly a lead role in hospital/community acquired infections, which is not different from our findings with *E. coli* and *Klebsiella* spp. being the most prevalent. *E. coli* had an occurrence frequency of 296 (60.9%) while 109 (22.5%) *Klebsiella* spp. were recorded. In a related study, Manjula et al. (2013) reported a high prevalence of *E. coli* (56.79%) and *Klebsiella* spp. (19.9%) isolated from patients in Karnataka region India having urinary tract infection. Similarly, in Dakar Senegal, *Klebsiella* spp. was reported to be the major ESBL-E isolated from patients (Ndir et al., 2016). Antibiotic resistance is a global problem which varies across countries as a result of hygiene levels in hospital and antibiotic management policies. As shown in this study, resistance of Enterobacteriaceae to regularly used antibiotics is unflinching and ever evolving. There was 86.8% resistance to amoxicillin, 35.2% resistance to amoxicillin + clavulanic acid. This rate of resistance could be attributed to selective pressure since these antibiotics has overtime been a first line drug in the treatment of bacterial infections. Isolates also displayed a remarkable resistance to cephalosporins tested. There was an average resistance of 58.5% to cefotaxime and cefepime, while resistance to ceftriaxone and cefoxitine was 61.9 and 26.1% respectively (Table 2). Mathlouthi et al. (2016) affirm this finding in their report of isolates from Tunisian and Libyan hospitals with 80% resistance to ceftazidime, cefotaxime, amoxicillin + clavulanic acid, amoxicillin and ciprofloxacin. Our report of 38.5% prevalence of ESBL-E in this study is relatively high; however a similar study by Ouedraogo et al. (2016) in Burkina Faso recorded a higher prevalence of 58%. This variation in findings could be explained by the size, duration and area where the two studies were conducted. The clinical impact of ESBL-producing pathogens on morbidity and mortality in infectious diseases in both children and adults as well as their economic burden are well documented (Lukac et al., 2015). Thus, ESBL-E is a threat that should be tackled head on. Resistance of ESBL-E to aminoglycoside was

Table 2. Susceptibility rate of 486 strains of *Enterobacteriaceae* to antibiotics in Ouagadougou.

Antibiotic	Susceptibility rate	
	S(%)	I +R (%)
Gentamicin	238 (49.0)	248 (51.0)
Amikacin	426 (87.7)	60 (12.3)
Tobramicin	221 (45.5)	265 (54.5)
Amoxicillin	64 (13.2)	422 (86.8)
Amoxicillin/ clavulanic acid	315 (64.8)	171 (35.2)
Cefoxitine	359 (73.9)	127 (26.1)
Ceftriaxone	185 (38.1)	301 (61.9)
Cefotaxime	201 (41.4)	285 (58.6)
Cefepime	202 (41.6)	284 (58.4)
Imipenem	485 (99.8)	1 (0.2)
Nalidixic acid	154 (31.7)	332 (68.3)
Ciprofloxacin	189 (38.9)	297 (61.1)
Triméthoprim-sulfaméthoxazole	91 (18.7)	395 (81.3)
Fosfomycin	406 (83.5)	80 (16.5)

S = susceptible, R = resistant, I = Intermediate.

Table 3. Distribution of clinical isolates according the collection site, sex, age and the clinical samples.

Variable	Collection site						Total	
	CHU-YO (N=312)		CHUP-CDG (N=94)		HOSCO (N=80)		(N=486)	
	E-ESBL (N)	Not E-ESBL (n)	E-ESBL (n)	Not E-ESBL (n)	E-ESBL (n)	Not E-ESBL (n)	E-ESBL [n(%)]	Not E-ESBL [n(%)]
Sex								
F n(%)	59	86	23	26	3	43	85 (17.5)	155 (31.9)
M n(%)	68	99	21	24	13	21	102 (21.0)	144 (29.6)
Total n(%)	127	185	44	50	16	64	187 (38.5)	299 (61.5)
Age (year)								
[0-15]	10	16	41	45	3	6	54 (11.1)	67 (13.8)
[15-30]	27	38	2	1	2	18	31 (6.4)	57 (11.7)
[30-45]	33	52	0	3	8	17	41 (8.4)	72 (14.8)
[45-60]	28	29	0	1	0	9	28 (5.8)	39 (8.0)
>60	29	50	1	0	3	14	33 (6.8)	64 (13.2)
Total	127	185	44	50	16	64	187 (38.5)	299 (61.5)
Pathological products								
Urines	80	124	23	32	13	54	116 (23.9)	210 (43.2)
Pus	34	44	17	12	0	2	51 (10.5)	58 (11.9)
VS	3	7	0	0	0	6	3 (0.6)	13 (2.7)
Stools	4	5	0	3	3	2	7 (1.4)	10 (2.1)
Blood	1	0	4	3	0	0	5 (1.0)	3 (0.6)
Other	5	5	0	0	0	0	5 (1.0)	5 (1.0)
Total	127	185	44	50	16	64	187 (38.5)	299 (61.5)

F = female M = Male; VS = Vaginal Swab; Other = Pleural fluid; E-ESBL = *Enterobacteriaceae*-producing Extended Spectrum B-lactamases.

observed. There was 71.7% resistance to gentamycin and 81.3% to tobramycin. This observation is in consonant with the report of Obeng-Nkrumah et al., (2013) that reported 91.2% of ESBL-E resistance to gentamycin in Ghana. The resistance of ESBL-E isolates to quinolones was 89.8% for nalidixic acid and 83.4% for

Table 4. Distribution of BLSE-producing strains according to the pathological products in Ouagadougou.

Strains	Clinical Samples [n (%)]						Total
	Urines	Pus	Blood	VS	Stools	PF	
E. coli	73	32	1	3	3	5	117 (62.6)
Klebsiella spp.	32	10	5	1	0	0	48 (25.7)
Proteus spp.	2	3	0	0	0	0	5 (2.7)
Enterobacter spp.	4	2	2	0	0	0	8 (4,3)
Citrobacter spp.	2	0	0	0	0	0	2 (1.1)
Salmonella sp.	1	1	0	0	3	0	5 (2.7)
Serratia spp.	1	0	0	0	0	0	1 (0.5)
Shigella flexneri	0	0	0	0	1	0	1 (0.5)
Total	115(61.5)	48(25.7)	8(4.3)	4(2.1)	7(3.7)	5 (2.7)	187 (100.0)

VS = Vaginal swab; PF = Pleural fluid.

Table 5. Susceptibility rate of 187 ESBL producing strains to antibiotics in Ouagadougou.

Antibiotics	Susceptibility rate		
	I (N, %)	R(%)	I+R(%)
Gentamicin	7(3.7)	127(67.9)	134 (71.7)
Amikacin	2(1.1)	12(6.4)	14 (7.5)
Tobramicin	4(2.1)	148(79.1)	152 (81.3)
Amoxicillin	0	182(97.1)	182 (97.1)
Amoxicillin/ clavulanic acid	7(3.7)	60(32.1)	67 (35.8)
Cefoxitine	1(0.5)	42(22.4)	43 (23.0)
Ceftriaxone	2(1.1)	177(94.6)	179 (95.7)
Cefotaxime	2(1.1)	179(95.7)	181 (96.8)
Cefepime	7(3.7)	166(88.8)	173 (92.5)
Imipenem	0	0	0
Nalidixic acid	9(4.8)	159(85.0)	168 (89.8)
Ciprofloxacin	10(5.3)	146(78.1)	156 (83.4)
Triméthoprim-sulfaméthoxazole	0	163(87.2)	163 (87.2)
Fosfomycin	5(2.7)	19(10.2)	24 (12.8)

S = susceptible, R = resistant, I = Intermediate.

ciprofloxacin. For other antibiotics, we observed a high rate of resistance to trimethoprim-sulfamethoxazole (87.2%). Both ESBL-E and non ESBL-E isolates were susceptible to imipenem and fosfomycin. Bourjilat et al. (2011) observed a similar trend in Morocco with all ESBL-producing isolates having total susceptibility to imipenem and fosfomycin. Also, Patwardhan and Singh (2017) in India reported 1,223 (96.5%) ESBL-producing Gram negative isolates that were susceptible to fosfomycin. This gives a glimpse of hope as this two antibiotics are still very much active against ESBL-E and can serve as a ready remedy when clinicians are confronted with multi-drug resistant ESBL-E. Conversely there should be caution in the use of these molecules as resistance to imipenem is beginning to emerge (Haidar et al., 2017). In Burkina Faso, as in many other African countries, the lack of antibiotic surveillance system, unfavorable hygiene conditions in hospitals, may be attributed to the spread of ESBL, as has been reflected in this study.

Conclusion

This study demonstrates the high prevalence of ESBL-producing Enterobacteriaceae in Ouagadougou. The spread of ESBL strains reduces the successful treatment ESBL bacterial infections. Nevertheless, ESBL bacteria remained susceptible to imipenem and fosfomycin, which are often drugs of choice for severe infections. This therefore highlights the need for routine detection and systematic reporting of ESBL bacteria in Burkina Faso to avoid therapeutic failures and the spread of these bacteria for effective management of bacterial infectious diseases. Clinicians must be cautious in the prescription

of antibiotics. Furthermore, antibiotic policy use is needed to limit the emergence and spread of ESBL strains.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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