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Full Length Research Paper

Production, optimization and characterization of extracellular amylase from halophilic *Bacillus lichineformis* AH214

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Twenty one moderately halophilic bacterial strains were isolated from seawater and sediment in Alexandria Eastern Harbour, Egypt. The isolates were screened for the production of four extracellular degradative enzymes. The majority of isolates (57.1%) possessed significant enzyme activities, 43% of them have potentiality to produce amylase enzyme. The most active isolate for the production of amylase enzyme was identified by using a 16S rRNA sequence analysis as *Bacillus lichineformis* AH214. Optimization of the fermentation medium components and environmental factors using One Variable at a Time Approach and Plackett-Burman design was applied to enhance the amylase production by *Bacillus lichineformis* AH214. The maximum microbial amylase production could be achieved using an optimized medium of the following composition (g/l): 1.0 g yeast extract, 0.05 g K_2HPO_4, 0.25 g $FeCl_3$, 15.0 g starch, 30.0 g NaCl, 0.75 g $MgSO_4.7H_2O$ and inoculums size of 1.5 ml/50 ml and incubated at optimum conditions of pH 7, agitation speed 160 rpm, time 30 h and temperature 40°C. On applying optimized medium in the fermentation process, an enzyme productivity of 13.44 U/mg protein was achieved with two fold increase compared to the basal one. The crude amylase produced by *Bacillus lichineformis* was stable up to 40°C, pH 7.5 and 1.5 M NaCl.

Key words: Halophiles, amylase, *Bacillus lichineformis*, Plackett-Burman, optimization.

INTRODUCTION

Enzymes are vitally important to existence of life itself, capable to act as biocatalyst for a wide variety of chemical reactions (Van der Maarel et al., 2002). Salts are required for all life forms and halophiles are distinguished by their requirement of high salinity conditions for growth. Based on definition of Das Sarma et al. (2012), the term of moderate halophiles was applied for microorganisms that grow optimally or very well at 0.85 to 3.4 M (5-20%) NaCl. The ability of halophiles to produce hydrolytic extremozymes has been much studied

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for its possible applications in industries (Moreno et al., 2013; Ali et al., 2014). Among bacteria, the use of halophiles in comparison to the extensive use of extremozymes from thermophiles and alkaliphiles is very low. Halophilic enzymes have thus far found applications in industries and biotechnology (Abdu Al-ZaZaee et al., 2011; Moreno et al., 2013). Mostly halophilic hydrolases such as amylases, cellulases, lipases, xylanases, and proteases have been reported from halophilic bacteria (Abdu Al-ZaZaee et al., 2011; Moreno et al., 2013; Ali et al., 2014). Amylases are among the most important industrial enzymes and also have great significance in biotechnological studies (Van der Maarel et al., 2002).

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Abdel-Fattah et al., 2013). Three kinds of amylase were divided by Ekunsaumi (2002) based on their ability to breakdown amylum. They were alpha amylase, beta amylase and amyloglucosidase. Amylase can be simply classified in two groups. (1) Endo–acting or endo–hydrolases e.g. α-amylase: -α-Amylases (1, 4-α-glucan-glucanohydrolases) are extracellular enzymes which hydrolyze α-1, 4-glycosidic bonds. These enzymes are endoenzymes, splitting the substrate in the interiors of the molecule. (2) Exo-amylase or exo-hydrolases e.g., β-amylases, glucosidase and α-glucosidase. Glucoamylases (α-1, 4-glucan-glucohydrolases) act on starch by splitting glucose units from the non-reducing end. β–glucosidase is usually of plant origin, but some microbes are also known to produce it (Parmar and Pandya, 2012).

Although amylases can be derived from several sources, including plants, animals, and microorganisms, microbial enzymes generally meet industrial demands (Abdel-Fattah et al., 2013; Alariya et al., 2013; Panneerselvam and Elavarasi, 2015). These enzymes were isolated from many microbial sources including bacteria, fungi and some actinomycetes, that are resistant to high salt concentration, temperature and pH (Abdu Al-ZaZaee et al., 2011).

Totay, α-amylases of microbial origin have replaced the chemical hydrolysis of starch in starch processing industry (Pandey et al., 2000). The major advantages of using microorganisms for the production of amylases are the economical bulk production capacity and, secondly, the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics (Abdel-Fattah et al., 2012; Panneerselvam and Elavarasi, 2015). The α-amylase is an important class of amylases which constitutes approximately 25 to 30% of its share in total enzyme market (Abdu Al-ZaZaee et al., 2011; Ali et al., 2014).

Bacillus sp.*, Halobacillus* sp.*, Haloferax mediterranei* and *Halothermothrix sp.* are known to be good producer of α-amylase. However, the production of α- amylases depends on the strain, composition of media, metal ions, pH, temperature, moisture and oxidative stress. Due to extensive industrial uses which withstand the high load of salt concentration about 10 to 25% and other industrial harsh conditions, enzymes from halophilic bacteria are frequently preferred (Abdu Al-ZaZaee et al., 2011).

Amylases have extensive applications in textile industries, detergent manufacturing processes pharmaceutical, and food industries. The microbial amylases could be potentially useful in various pharmaceutical, fine-chemical industries etc. With the event of new frontiers in biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry (Asgher et al., 2007; Liu and Xu, 2008; Shafiei et al., 2010; Abdel-Fattah et al., 2013; Ali et al., 2014).

Each application of amylase requires unique properties with respect to specificity, stability, and temperature and pH values dependence. Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications (Abdel-Fattah et al., 2013).

Enzyme overproduction can be achieved by both genetic manipulations and media engineering. As excretion of metabolism products is a part of survival strategy of microbes in certain environments, overproduction of enzymes by media manipulation may be considered a better strategy (Abdel-Fattah et al., 2013).

The classical method for medium optimization involves changing one independent variable, keeping the other factors constant OVAT (one-variable-at-a-time). This method is time-consuming and incapable of detecting the true optimum, due to the interactions among the factors and this limitation of a single factor optimization process can be eliminated by different techniques (Abdel-Fattah et al., 2013).

Unlike conventional optimization, statistical optimization methods present a more balanced alternative to the OVAT approach, since it takes into account the interaction of variables in generating the process response. Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response. Recently, the results analyzed by a statistical planned experiment are better acknowledged than those carried out by the traditional OVAT method. Some of the popular choices, applying statistical designs to bioprocessing, include the Plackett-Burman design (El-Sharouny et al., 2015).

Halophiles have been perceived as a potential source of industrially useful enzymes endowed with exceptional stabilities. The present study focuses on the (i) isolation of moderate halophilic bacteria from seawater and sediment samples collected along Alexandria Eastern Harbour, Egypt, (ii) screening for industrially important enzymes (especially amylase, lipase, cellulase and

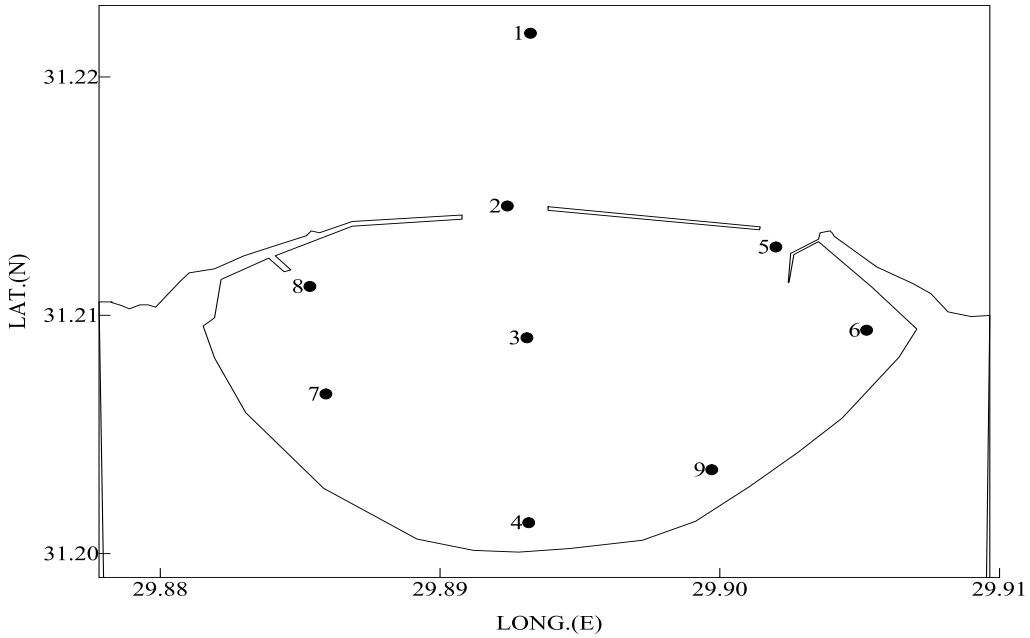

Figure 1. Location of sampling sits along Alexandria Eastern Harbour, Egypt.

protease) and (iii) studying the optimization of fermentation conditions and properties for amylase enzyme.

MATERIALS AND METHODS

Sampling

Sediment sample were collected from nine sites along Alexandria Eastern Harbour, Egypt (Figure 1), during spring 2012. Sampling was performed according to the World Health Organization manual for recreational water and beach quality monitoring and assessment (Clesceri et al., 2012).

Isolation of bacteria

Total heterotrophic marine bacteria (THB) were counted using standard pour plate method into Marine agar 2216 (MA), (Oxoid LTD, England). Plates were incubated at 30°C and final counts of colony forming units (CFU) taken after 24 to 48 h (Clesceri et al., 2012).

Halophilic bacteria were isolated using standard pour plate method into MH medium. The composition of the isolation medium was as follows: Yeast extract, 1% (w/v); peptone 0.5% (w/v); glucose, 0.1% (w/v); and Bacto Agar, 2% (w/v). This medium was supplemented with a balanced mixture of sea salts to give final concentrations of 10% (w/v). The pH was adjusted to 7.2 with 1 M KOH. The final counts of colony forming units (CFU) were taken after incubation at 30°C for 5 days in sealed plastic (Quesada et al., 1984).

Enzymatic profile of bacterial isolates

The ability of halophilic bacterial isolates to produce extracellular degradative enzymes was examined on MH medium agar plates (Quesada et al., 1984). The plates were amended with starch, olive oil, skimmed milk, or carboxymethylcellulose at 4 g/l to detect the production of amylase, lipase, protease, or cellulase, respectively. Formation of hydrolytic zone (clear zone, mm) around the colonies, resulting from polymer hydrolysis, was taken as evidence of hydrolytic activity (Moreno et al., 2013).

Selection of amylase producing bacteria

For selection of amylase- producing bacteria from marine isolates, qualitative determination of α-amylase was carried out using well cut or cup assay with some modifications (Sudharhsan et al., 2007). The agar plates were amended with 1% of starch and 1.5% of agar for well-cut assay. After agar solidification, around 10 mm diameter of well was cut out aseptically using cork borer. The well was filled with the culture filtrate (100 μl), incubated overnight at 35°C. The plates were flooded with Lugol solution (1% iodine in 2% potassium iodide w/v), for 1 min until the entire medium became colored in blue, then the hydrolytic zone around the well (clear zone) is measured. The negative control is maintained by adding sterile water in a separate well (Amoozegar et al., 2003).

Culture condition

Sterilized nutrient broth (20 ml) was inoculated with bacterial suspension of 24 h bacterial slant and incubated in orbital shaker at 150 rpm (35°C) until the absorbance at A_{600} nm reached to 0.15 (cell density about 1×10^6 CFU/ml).

Production of amylase

The production medium for amylase consisted (g/l): 3.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H$ O, 0.1 g K_2HPO_4, 0.5 mg $FeCl_3$, 20 g NaCl, 10.0 g starch and pH 7.5. The media was inoculated with 1% ($1x\ 10^6$ CFU/ml) and incubated with shaking (150 rpm) at 35°C for different time intervals in an orbital shaker incubator.

Enzyme assay for amylase

Amylase was determined by spectrophotometric method as described by Fisher and Stein (1961). According to procedure 1.0 ml of culture broth was taken in test tube in duplicate and 1.0 ml of substrate (starch) was added in test tube. The test tubes were incubated at 35°C for 15 min in water bath. Then 2.0 ml dinitro-salicylic acid reagent was added in each tube to stop the reaction and kept in boiling water bath for 15 min. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer. A unit of amylase activity was defined as the amount of amylase required to catalyze the liberation of reducing sugar equivalent to one ì mol of D-glucose per minute under the assay conditions (Miller, 1959).

Estimation of protein content

The total protein was estimated using Lowry's method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as a standard.

Bacterial identification

Genomic DNAs of the selected isolate was extracted with the genomic DNA extraction protocol of Gene Jet genomic DNA purification Kit (Fermentas). Polymerase chain reaction (PCR) using Maxima Hot Start PCR Master Mix (Fermentas).The amplifications were carried out in a thermal cycler (Multigene Optimax, Labnet international, Inc). The PCR thermocycler was programmed as follow: 95°C for 5 min for initial denaturation, 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. The PCR mixture contained 25 pmol of each primer, 10 ngchromosomal DNA, 200 mmol/LdNTPs and 2.5 U of *Taq* Polymerase in 50 µl of Taq polymerase buffer 10X Standard Taq Reaction Buffer.

The PCR Clean-Up of the PCR product was performed by using Gene JET™ PCR Purification Kit (Fermentas) at Sigma Scientific Services Company, Egypt, 2013. The sequencing of the PCR product was made by the GATC Company by using ABI 3730xl DNA sequencer with universal primers (16S 27F and 16S 1492R), (5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′). Genotypic characterization was performed using 16S sequence analysis. Multiple alignments with sequences of the most closely related members and calculations of levels of sequence similarity were carried out using BioEdit (software version 7) (Hall, 1999). Sequences of rRNA genes, for comparison, were obtained from the National Center for Biotechnology Information (NCBI) database.

Effect of different carbon and nitrogen sources on amylase production

This was studied by adjusting different carbon sources as starchy substrates (starch, rice kerench, wheat bran, potato cubes and potato peels), different sugars (maltose, glucose, sucrose, fructose, dextrose and lactose) and different nitrogen sources ($(NH4)_2SO_4$, NH_4Cl, $NaNO_3$, KNO_3, urea, peptone yeast extract and casein) in the production medium (Dharani, 2004; Ashwini et al., 2011).

Effect of incubation time, agitation, temperature and pH on amylase production

The bacterial isolate was grown on the production medium at different incubation time (6, 12, 18, 24, 30, 36 and 48 h), different speed (80, 120, 160, 180, 200 and 240 rpm), different temperatures (20, 25, 30, 35, 40, 45 and 50°C) and different pH (4, 5, 6, 6.5, 7, 7.5, 8, 9 and 10) (Ashwini et al., 2011).

Table 1. Factors examined as independent variables affecting amylase enzyme production and their levels in the Plackett-Burman experimental design.

Factors	Symbol	Levels		
		-1	0	1
Yeast extract	Ye	1	2	3
K_2HPO_4	K2	0.05	0.1	1.05
Starch	St	5	10	15
NaCl	Na	10	20	30
$MgSO_4.7\ H_2O$	Mg	0.25	0.5	0.75
$FeCl_3$	Fe	0.25	0.5	0.75
Size of inoculums*	IS	0.5	1	1.5

*cell density about 1×10^6 CFU/ml.

Optimization of growth medium using Plackett-Burman experimental design

The Plackett-Burman design was proved to be a powerful tool to rapidly determine the effects of medium constituents on amylase production. The Plackett-Burman experimental design (Plackett and Burman, 1946), a fractional factorial design was used in this research to reflect the relative importance of various growth media component factors on amylase activity in liquid cultures. In amylase assay experiment, seven independent variables were screened in eight combinations organized according to the Plackett-Burman design matrix (Tables 1 and 3). For each variable, the high (+1) and low (-1) levels were tested. Medium components are given in g/l and inoculum size was added in ml with culture (A_{600} = 0.15). All trials were performed in duplicates and the average of amylase activity results were treated as the responses. The main effect of each variable was determined by the following equation: $E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N$. Where Exi is the variable main effect, Mi+ and Mi- are the amylase production in trials where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. A main effect with a positive sign indicates that the high concentration of this variable is nearer to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance.

Verification experiments

A verification experiment was carried out in triplicates, the predicted optimum levels of the independent variables were examined and compared to the basal condition setting and the average of enzyme activity was calculated. Moreover, the Plackett-Burman reverse medium was applied.

Characterization of the crude enzyme

The effect of temperature and pH on the enzyme activity was determined. The standard assay conditions were used for 15 min at temperatures between 25 and 60°C. The pH of the reaction mixture was ranged from 4 to 10 using 50 mm buffers (Sodium acetate pH 3.6-6.5, Tris-HCl pH 7 -8.5 and glycine buffer 9-10).

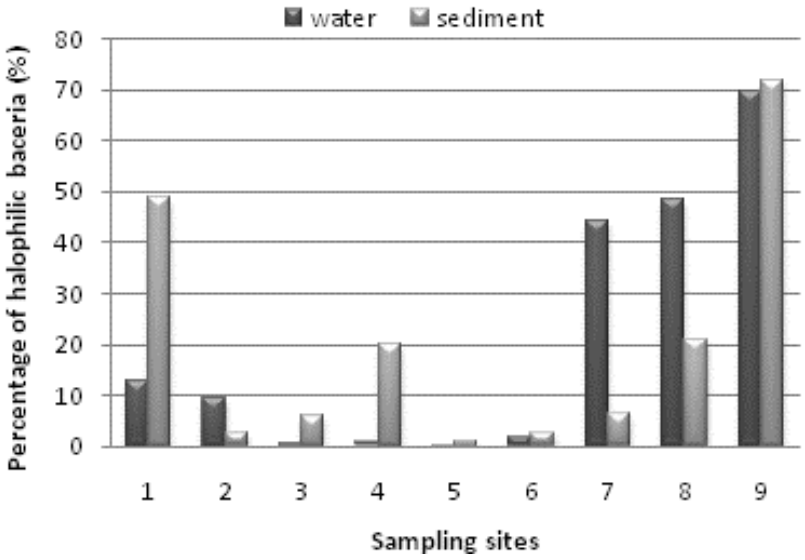

Figure 2. The percentage (%) of moderately halophilic bacteria with respect to the total heterotrophic bacteria isolated from seawater and sediments samples along Alexandria Eastern Harbour, Egypt.

Effect of salt tolerance of amylase

The crude amylase enzyme was incubated in 5 mM phosphate buffer (pH 7.5) containing different NaCl concentrations (0 to 5 M) for 2 and 12 h at 4°C, and enzyme activities were estimated in the same way as described above.

RESULTS

Isolation of halophiles

The distribution of halophiles in seawater and sediment along Alexandria Eastern Harbour of Egypt was investigated (Figure 2). The moderate halophiles bacteria was isolated at 10% NaCl, it ranged from 1.2×10^2 to 8.8×10^2 CFU/ml in seawater and from 7.0×10^3 to 2.8×10^5 CFU/g in sediment. The percentage of moderately halophilic bacteria with respect to the total heterotrophic bacteria ranged from 0.12 to 69.60% in seawater while it ranged from 0.88 to 48.88% in sediment.

Screening of hydrolase producers

Twenty one morphologically different colonies able to grow well at 10% NaCl were obtained from seawater and sediment samples, and tested for production of extracellular hydrolytic enzymes. The majority of moderate halophilic bacterial isolates (57%) expressed significant enzyme activity when applied on substrates like starch, olive oil, carboxy methyl cellulose or skimmed milk. Based on the preliminary screening experiment, twelve bacterial isolates have potentiality to produce one or more of the hydrolase enzymes, while most bacterial isolates (43%, nine out of twenty one isolates) have potentiality to produce amylase enzyme (Table 2).

Screening of amylase producers

In the present study a total of nine bacterial isolates were selected and qualitative determination of amylase activity was performed. Among these only three isolates showed the highest hydrolytic zone around the well, while the hydrolytic zone were 36, 28 and 19 mm for isolates 7W-1, 3S-3 and 2S-1, respectively. Based on the data obtained, isolate 7W-1 was selected for identification and study the production of amylase enzyme.

Identification of amylase producing bacterial isolate

For molecular characterization, DNA sequencing of 16S rDNA of the selected isolate (isolate 7W-1), showed a highest similarity of 99.7% to *Bacillus lichineformis* (Figure 3), isolate 7W-1 was identified as *Bacillus lichineformis* AH214 and the nucleotide sequence was deposited to National Center for Biotechnology Information (NCBI) data bank with the accession number of KT199247.

Pre-optimization experiments by one variable at a time approach

Effect of starchy substrates and incubation period on amylase production

The enzyme production varies with incubation period of

Table 2. Enzymatic profiles of selected bacterial isolates.

Isolate code	Amylase	Lipase	Cellulase	Protease
	Hydrolytic zones (mm)			
1S-1	25	0	0	0
2S-1	20	0	0	0
3S-3	14	0	0	0
7S-1	0	14	0	0
7S-2	19	29	17	0
7S-3	14	0	0	0
8S-2	13	0	0	0
9S-1	24	0	0	0
2W-1	21	24	19	0
3W-1	0	15	0	0
7W-1	28	21	16	0
8W-1	0	18	14	0

S, isolated from sediment samples; W**,** isolated from seawater.

Table 3. The Plackett-Burman experimental design matrix for seven factors.

Trials	ye	K2	st	Na	Mg	Fe	Is	Protein content (mg/ml)	Enzyme activity (U/mg protein)
1	-1	-1	-1	1	1	1	-1	3.14	9.17
2	1	-1	-1	-1	-1	1	1	2.44	9.38
3	-1	1	-1	-1	1	-1	1	2.63	9.47
4	1	1	-1	1	-1	-1	-1	2.43	8.78
5	-1	-1	1	1	-1	-1	1	3.23	12.49
6	1	-1	1	-1	1	-1	-1	3.45	9.90
7	-1	1	1	-1	-1	1	-1	3.02	9.17
8	1	1	1	1	1	1	1	3.45	12.05
9	0	0	0	0	0	0	0	3.55	10.25

the *B. licheniformis* cells. The results (Figure 4) indicated that the production of amylase enzyme increased gradually from 18 h (4.99 U/mg protein) and reached its maximum (6.99 U/mg protein) at 30 h of incubation, thereafter, the enzyme production started decreasing.

The different starchy substrates were used to substitute pure starch in the fermentation medium. The results given in Figure 4 Revealed that the maximum amylase production (6.99 U/mg protein) was recorded in starch supplemented medium and minimum amylase production was recorded in wheat bran (5.34 U/mg protein).

Effect of carbon source on amylase production

The addition of carbon source in the form of either monosaccharide or polysaccharides may influence the production of amylase enzyme. The influence of maltose was more effected (6.29 U/mg protein) than the other sugar sources tested. Glucose was the second best supplementary carbon source (5.23 U/mg protein). Lactose gave the lowest amylase enzyme activity (1.34 U/mg protein) (Figure 5).

Effect of nitrogen source on amylase production

The nitrogen sources are secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The effect of supplementary nitrogen sources on amylase production by *B. licheniformis* cells showed that yeast extract was the better nitrogen source for this isolate (8.12 U/mg protein) (Figure 6).

Effect of agitation speed, temperature and pH on amylase production

The production of amylase by *B. licheniformis* was

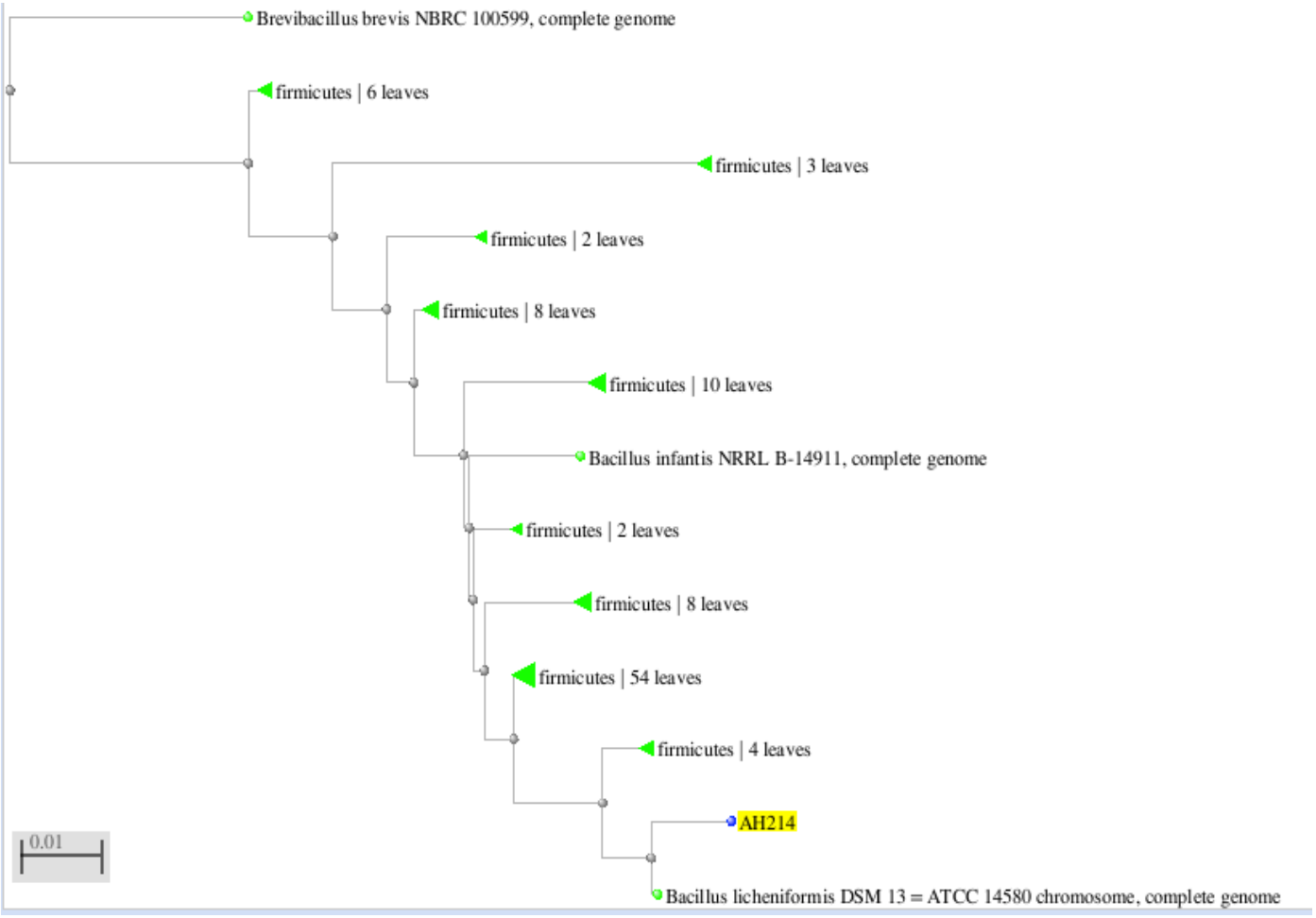

Figure 3. Phylogenetic relationship of strain *B. lichineformis* AH214 (isolate 7W-1) and the most closely related strains presented in the Genbank database.

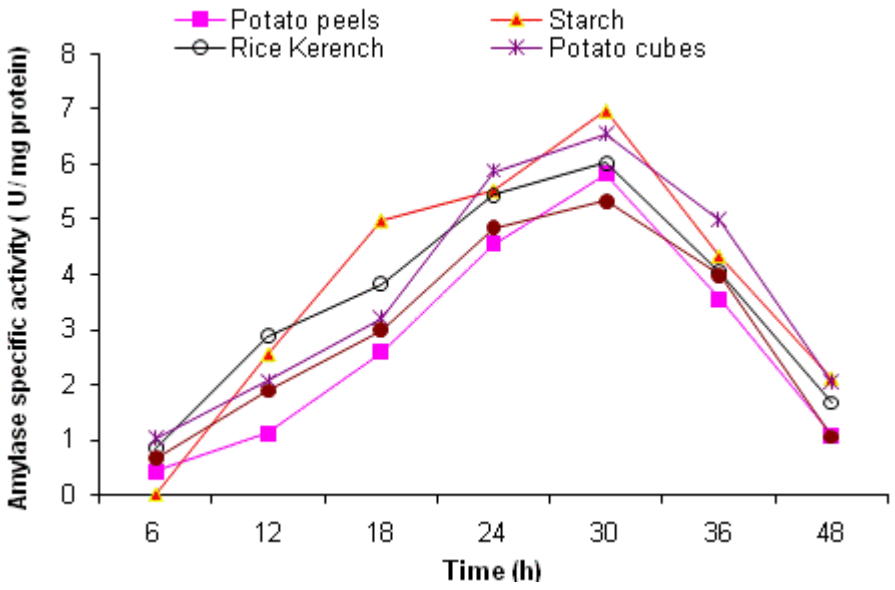

Figure 4. The effect of different starchy substrates and different incubation times on amylase production from *B. licheniformis.*

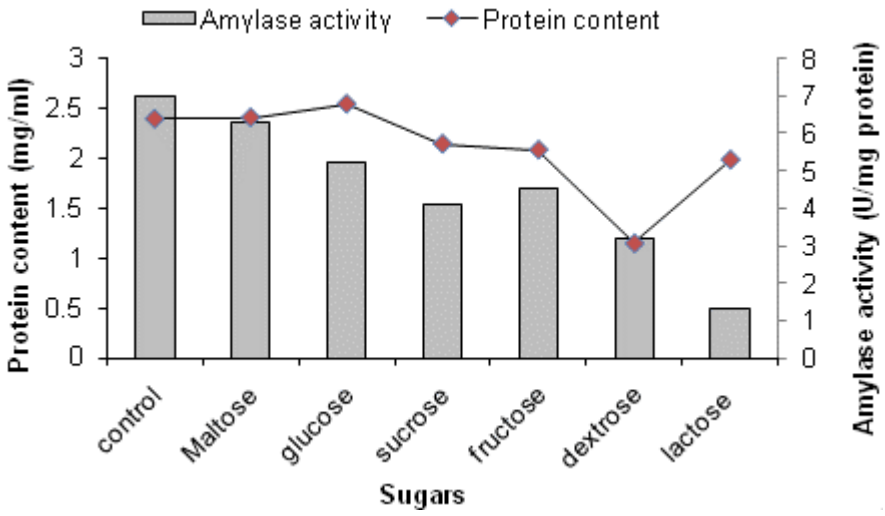

Figure 5. The effect of different carbon sources on amylase production from *B. licheniformis.*

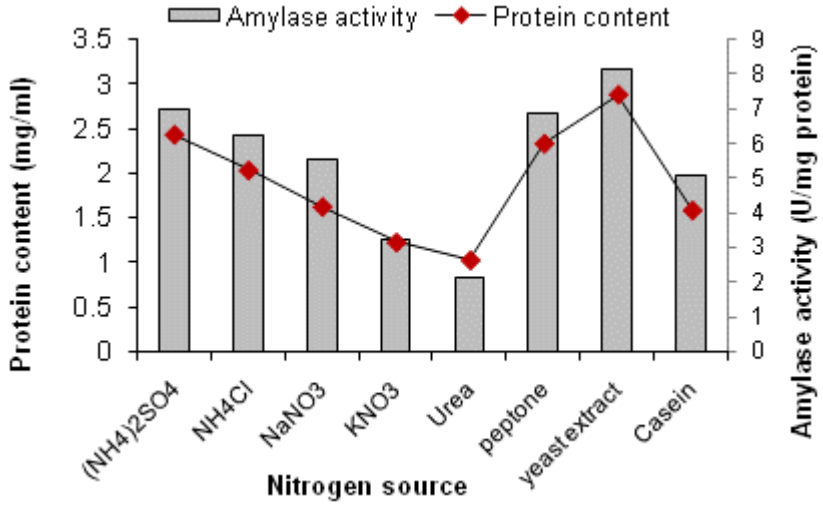

Figure 6. The effect of different nitrogen sources on amylase production from *B. licheniformis.*

investigated at different speed of agitation. The production of amylase enzyme increased gradually by increasing agitation and reached its maximum (8.16 U/mg protein) at 160 rpm of incubation, thereafter, the enzyme production started decreasing, compared by static incubation (6.56 U/mg protein) (Figure 7A)**.**

The results represented in Figure 7B showed that the optimum temperature which yielded the highest enzyme activity (10.4 U/mg protein) was 40°C. Higher or lower temperatures have an adverse effect on the amylase activity; the lowest activity (2.04 U/mg protein) was demonstrated at an incubation temperature of 20°C. Also, the protein content increased gradually by increasing the incubation temperature and reached its maximum value at 40°C.

The production of amylase was investigated at different pH values ranging from 4.0 to 10.0. The results presented in Figure 7C showed that enzyme synthesis of *B. licheniformis* was observed between pH 6.0 and 8.0. The pH 7 was the optimum pH value for amylase production (11.92 U/mg protein). When pH is altered below or above the optimum, the activity is decreased or becomes denatured.

Plackett-Burman design for optimization of *α*-amylase production by *Bacillus licheniformis*

Plackett-Burman design was employed to identify significant variables that enhance amylase production

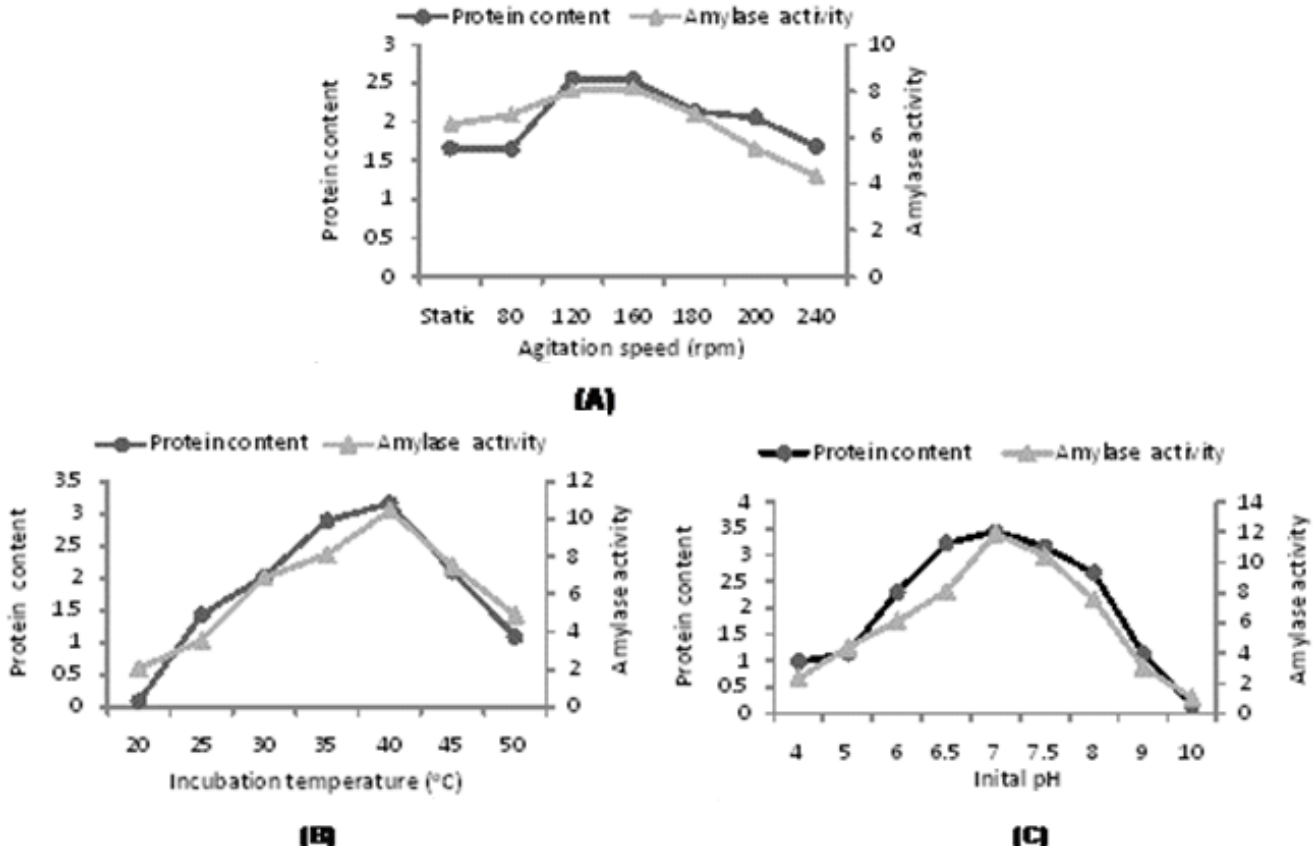

Figure 7. The effect of different (A) agitation speed, (B) incubation temperature and (C) pH on protein content (mg/ml) and amylase activity production (U/ mg protein) by *B. licheniformis.*

and to find out their probable optimal levels in a limited number of experiments. In this study, seven variables were analyzed with regard to their effects on enzyme production using a Plackett-Burman design. The independent variables were examined and their settings are shown in (Table 1).

The seven nutritional factors were yeast extract, K_2HPO_4, starch, NaCl, $MgSO_4.7\ H_2O$, $FeCl_3$ and inoculum size. The environmental factors were kept at optimum conditions of pH 7, agitation speed 160 rpm, incubation time 30 h and temperature 40°C. The seven variables were carried out by eight trials according to a design matrix (Table 3), which was based on the number of variables to be investigated.

The protein content and enzyme activity were measured (Table 3). For determination of variable significance, statistical t-values for equal unpaired samples were calculated with respect to observations. The necessary statistical analyses of this experiment are shown in Table 4. The main effect of each variable upon amylase activity was estimated and presented graphically in Figure 8. The production of amylase by *B. licheniformis* was positively affected by starch, NaCl, $MgSO_4.7H_2O$ and inoculums size, and negatively affected by yeast extract**,** K_2HPO_4 and $FeCl_3$, that is, the high concentration of yeast extract had the most significant negative effect on amylase production and the high concentration of starch had the most significant positive effect on amylase production by *B. licheniformis*. Therefore, decreasing the yeast extract concentration and increasing the starch concentration in the culture medium will enhance the extracellular amylase production.

According to the data obtained from the Plackett-Burman experimental results and all calculations related

Table 4. Statistical analysis of the Plackett-Burman experimental result for *B. licheniformis.*

Variable	Main effect	T-test
yeast extract	-0.046	-0.043
K_2HPO_4	-0.36	-0.344
Starch	1.701	**2.065**
NaCl	1.142	1.176
$MgSO_4.7H_2O$	0.191	0.178
$Fecl_3$	-0.219	-0.204
Size of inoculum	1.596	1.859

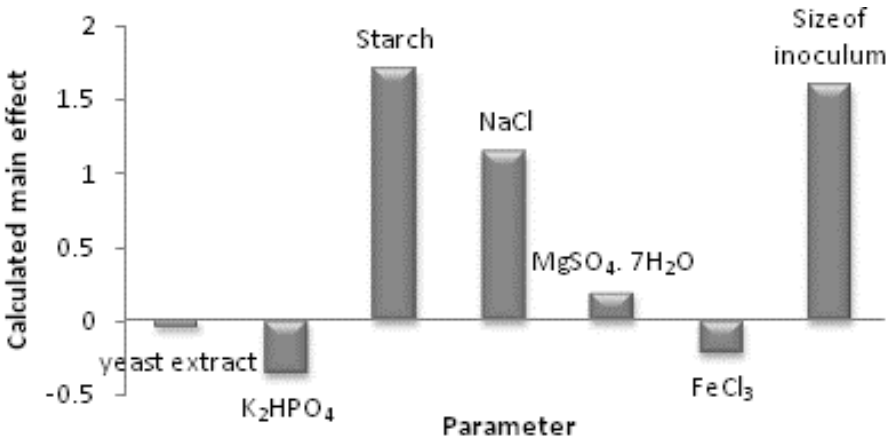

Figure 8. Elucidation of cultivation factors affecting amylase production by *B. licheniformis* using Plackett-Burman experimental design.

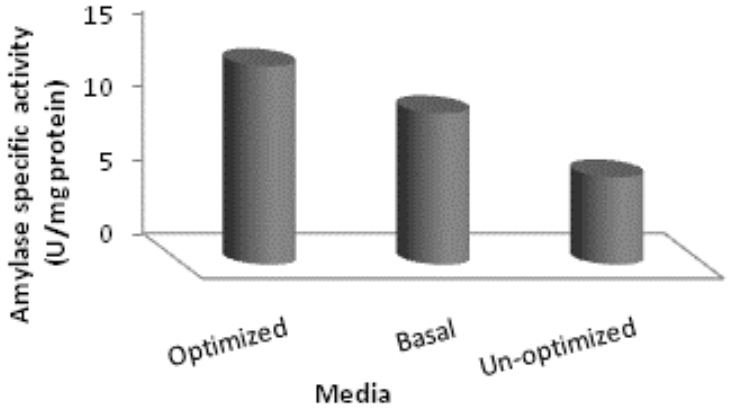

Figure 9. Verification experiment for amylase activity produced by *B. licheniformis.*

to this experimental design, it can be predicted that high microbial amylase production could be obtained using a medium formula of the following composition (g/l): 1.0 g yeast extract, 0.05 g K_2HPO_4, 0.25 g $FeCl_3$, 15.0 g starch, 30.0 g NaCl, 0.75 g $MgSO_4.7H_2O$ and inoculums size of 1.5 ml / 50 ml.

Verification experiment

In order to validate the obtained data and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates to predict the near optimum levels of independent variables. The data were examined and compared to the basal and anti-optimized medium. Data was revealed that the amylase production (13.44 U/mg protein) raised by 1.3 fold for *B. licheniformis* when growing in optimized medium (Figure 9).

Some properties of the crude amylase produced by *B. lichineformis*

Effect of enzyme and substrate concentration

This part deals with the study of some properties of crude enzyme included the effect of enzyme and substrate concentrations. From the proceeding experiments, an enzyme protein concentration of 1.28 mg/ml and substrate concentration of 7.5 mg/ml reaction mixture was the most favorable for maximum activity of the crude amylase enzyme produced by *B. lichineformis*

Effect of temperature and pH of the reaction mixture

To test the effect of temperature, the reaction was carried out for 15 min at 25, 30, 35, 40, 45, 50, 55 and 60°C. The results (Figure 10A) showed that the enzyme activity (36.11 U/mg protein) was stable up to 40°C, above this

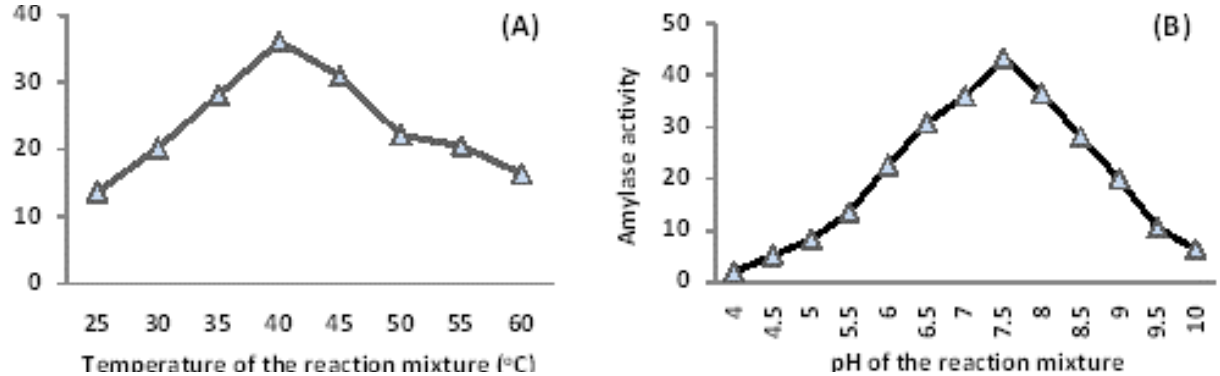

Figure 10. The effect of (A) reaction temperature and (B) reaction pH on amylase activity (U/ mg protein) by *B. licheniformis.*

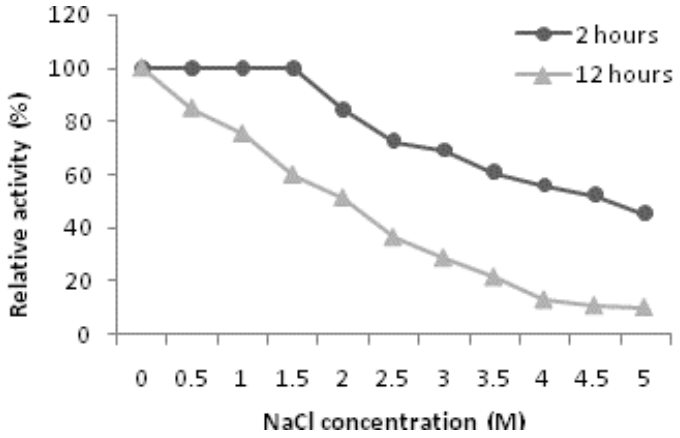

Figure 11. The stability of amylase enzyme activity by *B. licheniformis* at different NaCl concentration.

temperature, a rapid decrease in the enzyme activity was observed where only 45% of the activity was remained at 60°C. The effect of the pH of the reaction on the activity of crude amylase was studied using different buffers with different pH varied from 4 to 10. The data given in Figure 10B indicated that the optimum pH value of the reaction mixture was 7.5. At this value, the enzyme reached its maximal specific activity (43.42 U/mg protein). Above or below this value the activity of the tested crude enzyme decreased gradually.

Stability to NaCl

Effect of NaCl concentration on amylase activity and stability exhibited that the crude enzyme retained 100 and 60% of its activity for up 2 and 12 h, respectively, at 1.5 M (8.7%) NaCl. Also, 60% of relative activity retained for 2 h at 3.5 M of NaCl (Figure 11).

DISCUSSION

Halophilic bacteria are very abundant in the coastal regions, due to its potentiality in producing the extracellular hydrolytic enzymes; it has gained importance in industries for their commercial usage (Moreno et al., 2013). The variation of enzymatic profile was dependent on bacterial species and nature of substrate (El-Sharouny, 2015). The majority of moderate halophilic bacteria (57%) isolated from Alexandria Eastern Harbour of Egypt expressed significant enzyme activity when applied on substrates like starch, olive oil or carboxy methyl cellulose. Moreno et al. (2013) reported that some isolated strains harbored all the extracellular hydrolytic activities screened, except for the chitinase activity. In the present study, most bacterial isolates (43%) have potentiality to produce amylase enzyme. Generally, the strains producing amylolytic enzymes were the most diverse and abundant physiological group among the hydrolytic producers (Moreno et al., 2013).

In the present study the most active isolate for the production of amylase enzyme from the local marine environment was identified by using a 16S rRNA sequence analysis as *B. lichineformis* AH214. Most environmental isolates able to produce hydrolytic enzymes were Gram-positive bacteria, although the isolates were assigned to the family Bacillaceae, comprising species of the genera *Bacillus, Halobacillus* and *Thalassobacillus* (Moreno et al., 2013). Several researchers (Panneerselvam and Elavarasi, 2015; Paul et al., 2015; Khunt et al., 2011) produce amylase enzyme using *Bacillus* sp. isolated from moderate halophiles. This genus is well known as an extracellular enzyme producer and many industrial processes use species of this genus for commercial production of enzymes (Schallmey et al., 2004; Moreno et al., 2013).

The enzyme production by *B. licheniformis* cells reached its maximum (6.99 U/mg protein) at 30 h of incubation. Lowest enzyme activity on prolonged incubation could be due to inhibition and denaturation of the enzyme (Gautam et al., 2002). Short incubation time offers potential for inexpensive production of enzymes. In another study the optimum amylase enzyme activity produced by *B. licheniformis* 44 MB 82-G was recorded

after 96 h using glucose as carbon source (Tonkova et al., 1993). *Bacillus* sp. VS04 produced highest amylase enzyme after 72 h incubation (Vishnu et al., 2014).

Rao and Sathyanarayana (2003) reported that the different carbon sources have varied influence on the extracellular enzymes especially amylase strains. Also Bajpai and Bajpai (1989) found that the different carbon sources can greatly influence the production of amylase. Starch is generally accepted as nutritional component for induction of amylolytic enzymes. The maximum amylase production by *B. licheniformis* AH214 cells was achieved in the presence of starch and maltose as carbon source. Adeyanju et al. (2007) and Ashwini et al. (2011) used starch as a carbon source for amylase production from *B. licheniforms* and *B. mairini*. There are also reports on maltose as a best carbon source for amylase production from *Bacillus* sp. (Gurudeeban et al., 2011; Sivakumar et al. (2011). It was mentioned that induction of amylase requires starch and maltose as substrate, whereas the minimum enzyme activity and protein content were observed in the presence of dextrose (Ashwini et al., 2011). These results are similar to the findings of Haseltine et al. (1996) who observed that glucose represses the production of amylase in the hyper thermophilic archaeon *Sulfolobus solfataricus.* According to them glucose prevented amylase gene expression and not merely secretion of performed enzyme.

The present result explained that the yeast extract was the better nitrogen source for the highest yield of starch hydrolyzing enzyme by *B. licheniformis* AH214. Yeast extract is the best nitrogen source for amylas production, probably due to its high content in minerals vitamins, coenzymes and nitrogen components (Guerra and Pastrana, 2002; Roses and Guerra, 2009). The amylase production by *A. oryzae* was also reported as high in yeast extract and casein (Pederson and Neilson, 2000). Ramachandran et al. (2004) reported that peptone gave an increase in enzyme yield. Yeast extract and peptone is favored for the growth and synthesis of amylase by *Bacillus sp*. (Teodoro and Martins, 2000) also Vishnu et al. (2014) used yeast extract as a nitrogen source for *Bacillus* sp. VS 04.

Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the bioprocess are pH, temperature, aeration and agitation (Kunamneni et al., 2005). In the present study, *B. licheniformis* yielded the maximum amylase production at 40°C, 160 rpm, and pH 7. The present results are matched with the results obtained by other investigation on the α-amylase enzyme from *B. licheniformis* BT5.9 (Ibrahim et al., 2013). Alariya et al., (2013) reported that the all the four bacteria yielded maximum amylase production at 35 to 40°C. Another study by Shafiei et al. (2012) found the optimal temperature for the amylase activity was at 45°C.

According to Vidyalakshmi et al. (2009), the growth temperature plays an important role not only in the growth of bacteria but also in enzyme production. A cultivation temperature beyond the optimal one caused a reduction in the catalytic rate of amylase, as either the enzyme or substrate became denatured and inactive. Ibrahim et al., 2013 reported that the maximum production of the enzyme was obtained when the bacteria was agitated at 100 rpm. The increase in enzyme production could be attributed by increased oxygen transfer rate, increased surface area of contact with the media components and better dispersability of the substrate during fermentation under agitated condition (Elibol and Ozer, 2001).

The pH of the growth medium plays an important role in terms of inducing enzyme production and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannan, 2006). Different organisms have different pH optima and decrease or increase in pH on either side of the optimum value results in poor microbial growth and reduction in enzyme production (Gangadharan et al., 2006; UL-Haq et al., 2002). Gangadharan et al. (2006) reported that pH 4 was the best for the production of amylase by *Bacillus amyloliquefaciens*. UL-Haq et al. (2002) reported pH=7.5-8.0 to be the best for the production of alpha amylase by *Bacillus subitlis.* Plackett-Burman designed an efficient technique for medium component optimization (El-Sharouny et al., 2015). Results obtained in this study are in accordance with others findings where it reported that soluble starch and yeast extract play an important role in enhancing the α-amylase activity (Abdel-Fattah et al., 2013). Also, inoculum size has significant effect on the enzyme production. Therefore, a suitable inoculum size is needed to have the highest enzyme production as lower inoculum size was able to slow down the biomass proliferation. Thus, the degradation of the substrates by the microbes is slower and affects the metabolite production (El-Sharouny et al., 2015). Data in the present results revealed that the amylase production raised by 1.3 fold for *B. licheniformis* AH214 when growing in optimized medium. These results agreed with those of Abdel-Fattah et al. (2013) who reported that the optimal value of the enzyme activity by thermotolerant *B. licheniformis* AI20 is more than two folds of the basal medium of optimization process.

The enzyme activity by *B. licheniformis* (43.42 U/mg protein) was stable up to 40°C, and pH 7.5, above or below this values the activity of the tested crude enzyme decreased gradually. Also the crude enzyme retained 100 and 60% of its activity for up 2 and 12 h, respectively, at 1.5M (8.7%) NaCl. In the last years, several extracellular halophilic α-amylases have been purified from moderate halophiles. This enzyme being relatively stable at pH 6.5-7.5 and temperature 45°C. The purified enzyme was highly active in a broad range of NaCl concentrations (0-4 M) with optimal activity at 0.25 M NaCl. This amylase was highly stable in the presence of 3-4 M NaCl (Shafiei et al., 2012). The α-amylase by a

thermotolerant *B. licheniformis* AI20 isolate had an optimal temperature and pH of 60-80°C and 6-7.5, respectively (Abdel-Fattah et al., 2013). The α-amylase was highly stable over a broad range of temperatures (30-90°C), pH (6.0-12.0), and NaCl concentrations (0-20%) (Moreno et al., 2013). Many other amylases from moderately halophilic *Bacillus* showed to exhibited higher optimal salinity with more NaCl stability than that obtained in our study.

Conclusion

The marine environment is a good source for valued microflora hat need to be explored. The present study explored our natural environments, searching for halophilic marine bacteria producing extracellular degradative enzymes. The findings of the present study suggested that the halophilic *B. licheniformis* AH214 isolated from the local marine environment could be used for amylase production. Optimization of the fermentation medium components, environmental conditions and inoculums size for the isolate was applied using One Variable at a Time approach and Plackett-Burman design, leading two fold increased in enzyme activity**.** The produced crude amylase enzyme was stable up to 40°C, pH 7.5 and 1.5 M NaCl.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Cloning, over-expression, and characterization of a new carboxypeptidase A gene of *Bacillus pumilus* ML413 in *Bacillus subtilis* 168

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Carboxypeptidase A (CPAs) are a well-studied group of zinc-containing exopeptidases that facilitate the breakdown of proteins and peptides during metabolism. Carboxypeptidase A is typically produced in mammalian pancreatic, brain and other tissues. A new gene encoding carboxypeptidase A in the prokaryote *Bacillus pumilus* was amplified by polymerase chain reaction (PCR), ligated into the shuttle vector pMA5, and cloned in a GRAS bacteria-*Bacillus subtilis* 168 host. This gene sequence contained a 1621 bp open reading frame that encodes a protein of 540 amino acids. The optimum pH and temperature for enzyme activity were 7.5 and 50°C, respectively. The enzyme was quite stable at neutral pH and maintained about 65% activity following a 24 h incubation at 40°C. The Km of this CPA was 0.1 mM, much higher than in mammalian species. Glycerol, ammonium sulfate, and sodium citrate improved enzyme activity under optimal culture condition. The carboxypeptidase activity in recombinant *B. subtilis* 168 reached a maximum of 179 U ml^{-1} in a 5 L fermentator when cultured on improved medium. The over expression of carboxypeptidase A in *Bacillus subtilis* has commercial applications.

Key words: *Bacillus pumilus, Bacillus subtilis* 168, over-expression, orthogonal arrays, carboxypeptidase A, metallocarboxypeptidase.

INTRODUCTION

Carboxypeptidases (CPs) catalyze the release of C-terminal amino acids from proteins and peptides (Sebastian Tanco et al., 2013). CPs serve many important functions in a variety of organisms since originally isolated from bovine pancreatic tissue in 1929 (Suwen et al., 2002). Some non-digestive zinc carboxypeptidases are involved in hormone and neuropeptide processing, bioactive peptide activation or inactivation, or functional modulation of regulatory proteins (Joshi et al., 1999). Thus, CPs are widely used

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in the pharmaceutical, food and other industries. For example, they are used to remove ochratoxin A (Abrunhosa et al., 2007) and as a debitterizing reagent in the hydrolysis of soybean protein (Fang et al., 2005). Carboxypeptidases are classified into several types, carboxypeptidases that have a stronger preference for those amino acids containing aromatic or branched hydrocarbon chains are called carboxypeptidase A (A for aromatic/aliphatic), carboxypeptidases that cleave positively charged amino acids (arginine, lysine) are called carboxypeptidase B (B for basic), metallo-carboxypeptidase that cleaves a C-terminal glutamate from the peptide N-acetyl-L-aspartyl-L-glutamate is called "glutamate carboxypeptidase´´.

Carboxypeptidase A (CPA), a type of metallocarboxypeptidase, preferentially removes C-terminal amino acid residues in the presence of aromatic or branched aliphatic side chains with the aid of a Zn^{2+} ion (Austin et al., 2011). CPA has been widely studied, including the structures of the substrate-binding sites and the mechanism of catalysis (Vertesi et al., 1999). Many characteristics of CPA have been reported in literature (Kumar et al., 2014; Elena et al., 2007; Li and Solomon, 1997; Kazuhisa and Kamisosoyama, 2000). Proposed commercial applications of CPA include its use in the hydrolysis of cheese whey protein and other protein hydrolysates. However, commercial production of CPA involves its isolation from pancreatic tissue which limits production (Lyons et al., 2008). CPA has been studied in numerous mammalian species including human, rat, bovine, swine, goat and buffalo. CPA has been studied in avian species (Kazuhisa and Kamisosoyama, 2000). CPA production by the fungi *Aspergillus niger* and *Metarhizium anisopliae* have been reported by Abrunhosa and Venancio (2007) and Austin et al. (2011), respectively. While most studies have focused on CPA characterization and applications, few studies have focused on improving the production of CPA. Heterologous expression and production of CPA in engineered strains of bacteria could increase the production of CPA.

Bacillus pumilus strains are highly resistant to UV radiation and hydrogen peroxide, making them good model bacteria for studying of industrial production strains. Nagamori et al. (1991) examined the enzymatic properties of dipeptidylcarboxypeptidase (DCP) isolated from *B. pumilus*; they found that the presence of chloride ion is essential for the hydrolysis, enzyme is readily inhibited by Ethylenediaminetetraacetic acid (EDTA) but restored by Co^{2+}, Mn^{2+}, and Zn^{2+}. However, there is no information on the characterization of carboxypeptidase A from *B. pumilus,* or from other prokaryotic bacteria*. Bacillus* species have been major workhorse industrial microorganisms with roles in applied microbiology (Schallmey et al., 2004). Engineered strains of *B. subtilis* are attractive as industrial organisms due to their rapid growth rate that result in short fermentation cycle times and their capacity to secrete proteins into the extracellular medium (Gao et al., 2013).

In this study *B. subtilis* 168 was utilized as a host for the heterologous expression of a gene encoding carboxypeptidase A from *B. pumilus*. The CPA was also characterized.

MATERIALS AND METHODS

Strains, plasmids, and chemicals

B. pumilus ML413, *E.coli* JM109, *B. subtilis* 168 and vector pMA5 were preserved in our laboratory. Plasmid pMA5 and *B. subtilis*168 were used as the expression vector and host cell, respectively. The vector pMD18-T, restriction enzymes, T4 DNA ligase, and ExTaq DNA polymerase were purchased from TaKaRa Bio Co., Ltd. (Dalian, China). The Mini Chromosome Rapid Isolation Kit, Mini Plasmid Rapid Isolation Kit, and Mini DNA Rapid Purification kit were obtained from Generay Biotech Co., Ltd. (Shanghai, China). AKTA prime plus and Butyl Sepharose HP columns were purchased from GE Healthcare, Inc. (Little Chalfont, UK). PCR primers and Bradford reagent kits were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Hippuryl-L-phenylalanine was purchased from Sigma-Aldrich Co., Ltd. (St. Lous, MO, USA). All other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Medium and culture conditions

B. pumilus, *E.coli* JM109 and *B. subtilis* 168 were grown at 37°C in Luria-Bertani (LB) medium during the construction of strains. Ampicillin (100 µg/ml) or kanamycin (50 µg/ml) were added to the growth medium as required. The basal fermentation medium for producing CPA was Terrific Broth (TB) medium (Gao et al., 2014) containing (g/L): yeast extract, 24; tryptone, 12; K_2HPO_4, 9.4; KH_2PO_4, 2.2; glycerol, 5, at pH 7.2. Optimization of medium was carried out based (by completing the TB medium with carbon sources, nitrogen sources, and inorganic salts) on the TB medium. Liquid cultures were incubated at 37°C at 200 rpm.

Cloning and construction of recombinant plasmid pMA5-*cpa*

Total genomic DNA of *B. pumilus* ML413 was extracted using a mini chromosome rapid isolation kit. The *cpa* gene was amplified from total chromosomal DNA isolated from *B. pumilus* ML413 by polymerase chain reaction (PCR) using ExTaq DNA polymerase, with *cpa* forward (5'-CGGGATCCCGATGTCCAAACAGAAAAGCACCATT-3') and reverse (5'-CACGCGTCTTAGTGGTGGTGGTGGTGGTGTTTTATACCTAACG T-3') primers (underlined sequence = restriction sites), which were designed using the hypothetical (yes) M14 family carboxypeptidase gene of *B. pumilus* SAFR-032 (accession number CP000813.1) available from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The restriction sites of the forward and reverse primers were *Bam*HI and *Mlu*I, respectively. Procedures for PCR were carried out as: 94°C for 5 min followed by 34 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min. PCR amplicons were checked by agarose electrophoresis and purified from the gel using the Mini DNA rapid purification kit. The purified fragment was ligated directly into the cloning vector pMD18-T, which was then used to transform chemically competent *E. coli* strain JM109. The presence of the correct plasmid was verified

using Ba*m*HI and Ml*u*I restriction digests. The digested fragment was purified and then ligated with pMA5, which had already been digested using Ba*m*HI and Ml*u*I restriction enzyme. The ligation mixture was used to transform *E.coli* JM109 competent cells. Restriction digestion analysis was carried out to verify the presence of the recombinant plasmid pMA5-*cpa* gene. Nucleotide sequence of *cpa* insert was analyzed by Sangon Biotech. Protein and nucleotide sequence comparisons were performed using the BLAST server available from the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and then this recombinant plasmid was transformed into chemically competent *B. subtilis* 168 cells for Carboxypeptidase A production using the procedure described by Spizizen (Jia et al., 2013).

Expression of *cpa* in *B. subtilis*

Recombinant *B. subtilis* 168 was cultivated overnight (12 h) on selective LB medium supplemented with 50 µg/ml kanamycin. The cells were then harvested by centrifugation at 10,000 *g* for 10 min at 4°C, and the supernatant was used for a CPA enzyme activity assay. The precipitated pellets were washed twice with 5 ml of 50 mM Tris/HCl buffer (pH 7.5), then suspended in 5 ml of the same buffer supplemented with 50 µl of lysozyme solution and kept on ice for 2 h. The mixture was then sonicated 10 times for 2 s with 5 s cooling intervals. Cell extracts were centrifuged for 30 min at 10,000 *g* at 4°C in a SIGAMA 4K-15 centrifuge (Sigma-Aldrich Co., Ltd., Shanghai, China) to remove cell debris. The supernatant was used for crude enzyme activity assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12% acrylamide), or stored at -20°C for further analysis.

Enzyme activity assay

Enzyme activities of the intracellular and extracellular portions of the protein were measured spectrophotometrically by recording the increase in absorbance at 254 nm that accompanies the hydrolysis of the peptide substrate hippuryl-L-phenylalanine as described by (Tardioli et al., 2003) with slight modifications. Assays were performed using a UV-1200 spectrophotometer (Pharmacia Biotech), with thermostatically controlled at 25°C. A 100 µl aliquot of enzyme solution was added to the assay solution (1.45 ml of 1 mM hippuryl-L-phenylalanine prepared in 50 mM Tris-HCl buffer, pH 7.5) supplemented with 40 µmol $ZnSO_4.7H_2O$, and the increase in absorbance was measured for 5 min. The amount of phenylalanine released by CPA action was calculated from the millimolar extinction coefficient at 254 nm (0.36 l $mmol^{-1}$•cm^{-1}), and enzymatic rate was expressed as concentration of product formed per min (µmol L^{-1} min^{-1}). One unit (U) was defined as the amount of enzyme that hydrolyses 1.0 µmol of hippuryl-L-phenylalanine per min at 25°C. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard (David et al., 2004).

Enzyme purification

All purifications were performed using an ÄKTA Prime Plus (GE Healthcare Bio-sciences procedures). The CPA-containing supernatant was filtered through a 0.2 µm cellulose acetate membrane (Corning Incorporated, NY, USA) and applied to a 2.5 ml HisTrap column (AmershamBioSciences) equilibrated in buffer A with 50 mM Tris-HCl (pH 7.4). Then the enzyme was eluted in a linear gradient with a change of imidazole concentration (500 mM) using a flow rate of 1.0 mL min^{-1}. The active fractions were collected (Figure 7) and dialyzed for CPA enzyme assay or storing at -20°C.

Enzyme characterization

Optimum pH was determined by measuring the activity of purified CPA at pH 3.0 to 10.0 (pH 3.0 to 6.5, 50 mM citrate-sodium citrate buffer; pH 6.5 to 8.0, 50 mM Tris-HCl buffer; pH 8.0 to 10.0, 50 mM glycine-NaOH buffer), while the optimum temperature was examined in 50 mM Tris-HCl buffer (pH 7.5) using the standard reaction mixture. The pH and thermal stability of CPA was determined by incubation at different pH levels at 4°C for 24 h and at various temperatures for 24 h, respectively. Effects of various compounds on enzyme activity were examined in the standard reaction mixture supplemented with various cations and EDTA at concentrations of 1 mM. Enzyme in the absence of metal ions or EDTA served as a control. Kinetic parameters were determined in 50 mM Tris-HCl buffer (pH 7.5) at 25°C by changing the concentration of the substrate. Eadie-Hofstee plots were used to calculate kinetic parameters K_m and V_{max} according to the enzyme reactions.

Single-factor-at-a-time experiments for medium optimization

To determine the effect of carbon, nitrogen sources and inorganic salt on enzyme activity, the growth medium was supplemented with sucrose, maltose, lactose, glucose, galactose, glycerol and soluble starch as carbon sources, ammonium chloride, ammonium sulfate, ammonium acetate and urea as nitrogen sources and sodium chloride, potassium chloride, sodium nitrate, potassium nitrate and sodium citrate as inorganic salts. Each source was used at a concentration of 0.5% (w/v). Enzyme activity was determined after 12 h of incubation at 37°C and 200 rpm (revolutions per minute).

Orthogonal array methodology for medium optimization

To examine the interactions among nutritional components of fermentation medium and to optimize their concentrations for CPA production, orthogonal arrays were used as reported by Vijayalakshmi et al. (2011). An L9 orthogonal array in three levels was used consisting of 9 different experimental trials for the medium optimization to increase CPA activity (Tables 3 and 4). The design for the L9 orthogonal arrays was developed and analyzed using “zhengjiaozhushou” Chinese software. Zhengjiaozhushou design is an independent quadratic design in that it does not contain an embedded factorial or fractional factorial design. In this design the treatment combinations are at the midpoints of edges of the process space and at the center. These designs are rotatable (or near rotatable) and require 3 levels of each factor. The designs have limited capability for orthogonal blocking compared to the central composite designs.

5 L fermentation for CPA production

The recombinant *B. subtilis* 168 harboring plasmid pMA5-*cpa* was scaled up to 2 L in optimized medium in a 5 L bioreactor (Shanghai Baoxing Bioengineering Equipment Co., ltd). Inoculum (100 ml) was added to the bioreactor. The agitation rate was set to 300 rpm at 37°C. For controlled pH cultivations, the pH was maintained at 7.2 by addition of 0.5 M NaOH and 1 M HCl solution. After 8 h incubation, the parameters like optical density of cell (OD_{600}), enzyme activity, and protein concentration were determined at

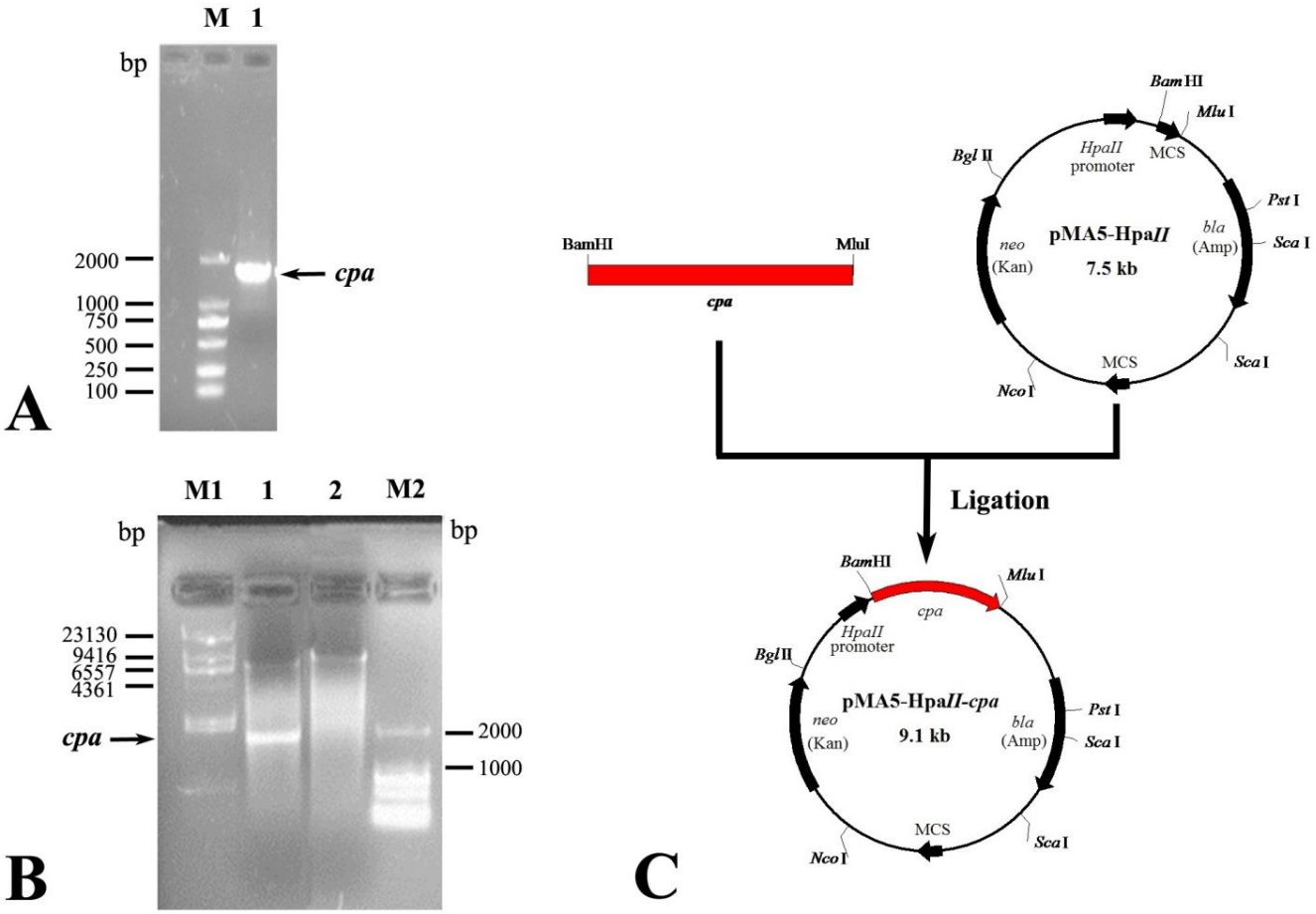

Figure 1. PCR and construction of recombinant vector pMA5-*cpa*. (A) Lane M, DL2000 DNA marker; lane 1, *cpa* PCR product. (B) Lane M1, λ Hind III DNA marker; lane 1, recombinant vector digested with *Bam*HI and *Mlu*I; lane 2, recombinant vector digested with BamHI; lane M2, DL2000 DNA marker. (C) Ligation of the *cpa* gene with shuttle vector pMA5. The *cpa* gene predigested with BamHI and MluI was ligated into the BamHI and MluI sites of the pMA5 vector.

regular intervals.

RESULTS AND DISCUSSION

Cloning, construction of recombinant plasmid pMA5-*cpa*

The carboxypeptidase A (CPA) gene was successfully amplified from the genomic DNA of *Bacillus pumilus* ML413 strain by PCR. Electrophoresis revealed that the amplicon consisted of a 1621 bp fragment (Figure 1A). BLAST analysis showed that the gene sequence had a high level of similarity with that of several Peptidase M14 containing strains published in the NCBI database including *B. pumilus* strain W3 (CP011150.1) and *B. pumilus* strain MTCC B6033 (CP007436.1) with similarities of 99 and 98%, respectively. The PCR product was ligated with pMD18-T to create pMD18-*cpa*, and then digested with *BamHI* and *MluI*. The digestion product was cloned into the expression vector pMA5 under the control of the *HpaII* promoter, resulting in recombinant vector pMA5-Pept (Figure 1C). The recombinant plasmid was digested with *BamHI* and *MluI* to produce two major fragments with sizes of 1621 and 7500 bp (Figure 1B). This result confirmed the successful construction of the recombinant plasmid.

Expression of the recombinant vector in *B. subtilis 168*

The resultant pMA5-*cpa* was used to transform the expression host *B. subtilis* 168 to construct a recombinant *B. subtilis* strain 168/pMA5-*cpa*. The recombinant *B. subtilis* could express CPA under the control of the *HpaII* promoter. For production of CPA, the

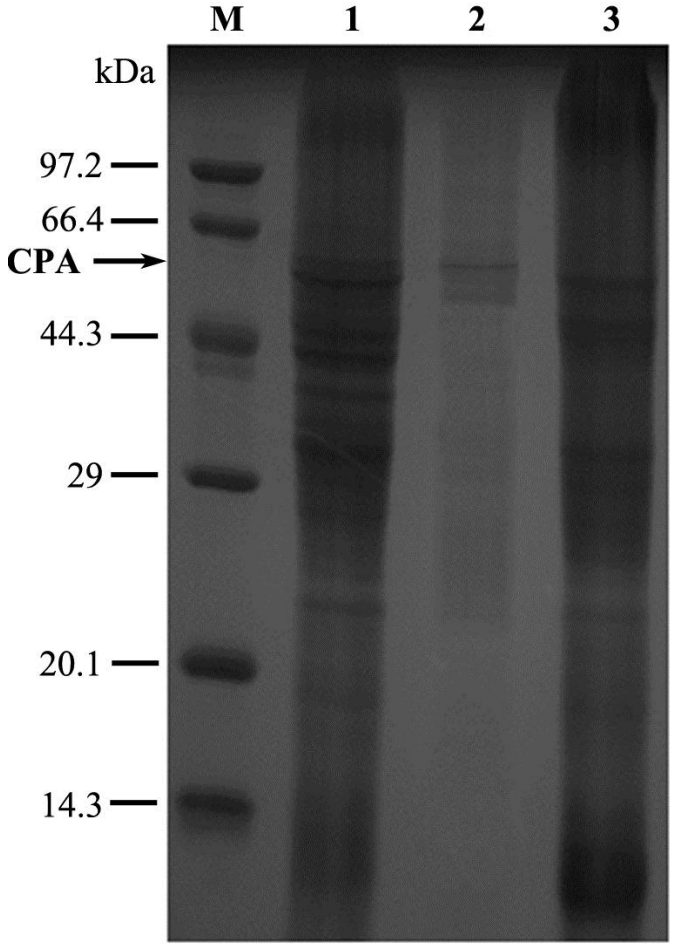

Figure 2. SDS-PAGE analysis of recombinant *B. subtilis* 168/pMA5-*cpa*. Lane M, protein marker; lane1,*B. subtilis* 168/pMA5-*cap* cell extract; lane 2, Recombinant pure CPA; lane 3, *B. subtilis* 168 cell extract as control.

recombinant *B. subtilis* was used to inoculate 50 ml of LB medium (pH 7.2) supplemented with kanamycin (50 µg ml^{-1}) in a 250 ml flask, and incubated at 37°C in a rotary shaker at 200 rpm for 12 h. The recombinant *B. subtilis* showed intracellular and extracellular enzyme activities of 29.49 and 0 U ml^{-1}, respectively. The total enzyme activity was substantially higher than the 7 U ml^{-1} of the host *B. subtilis* 168. A band of 59 kDa corresponding to CPA was produced by the recombinant *B. subtilis* (Figure 2). Unlike other mammal digestive carboxypeptidases which are activated through limited proteolysis in the duodenum (Pedro José et al., 2002), our active recombinant CPA was expressed directly. However, the recombinant CPA could not be expressed outside the host *B. subtilis*, while it performed as an extracellular enzyme in *B. pumilus* (data not shown). Gao et al. (2013) reported that TB medium was suitable for the production of enzymes by recombinant engineered strains. Hence, we cultured the recombinant *B. subtilis* into 50 ml TB medium using the other conditions as described for the LB medium. Intracellular and extracellular enzyme activities of CPA in TB were 45 and 0 U ml^{-1}, respectively.

Table 1. Enzyme activity of purified CPA.

Step	Protein (mg ml^{-1})	Activity (U ml^{-1})	Specific activity (Umg^{-1})
Affinity chromatography	4.01	179	45

Purification of the enzyme

For affinity purification of recombinant proteins expressed in *B. subtilis* 168, a His tag (T*gg*, T*gg*, T*gg*, T*gg*, T*gg*, T*gg*) was inserted into the sequence of the reverse primer. Affinity tags are useful tools for the production of recombinant proteins because of the easier detection and purification of their fusion partners. Affinity tags may also have a beneficial impact on the yield of recombinant proteins, increase the recombinant protein's solubility, and promote their proper folding (Bown et al., 2004). This facilitates the further study of these proteins. Recombinant CPA was purified by metal affinity chromatography, making use of a His tag at the C terminus of CPA. The SDS-PAGE of lane 2 in Figure 2 indicated that this recombinant CPA was successfully purified with a band of 59 kDa. As a result, the purified enzyme showed a specific activity of 45 U mg^{-1} (Table 1). The specific activity of the recombinant CPA approached the activity of commercial CPA derived from bovine pancreas (52 U mg^{-1}) (Vertesi et al., 1999). This suggests a potential commercial use of our recombinant CPA. SDS-PAGE analysis indicated that the subunit of the enzyme had a molecular mass of about 59 kDa (Figure 2), when the gel was stained with CBB R-250.

Characterization of the Enzyme

The optimum pH values of CPA for hydrolyzing hippuryl-L-phenylalanine was pH 7.5 (Figure 3a). The optimum pH value reported here was different from values reported for carboxypeptidase from Archaeon *Thermococcus sp*. NA1 (pH 6) (Hyun Sook Lee et al., 2006), crayfish carboxypeptidase from *Astacus fluviatilis* (pH 6.5) and carboxypeptidase III from germinating triticale grains (pH 4.6) (Drzymala et al., 2009); but similar to immobilized carboxypeptidase A and carboxypeptidase A from the pancreas of the catfish *Parasilurus asotus* (Yoshinaka et al., 1985).

In the present study, the recombinant CPA showed maximum activity at 50°C, and then abruptly decreased at higher temperatures (Figure 3b). This was in agreement

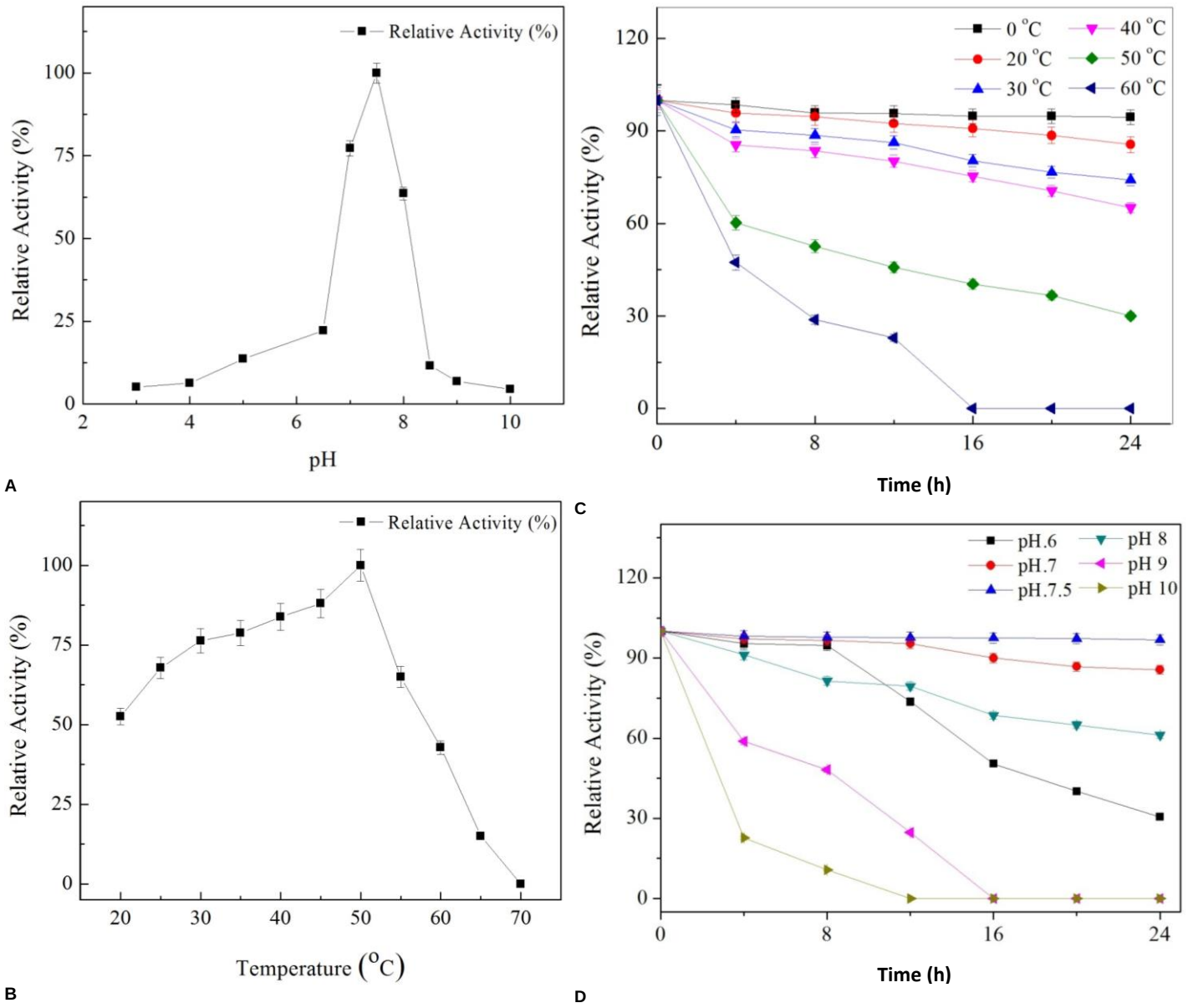

Figure 3. Effects of pH and temperature on recombinant CPA enzyme. (a) Optimum pH was carried out in 50 mM citrate sodium/citrate buffer (pH 3 to 6.5), 50 mM Tris/HCl buffer (pH 6.5 to 8), and 50 mM glycine/NaOH buffer (pH 8 to 10), at a temperature of 37°C. (b) Optimum temperature was carried out in 25 mM Tris/HCl buffer (pH 7.5) with the temperature range of 20 to 70°C. (c) Effect of temperature on stability of the CPA was determined between 0°C and 60°C by incubating the enzyme for a certain time in 25 mM Tris/HCl buffer (pH 7.5). (d) Effect of pH on the stability of the CPA was determined by incubating the enzyme at different pH at 4 °C for 24 h.

with earlier reports of the optimum temperature of carboxypeptidase A from chicken pancreas (Kazuhisa et al., 2000). The thermal stability of our recombinant CPA is shown in Figure 3c. The recombinant CPA was stable when the temperature was less than 40°C. The activity remained at 86 and 65% at 20 and 40°C after 24 h storage, respectively. The effectiveness of pH on the stability of the CPA was examined in the range of pH 6 to 10 (Figure 3d). The recombinant CPA was found to be stable only at pH 7.5 and the activity was 98% after 24 h incubation. The influences of metal ions and EDTA on the CPA activity are as shown in Figure 4. These results indicated that the CPA enzyme was activated by Li^{+} (117%), Cu^{2+} (115%), Co^{2+} (110%) and Ca^{2+} (107%). Mg^{2+} had no effect on CPA activity. Fe^{2+} (74%) and EDTA (57%) inhibited CPA activity. Some metal ions, notably cobalt, slightly enhanced the enzyme activity of the carboxypeptidase involved in the proteolytic cleavage of the influenza haemagglutinin as reported by Garten et al. (1983).

The K_m and K_{cat} values for recombinant CPA using hippuryl-L-phenylalanine as substrate were 0.1 mM and

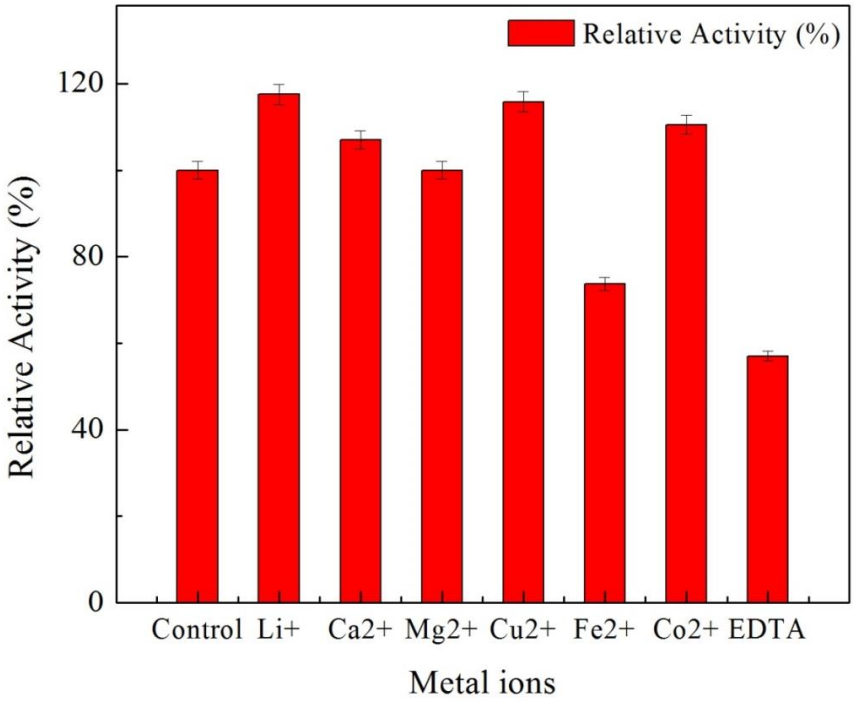

Figure 4. Effects of different metal ions and EDTA at the same concentration (1 mM) on CPA activity (enzyme with absence of metal ions or EDTA served as the control).

Table 2. Kinetic parameters of recombinant CPA with hippuryl-L-phenylalanine as substrate.

Substrate (1mM)	K_m (mM)	Vm (μM s^{-1})	K_{cat} (s^{-1})	K_{cat}/k_m(mM^{-1} s^{-1})
Hippuryl-L-phenylalanine	0.1	6.36	63.6	636

63.6 s^{-1}, respectively (as shown in Table 2). These values are similar to those of CPA from chicken pancreas, but lower than the values reported for ostrich carboxypeptidase (0.41 mM), bovine carboxypeptidase (1.75 mM), and the value reported by Cheng et al. (1999) for carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus* (0.9 mM). These results indicate that substrate specificity of our recombinant CPA shows a higher affinity for hippuryl-L-phenylalanine when compared to that of mammals and other avian species. This suggests a potential commercial application.

Single-factor-at-a-time optimization strategy

The production of enzymes from microorganisms is strongly influenced by the composition of the medium, with carbon and nitrogen sources being the most important factors. Of the seven carbon (Figure 5a) and five nitrogen sources (Figure 5b) tested, glycerol and ammonium sulfate resulted in the highest relative activities of 197.21 and 175.85%, respectively. Lower relative activities were observed with lactose (77%) and ammonium chloride (94%). The carbon and nitrogen sources used to increase production of recombinant CPA in this study are different than those reported by (Imtiaz az et al., 2013) for the production of alkaline protease by *B. subtilis*, or other recombinant enzymes using *B. subtilis* as a host (Jia et al., 2013). The highest relative activity of carboxypeptidase (162.28%) was observed when sodium citrate was added to the culture medium as an inorganic salt source (Figure 6c). Sodium citrate was added in its simple form, so it was readily available for protein synthesis. A lower level of activity was observed when medium contained sodium nitrate or potassium nitrate. The inhibitory effects of nitrate were reported by Bhutto et al. (2011).

Optimization of enzyme activity using orthogonal array (OA) methodology

The orthogonal array methodology was used to investigate the influence of glycerol, ammonium sulfate

Table 3. Factors and levels listed for compatibility testing.

Factor	Level 1	Level 2	Level 3
Glycerol	G1	G2	G3
Ammonium sulfate	As1	As2	As3
Sodium citrate	Sc1	Sc2	Sc3

Table 4. Orthogonal array constructed for the compatibility testing.

Test number	Glycerol	Ammonium sulfate	Sodium citrate
Test 1	G1	As1	Sc1
Test 2	G1	As2	Sc2
Test 3	G1	As3	Sc3
Test 4	G2	As1	Sc2
Test 5	G2	As2	Sc3
Test 6	G2	As3	Sc1
Test 7	G3	As1	Sc3
Test 8	G3	As2	Sc1
Test 9	G3	As3	Sc2

and sodium citrate concentrations on CPA production. There are three independent variables (Glycerol, G1= 0.25%; G2 = 0.5%; G3 = 0.75%), (ammonium sulfate, As1 = 0.25%; As2 = 0.5%; As3 = 0.75%), and (sodium citrate, Sc1 = 0.25%, Sc2 = 0.5%; Sc3 = 0.75%). The order of factor effects on CPA production was found to be glycerol > sodium citrate > ammonium sulfate as shown in Table 5. The results of the optimization medium on CPA activity in 5 L fermenter are shown in Figure 6. The optimized medium contained yeast extract 24 g L^{-1}, tryptone 12 g L^{-1}, K_2HPO_4 9.4 g L^{-1}, KH_2PO_4 2.2 g L^{-1}, glycerol 5 g L^{-1}, ammonium sulfate 5 g L^{-1} and sodium citrate 7.5 g L^{-1}. The predicted level of CPA activity was 45 U ml^{-1} in a 50 ml flask culture. This increased to 179 U ml^{-1} of recombinant CPA after scaling up to 2 L in a lab scale bioreactor (12 h after inoculation). The optimized medium resulted in a 4-fold higher level of activity when compared to TB basal medium and 6-fold higher activity when compared to LB medium. The activity curve of the recombinant CPA in a bioreactor was similar to the curve of the OD_{600} (optical density, of sample measured at a wavelength of 600 nm), this could be due to the fact that the decline growth phase of the recombinant *B. subtilis* began after 16 h in such an abundant nitrogen source medium. Enzyme activity is generally directly related to the incubation period and metabolite concentrations as reported by Imtiaz et al. (2013).

Conclusion

A new carboxypeptidase A gene isolated from *B. pumilus* was successfully cloned into a Generally Recognized As Safe (GRAS) bacterium-*B. subtilis* 168. This enzyme was characterized after purification. The recombinant CPA was expressed in a mature form with a molecular weight of 59 kDa. This was in contrast to other mammalian digestive CPAs which need be activated through limited proteolysis with a scission of a ~100 residue N-terminal prosegment. Culture conditions, including incubation period, had a profound effect on the production of enzyme. Glycerol and ammonium sulfate provided the carbon and inorganic nitrogen sources, respectively. The recombinant carboxypeptidase A showed some characteristics in common with CPA isolated from other sources. The CPA has a broad specificity for C-terminal amino acid residues with aromatic or branched aliphatic. With a high activity of 179 U ml^{-1} in 5 L bioreactor, the recombinant CPA expressed in the GRAS bacterium-*B. subtilis* proves promising as a processing aid for protein hydrolysis in order to improve flavor and reduce bitterness in food protein hydrolysates.

Conflict of interest

The authors have not declared any conflict of interest

ACKNOWLEDGEMENTS

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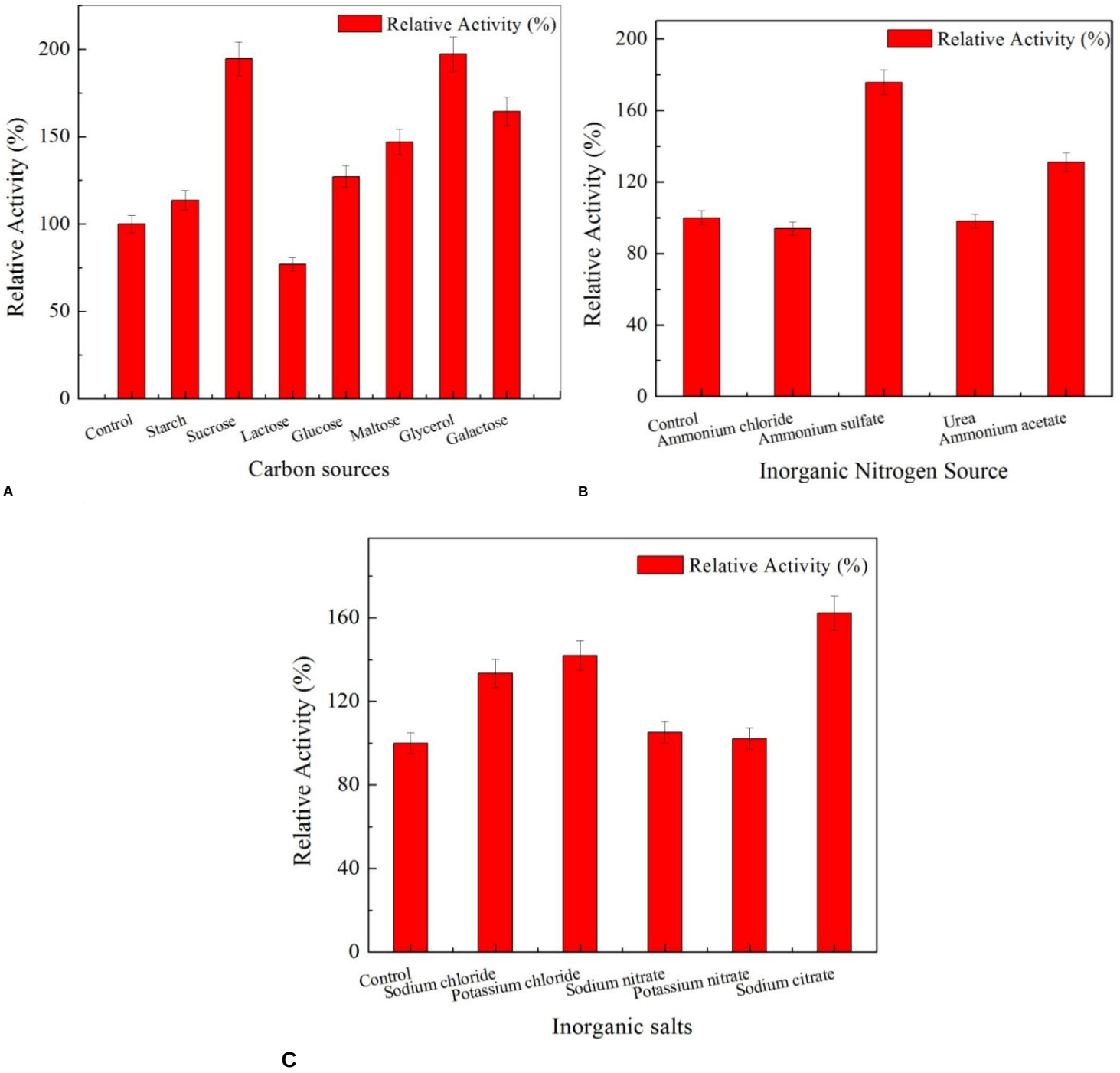

Figure 5. Effects of carbon, inorganic nitrogen sources, and inorganic salt for the medium on enzyme activity. a. Effect of carbon sources. b. Effect of inorganic nitrogen sources. c. Effect of inorganic salt.

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Table 5. Result of compatibility testing.

Test number	1	2	3	Result
	G	As	Sc	
Test 1	1	1	1	16.35
Test 2	1	2	2	15.14
Test 3	1	3	3	17.01
Test 4	2	1	2	25.11
Test 5	2	2	3	34.23
Test 6	2	3	1	17.95
Test 7	3	1	3	22.51
Test 8	3	2	1	18.25
Test 9	3	3	2	31.01
K 1	16.167	21.323	17.517	
K 2	25.763	22.540	23.753	
K 3	23.923	21.990	24.583	
Factors effects	9.596	1.217	7.066	

G: glycerol; As: Ammonium sulfate; Sc: Sodium citrate.

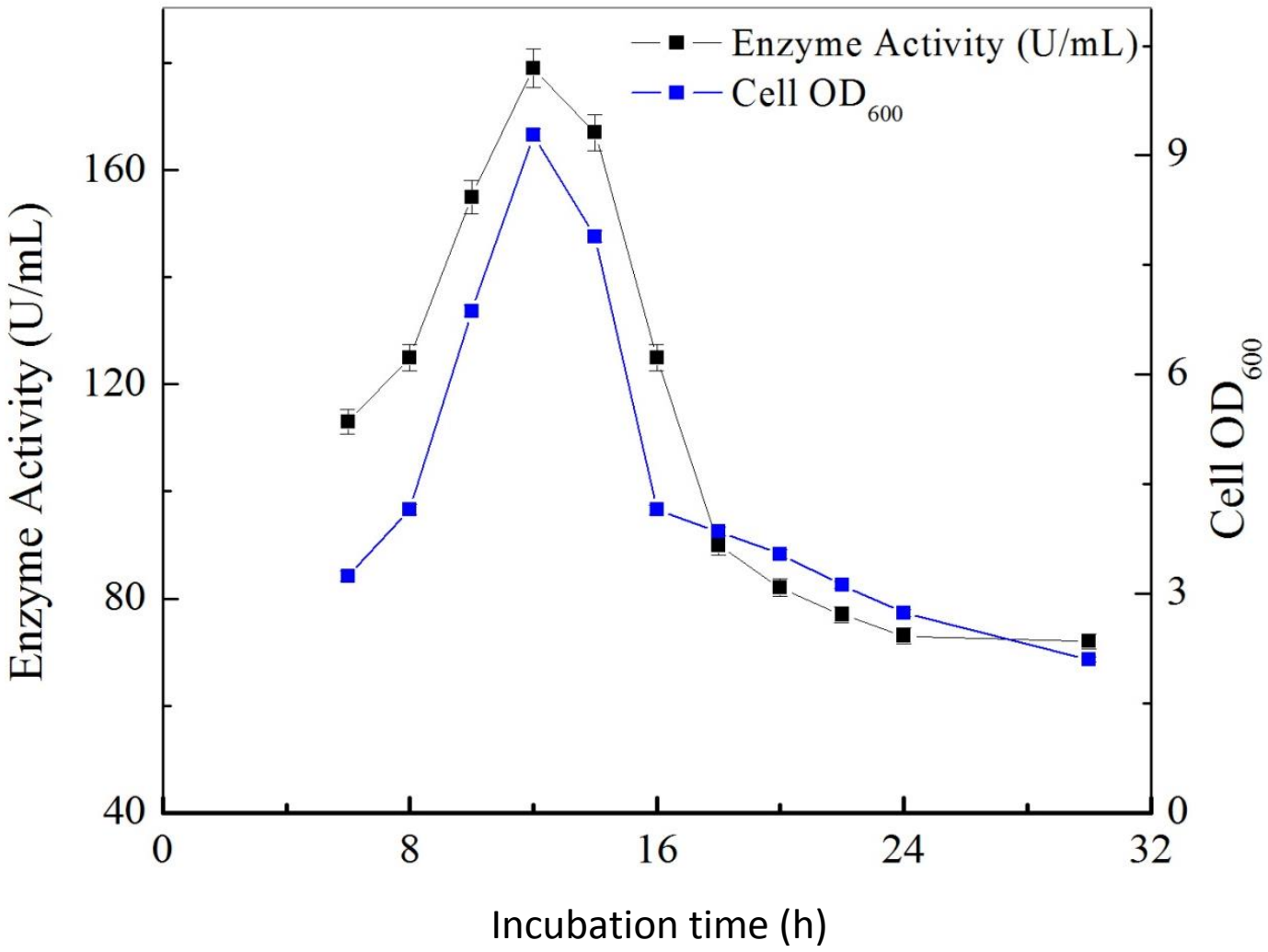

Figure 6. Trend curve of recombinant CPA activity and biomass (OD_{600}) using the optimum medium in a 5 L fermentator.

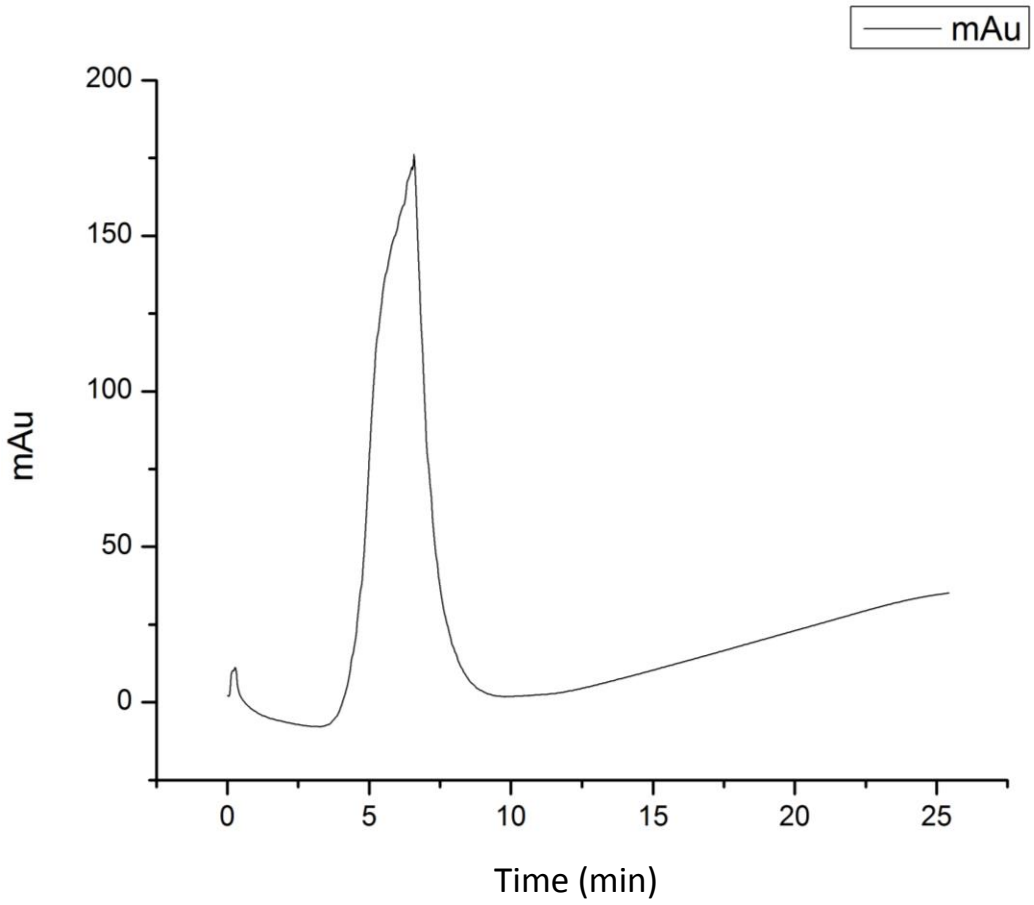

Figure 7. Graph of protein purification.

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Molecular serotype and evolutionary lineage of *Listeria monocytogenes* isolated from different Nigerian food items

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The molecular serotypes and the evolutionary lineage of *Listeria monocytogenes* isolated from various foods in Nigeria are yet to be documented. Consequently, popular uncooked food items known locally as *Okazi Utazi, Onugbu, Ogbono, Garri* and *Egusi* obtained from plants botanically known as *Gnetum africanum, Gongronema latifolium, Vernonia amygdalina, Irvingia gabonensis, Manihot esculanta Crantz* and *Colocynthis Citrullus,* respectively were analyzed. Molecular serotype of three chosen isolates was determined using multiplex polymerase chain reaction (PCR) serotyping before analysis based on *prfA* virulence gene cluster of *L. monocytogenes* was carried out to establish the evolutionary lineage. There was no *L. monocytogenes* detected in foods from *I. gabonensis, M. esculanta Crantz* and *C. citrullus.* However, the vegetables from *G. africanum, G. latifolium* and *V. amygdalina* showed the presence of the organism and chromogenic tests carried out on the three strains chosen from oxford formulation media indicated that they were not other non-pathogenic strains of *Listeria*. The V3 region of 16S rRNA gene of one strain showed that a close relative of the isolate is a strain implicated in an outbreak of listeriosis. Leafy vegetables could be a major vehicle for transmission of *L. monocytogenes* in Nigeria since this pathogenic bacterium occured in different vegetables analyzed.

Key words: *Listeria monocytogenes,* serotype, lineage, pathogenic potential, Nigerian food.

INTRODUCTION

Listeria monocytogenes is a bacterial foodborne pathogen that can cause listeriosis especially among the young, old aged, pregnant and immunocompromised persons (Cartwright et al., 2013). The presence of *L. monocytogenes* in food remains a major challenge because it is psychrotrophic, relatively tolerant of high

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solute concentrations, resists desiccation and therefore can overcome mild food preservation techniques (McLauchlin et al., 2014). Despite the world-wide reports of outbreaks of food-borne listeriosis, the occurrence of *L. monocytogenes* is still not widely reported in Nigeria due to lack of a comprehensive surveillance system for food-borne pathogens (Nwaiwu, 2015).

Common popular Nigerian foods found in South eastern region namely *Okazi* (also called *Afang* in another region)*, Utazi, Onugbu, Ogbono, Garri* and *Egusi* can be sourced from plants with botanical names *Gnetum africanum, Gongronema latifolium, Vernonia amygdalina, Irvingia gabonensis, Manihot esculanta Crantz* and *Colocynthis Citrullus,* respectively*.* Leaves of *G. africanum* are eaten as a vegetable and also used widely as an ingredient in soups and stews and are important for their nutritional and therapeutic properties (Ali et al., 2011)*.* Also popular is *G. latifolium,* a tropical rain forest plant used as a spice and vegetable and generally believed to possess many medicinal properties (Ugochukwu and Babady, 2002). Equally regarded to have medicinal properties are leaves of *V. amygdalina* which has several health benefits and also used for preparing local bitter leaf soup (Farombi and Owoeye, 2011). In Nigeria *I. gabonensis* is known as the wild mango seeds and it is usually used as a conventional soup thickner (Ekundayo et al*.,* 2013). Another popular soup condiment highly rich in protein is *Egusi* made from melon seeds of *C. citrullus* (Bankole et al*.,* 2006). Most soups are normally eaten with *Garri,* a popular food in Nigeria rich in carbohydrate and derived from the fermentation of mash obtained from the enlarged root of the cassava plant *M. esculanta Crantz* (Okafor et al., 1999).

Reports regarding foodborne listeriosis have increased due to the ability of *L. monocytogenes* to survive in harsh conditions (Hernandez-Milian and Payeras-Cifre, 2014). In order to reduce occurrence, Gillespie et al. (2006) suggested that dietary advice on avoiding high-risk foods should be provided routinely for the elderly and immune-compromised, not just to the pregnant women, these groups are associated with 15% out of 3088 cases of listeriosis reported in England and Wales between 1990-2010 (Awofisayo et al., 2015). In a serological study in Nigeria among different animal species (Oni et al., 1989), the prevalence of agglutinins to 5 serotypes of *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a and 4b) has been reported but molecular serotypes and evolutionary phylogeny of *L. monocytogenes* from Nigerian foods, animal or environment are still unknown. Although serological (agglutinins) typing correlates well with molecular lineage assignments (Nadon et al., 2001), molecular serotyping methods are now acceptable replacements for sero-grouping procedures (Nightingale et al., 2007).

Many different approaches are now used to evaluate occurrence and evolution of *L. monocytogenes* serotypes. Yin et al*.* (2015) performed multilocus sequence typing (MLST) and multi virulence locus sequence typing (MVLST) for 86 *L. monocytogenes* strains derived from 8 countries from 1926 to 2012 in order to understand the molecular evolution and genetic characteristics of the organism. The scientists identified a total of 13 clonal complexes and noted that polymorphism of housekeeping genes of isolates belonging to one of the clones increased rapidly over time. Furthermore, the phylogenetic analysis carried out showed that serotype 1/2b and 4b strains had an interval-type evolution pattern, while serotype 1/2a and 1/2c strains had a progressive-type evolution pattern. In another study, Pászti et al*.* (2014) used the molecular serotyping method of Doumith et al*.* (2004) and pulsed-field gel electrophoresis (PFGE) to serotype and characterize *L. monocytogenes* from human infections and found 2 serogroups and many pulsotypes. Other workers have used ribotyping and automated ribotyping (Matloob and Griffiths, 2014), Fourier transform infrared spectroscopy and chemometrics (Nyarko et al., 2014) and fluorescent amplified fragment length polymorphism (Amar, 2014) to differentiate *L. Monocytogenes* strains.

Ferreira et al. (2014) has highlighted that the current available data clearly indicate that *L. monocytogenes* persistence at various stages of the food chain contributes to contamination of finished products and continued efforts to integrate data on *L. monocytogenes* persistence is still needed to advance our understanding on the persistence of this important pathogenic bacterium. To this direction, the aim of this study was to establish the molecular serotypes and lineages of *L. monocytogenes* that may be found in common food items in south eastern Nigeria.

MATERIALS AND METHODS

Food items and detection of *L. monocytogenes*

Ground food items of *I. gabonensis, M. esculanta Crantz* and *C. citrullus* and sundried vegetables from *G. africanum, G. latifolium* and *V. amygdalina* (Figure 1) were purchased from a market in Owerri City, south eastern Nigeria after which standard presumptive identification as described by HPA (2007) was carried out. In this study, all media were prepared according to manufacturer's instructions and for primary detection, 25 g of each food item were weighed out into a stomacher bag and made up with 225 ml of half Fraser broth (Oxoid, Basingstoke, United Kingdom; CM895 base plus SR166E supplement) and then homogenized and incubated for 24 h at 30°C.

The incubated half Fraser broth was used for the secondary enrichment by inoculating 0.1 ml into 10 ml of Fraser broth (Oxoid, CM895 base plus SR156 supplement) in a universal bottle. Incubation was carried out at 37°C for 48 h after which 0.1 ml of broth was spread-plated onto *Listeria* oxford formulation (Oxoid; CM856 base and SR140 supplement) agar plates. Colonies that showed typical *L. monocytogenes* morphology were sub-cultured on brain heart infusion (BHI) agar (Oxoid, CM1135) to get pure cultures after which standard Gram stain, motility (Vatanyoopaisarn et al*.*, 2000) and catalase tests were carried out. The isolates were stored at -85°C for future use.

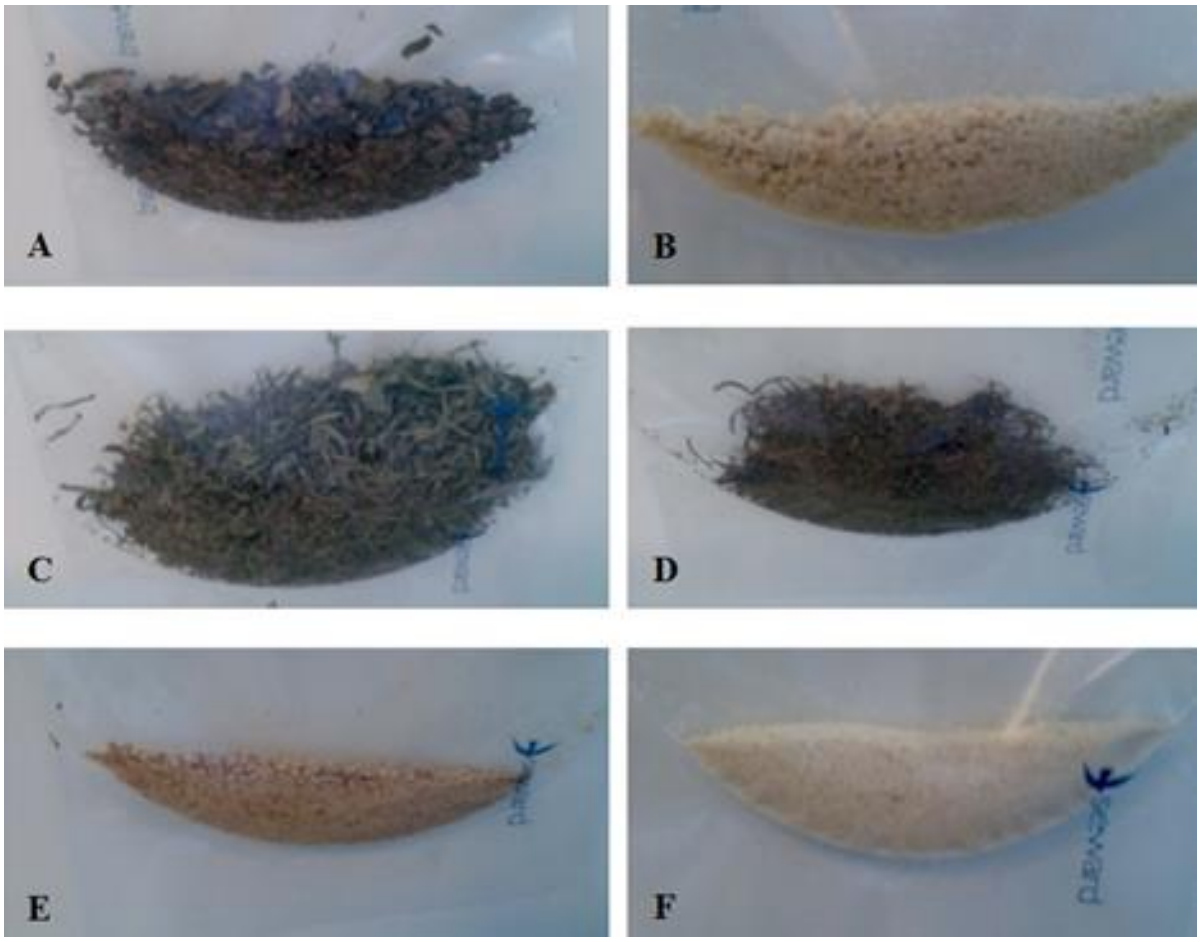

Figure 1. Nigerian food samples in stomacher bags just before homogenization with half Fraser broth. These were *Utazi* from *G. latifolium* (A), *Egusi* from *C. citrullus* (B), *Okazi* from *G. africanum* (C), *Onugbu* from *V. amygdalina* (D), *Ogbono* from *I. gabonensis* (E) and *Garri* from *M. esculanta Crantz* (F).

Separating *L. monocytogenes* from non-pathogenic *Listeria* species

To confirm that selected strains were not non-pathogenic *Listeria*, tests were performed with SwabSURE-ListeriaP kit (TSC, Heywood, United Kingdom). According to the manufacturers, the protocol works by sampling a pre-moistened swab dosed in the kit's TSC neutralising buffer and then followed by incubation for 24-48 h. The appearance of a turquoise blue colour indicates a positive presence for pathogen specific Phospholipase C enzyme. In order to satisfy manufacturer's requirement for tests to be carried out only on surfaces, a test surface to mimic a food contact surface was prepared with a microscope slide as previously described (Mafu et al., 2011) with modification. Instead of cutting glass coupons from the slide, the whole glass microscope slide with plain cut edges measuring 76 × 26 mm (Fisher Scientific) was used. After inoculating a loop of cells from a colony of the test strain to 5 ml of BHI broth in a sterile test tube, the test tube was incubated in a test tube shaker (New Brunswick Scientific) at 30°C for 18 h after which a 100 µl of the broth culture was placed in the middle of the microscope slide and then spread out with an inoculating loop. The slide was air dried for 3 h after which recovery of *L. monocytogenes* from the surface of the slide was carried out according to manufacturer's instructions.

The kit's pre-moistened swab with foam tip was removed from its holding tube making sure that it did not touch any surface before swabbing the surface of the microscope glass slide in 3 planes (horizontal, vertical and diagonal) whilst rotating swab between forefinger and thumb. After swabbing the surface of the slide, the swab was inserted into the media tube placed on a test tube rack and then incubated horizontally at 37°C for 48 h after which the tube was observed for change of colour.

Resuscitation of control strains

Four strains of *L. monocytogenes* with known serotype and lineage were used as control strains for this study, among them two reference strains were included (Table 1). The strains were maintained under cryo preservation at -85°C and these were obtained from the culture collection of Division of Food Science, University of Nottingham, UK. Cryo preservation beads containing the strains were removed from the -85°C freezer and streaked on to BHI media. Colonies that emerged after 48-72 h incubation at 37°C were used. To ensure that there was no contamination during recovery of the strains, the organisms were Gram stained and also grown on *Listeria* selective Oxford medium to confirm typical colonies of *L. monocytogenes*.

Table 1. Control strains representing the major serotypes of *L. monocytogenes* used in this study.

Strain	Serotype	PCR lineage	Source	Reference
Lm 4	1/2b	I	Bovine	Lawrence et al. (1995)
Lm 27	1/2c	II	Food environment	Lawrence et al. (1995)
Lm 10403S*	1/2a	II	Human	Bishop and Hinrichs (1987)
Lm ATCC23074*	4b	I	Human	ATCC catalogue

*****Reference strain.

Extraction of genomic DNA

This was carried out as described by Pitcher et al. (1989). To the sterile test tubes, 5 ml of BHI broth was added and inoculated with a colony of of *L. monocytogenes*. The test tubes were placed in a test tube shaker (New Brunswick Scientific) and incubated at 37°C for 24 h. A slight modification to the protocol was introduced by pelleting 1.5 ml of the incubated culture in an Eppendorf at 13000 xg for 1 min with a microfuge (Biofuge 13; Heraeus Sepatech) instead of centrifuging at 1000 xg for 15 min to obtain a suitable cell pellet. The process was repeated to obtain a bigger pellet following which 1 ml of ice cold lysis buffer (25 mM Tris-HCl 8.0, 10 mM EDTA, 50 mM sucrose, Fisher Scientific, UK) was added. The mixture was then treated with 50 $mgml^{-1}$ lysozyme (Sigma) and incubated for 1 h at 37°C after which 0.5 ml lysis solution (5 M guanidium thiocynate, 0.1 M EDTA, Fisher Scientific; 0.5% Sakosyl, Sigma) was added, mixed well and left at room temperature for 5 min. The lysate was cooled on ice for 30 min and 0.25 ml of ammonium acetate was added, vortexed and incubated again on ice for another 10 min. Chloroform : isoamyl alcohol (24:1 ratio, 0.5ml, Fisher Scientific, UK) was added, vortexed and spun for 10 min at 13000 xg.

The upper phase 850 µl was removed and introduced into a clean Eppendorf tube following which exactly 0.54 volumes of cold isopropanol were added. The mixture was centrifuged at 13000 xg for 20 s and the visible pellet was washed 3 times in 70% ethanol. The pellet was resuspended in 50 µl TE (10mM Tris, 1mM EDTA pH7.0). To remove RNA, 1 µl (50 $mgml^{-1}$) of lyophilized RNAse was added to the suspension and incubated at 37°C for 30 min. The integrity of the DNA was checked by horizontal electrophoresis in 0.7% agarose in TAE buffer (40 mM Tris base, 20 mM glacial acetic acid 1 mM EDTA, pH 8, Fisher Scientific) containing ethidium bromide (0.5 $mgml^{-1}$) and viewed under ultraviolet light (ImageMaster® VDS system (Amersham Biosciences,No.80-6254-80). The concentration (ng/µl) was measured by placing 1 µl of DNA sample onto NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.,USA) after which the DNA was stored at -20°C till its subsequent use in PCR reactions.

Molecular serotyping

Serotyping was carried out using the multiplex PCR protocol described by Doumith et al. (2004) using the primer pairs No. 5-9 (Table 2). A final volume of 50 µl containing 1 U of Taq polymerase (ABgene), 10 ng DNA template, 0.2 mM deoxyribonucleoside triphosphate (Promega), 2 mM $MgCl_2$ (ABgene) and 5 µl of 1X PCR buffer (ABgene) was used. Primers were added at the following concentration: 1 µM for *lmo*0737, *ORF*2819 and *ORF*2110; 1.5 µM for *lmo*1118 and 0.2 µM for *prs.* PCR was performed with an initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 0.40 min, 53°C for 1.15 min and 72°C for 1.15 min and one final cycle of 72°C for 7 min in a thermocycler (Techne 312). Following PCR reaction, the product (5 µl) was mixed with 2 µl of loading buffer and separated on a 2% agarose gel in TAE buffer. Staining with ethidium bromide enabled visualization of PCR fragments under UV light. After scoring the PCR products against a 100bp ladder, serotypes were assigned to the test strains based on genes amplified by the corresponding reference strain.

Evolutionary lineage analysis

Lineage group identification based on *prfA* virulence gene cluster of *L. monocytogenes* developed by Ward et al*.* (2004) was used by amplifying allelic specific oligonucleotides (ASO) using primer pair No. 2-4 (Table 2). Amplifications were performed in 50 µl volumes with 0.5 µM concentrations of each primer, 2 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate, 0.5 U of Taq polymerase (Abgene) and 100 ng of DNA. Amplifications consisted of 25 cycles of 15 s at 94°C, 10s at 56°C and 10 s at 72°C following which amplification products were resolved in 2% (wt/vol) agarose gel and scored relative to 100 bp DNA size ladder before evolutionary lineages were assigned to the test strains based on genes amplified by the corresponding reference strain.

Sequencing of the V3 region of 16S rDNA

Sequencing for an isolate of interest was carried out by PCR amplification of the V3 region of the 16s rDNA (Muyzer et al., 1993; Coppola et al*.,* 2001) using primer pairs No. 1 (Table 2). A 1 µl of DNA template (100 ng/µl) was used and the reaction mixture (50 µl) for amplification contained 1.25 U of Taq DNA polymerase (Fisher Scientific) 5 µl of 10X PCR buffer (Fisher Scientific,), 0.2 mM of the deoxynucleotide triphosphates, 0.2 pmol/µl of forward and reverse primers and 2.5 mM of magnesium chloride.

DNA was denatured for 5 min at 94°C followed by initial annealing temperature of 66°C, which was decreased 1°C every cycle for 10 cycles after which another 20 cycles were performed at 56°C. Elongation was performed at 72°C for 3 min before a final extension at 72°C for 10 min was carried out. The PCR fragments generated were cut out from the gel and cleaned with a purification kit (Qiagen) and then sequenced (MGW Eurofins, Germany). Sequence generated was used for a search on the databases of National Center for Biotechnology Information (http://ncbi.nlm.nih.gov) using the BLAST program after which the sequence was submitted to EMBL archives and given accession number LN832629.

RESULTS AND DISCUSSION

Detection and identification of *L. monocytogenes*

It is known that the ability of *L monocytogenes* to hydrolyze esculin to esculetin (Qadri et al., 1980) and the

Table 2. Primers pairs used in this study.

Gene	Forward (top line) and reverse primers (5'- 3')	≈PCR product (bp)	Reference
V3 16S rDNA	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTCG	200	Coppola et al. (2001)
*actA*1	AATAACAACAGTGAACAAAGC TATCACGTACCCATTTACC	373	Ward et al. (2004)
*plcB*2	TTGTGATGAATACTTACAAAC TTTGCTACCATGTCTTCC	564	"
*actA*3-f *plcB*3-r	CGGCGAACCATACAACAT TGTGGTAATTTGCTGTCG	277	"
lmo0737	AGGGCTTCAAGGACTTACCC ACGATTTCTGCTTGCCATTC	691	Doumith et al. (2004)
lmo1118	AGGGGTCTTAAATCCTGGAA CGGCTTGTTCGGCATACTTA	906	"
ORF2110	AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGA	597	"
ORF2819	AGCAAAATGCCAAAACTCGT CATCACTAAAGCCTCCCATTG	471	"
prs	GCTGAAGAGATTGCGAAAGAAG CAAAGAAACCTTGGATTTGCGG	370	"

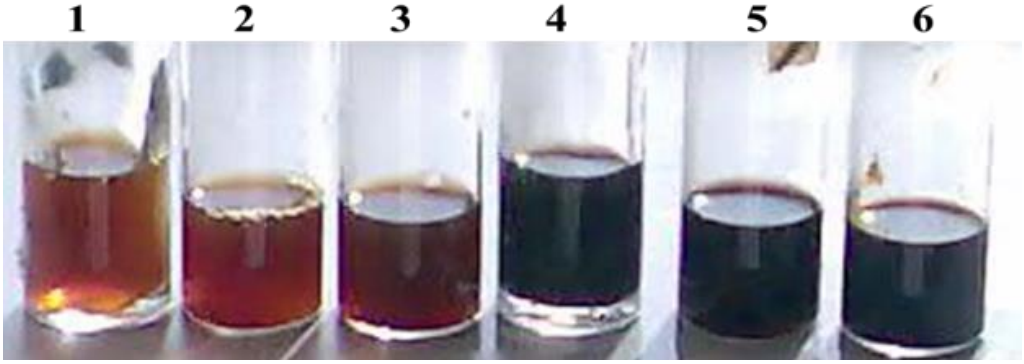

Figure 2. Secondary enrichment with Fraser broth. 1, Ogbono (from *I. gabonensis*); 2, egusi (*C. citrullus*); 3, garri(*M. esculanta Crantz*); 4, utazi (*G. latifolium*); 5, Okazi (*G. africanum*); 6, Onugbu (*V. Amygdalina*).

subsequent reaction of esculetin with ferric ions which results in blackening of media is used for the identification of the organism. After secondary enrichment, there was complete blackening of the Fraser broth for food samples from *G. latifolium, G. africanum, V. Amygdalina* whereas broth samples of food from *I. gabonensis, M. esculanta Crantz* and *C. citrullus* did not blacken (Figure 2).

All broths were subcultured on Oxford agar before discarding, irrespective of colour change. After incubation at 30°C for 48 h colony types that showed black colouration of media were selected and Gram

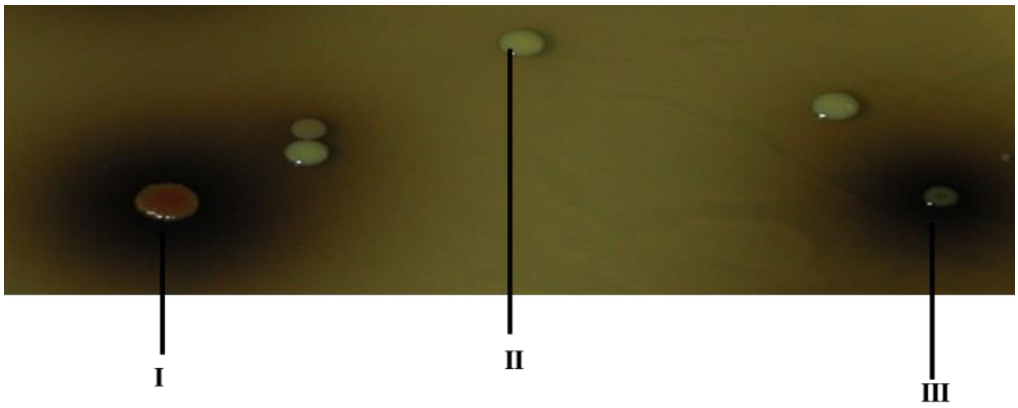

Figure 3. Three colony types isolated from leaves of *G. latifolium* growing on Oxford formulation agar showing aesculin (I and III) and non- aesculin (II) hydrolyzing colonies after 48 h incubation at 37°C.

stained. Colonies (Colony III, Figure 3) consistent with *L. monocytogenes* morphology were found to be Gram positive rods, had a doughnut like appearance on Oxford media and were about 0.5 µm in width and 1-1.5 mm in length whereas staining of aesculin producing colonies with non-conforming *L. monocytogenes* morphology (Colony I, Figure 3) showed Gram positive cocci shaped organisms similar to *Enterococcus* species that are known to be also capable of aesculin hydrolysis (HPA, 2010).

This confirms that relying on black coloration of Oxford media for selection and enumeration purposes as shown in some reports (Nwaiwu, 2015), may result in false positives (Fraser and Sperber, 1988). No isolate fitting *Listeria* morphology was found in food items obtained from *I. gabonensis, M. esculanta Crantz* and *C. Citrullus.* One potential *L. monocytogenes* isolate which was catalase positive and showed tumbling motility at 25°C (Vatanyoopaisarn et al., 2000) was selected for each food sample from the vegetables *G. latifolium, G. africanum, V. amygdalina* and designated LmNG1, LmNG2 and LmNG3, respectively and stored at -85°C for future use. Overall, only these 3 isolates were presumptively identified as *L. monocytogenes* and selected for molecular processing.

Assessing pathogenicity potential of isolated strains

To indicate if isolates are pathogenic *L monocytogenes*, a test was carried out using SwabSure ListeriaP kit, a chromogenic kit described by the manufacturers as being capable of differentiating pathogenic *L monocytogenes* and *L. ivanovii* from other commensal microorganisms including *L. innocua.* Test with the kit after swabbing the surface of a microscope glass slide containing dried cultures produced the turquoise blue colour for positive identification indicating the presence of pathogen specific phospholipase C enzyme (Notermans et al., 1991).

Molecular analysis of selected strains

According to Gasanov et al. (2005) early typing methods differentiated isolates based on phenotypic markers (motility, hemolysis, sugar fermentation) but these phenotypic typing methods are being replaced by molecular tests, which reflect genetic relationships between isolates and are more accurate. Further confirmation and determination of the serotypes and lineage of the isolated strains were ascertained by PCR multiplex serotyping and lineage analysis. These methods are still widely used (Warren et al., 2015) by other investigators. Strains representing the four major serotypes 1/2a, 1/2b, 1/2c and 4b of *L. monocytogenes* (Liu and Busse, 2009) and strains representing Lineage I and II were used as control (Table 1).

Genomic DNA template which had approximately 2.0 (260/280 nm) absorption ratio after Nano-Drop nucleic acid measurements was used for all PCR reactions. The result of the multiplex PCR serotyping carried out is shown in Figure 4. The control and test strains amplified *prs* gene (approximately 370 bp). In addition to *prs*, the control strain that is 4b amplified *ORF*2110 and *ORF*2819 while strains that were serotype 1/2a amplified *lmo* 0733. The strain that is 1/2c also amplified *lmo* 0733 in addition to *lmo*1118 and strains of 1/2b serotype amplified *ORF*2819. A summary table of the result gained is in Table 3. When all the amplification patterns were considered using the grouping scheme shown by Doumith et al. (2004), it was found that *L. monocytogenes* isolated from food item of *G. latifolium* and that of *G. africanum* were Group 3 (1/2b, 3b,7) whereas the strain from *V. amygdalina* food sample was grouped into Group 1 (1/2a, 3a). There was no prevalence found in groups 2 (1/2c, 3c) or 4 (4b, 4d and 4e). The amplification patterns corresponded to the control strains Lm4 (1/2b) and Lm10403S (1/2a) which indicated that the new strains LmNG1 and LmNG2 are1/2b serotype whereas LmNG3 is serotype 1/2a.

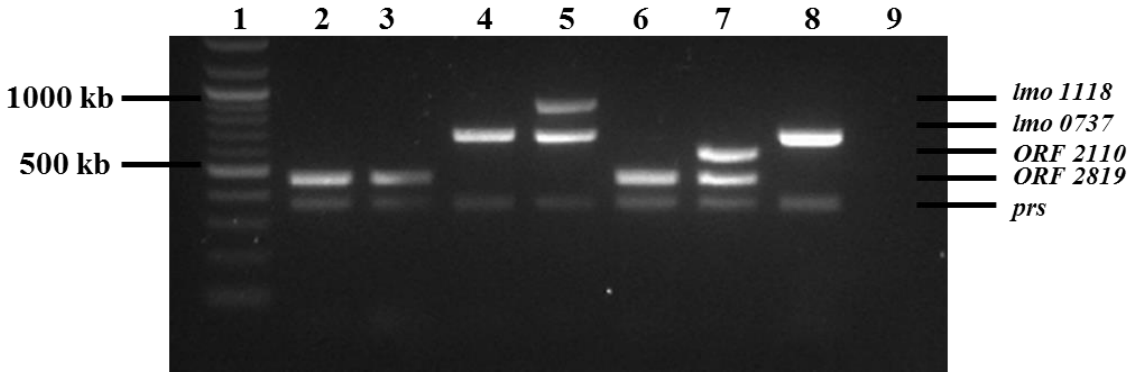

Figure 4. Multiplex PCR serotyping by amplification of different *L. monocytogenes* genes. The PCR products were separated on 2.0% agarose gel and scored against a 100 bp ladder, Lane 1 = 100 kb ladder; lane 2 = LmNG1; lane 3 = LmNG2; lane 4 = LmNG3; lane 5 = LM27; lane 6 = Lm4; lane 7 = LmATTC23074; lane 8 = Lm10403S; lane 9 = negative control.

Table 3. Summary of serotype assignment based on multiplex PCR serotyping of *L. monocytogenes* strains isolated from Nigerian vegetables. Serotypes were assigned to the strains based on the pattern of genes amplified. Strains of known serotypes were used as control.

Strain	Multiplex PCR fragment amplification					Sero group classification	Source
	***lmo*1118 (906 bp)**	***lmo*0737 (691 bp)**	***ORF*2110 (597 bp)**	***ORF*2819 (471 bp)**	***Prs* (370 bp)**		
ATCC23074	-	-	+	+	+	4b	UoN
Lm4	-	-	-	+	+	1/2b	UoN
Lm27	+	+	-	-	+	1/2c	UoN
Lm10403S	-	+	-	-	+	1/2a	UoN
LmNG1	-	-	-	+	+	1/2b	*G. latifolium*
LmNG2	-	-	-	+	+	1/2b	*G. africanum*
LmNG3	-	+	-	-	+	1/2a	*V. amygdalina*

+ = Amplified; -, not amplified; UoN = University of Nottingham.

The lineage classification performed showed that strains that are serotype 1/2b and 4b amplified fragments of *actA1* **(**373bp) expected for lineage I, while strains that are serotypes 1/2a and 1/2c amplified fragments of *plcB2* (564bp) expected for lineage II (Figure 5). The lineage classification of all the strains under study is shown in Table 4. There was no amplification of lineage III specific sequence showing that no lineage III strain was found in this study. Isolates LmNG1 and LmNG2 were found to be lineage 1 whereas LmNG3 was found to be lineage II. Isolates LmNG1 and LmNG2 had the same amplification with control strain Lm4 (1/2b) whereas strain LmNG3 had the same lineage with reference strain Lm10403S (1/2a).

Over 90% of the human listeriosis cases are caused by *L. monocytogenes* serotypes 1/2a,

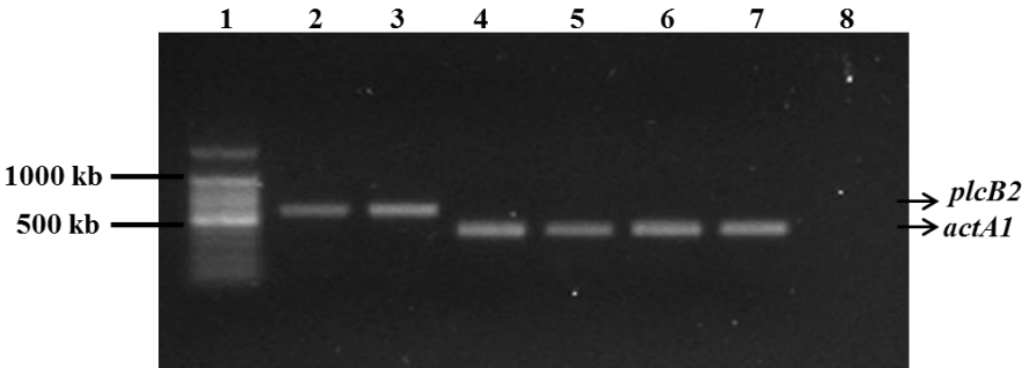

Figure 5. Allelic specific oligonucleotide amplification to determine evolutionary lineage of new isolates from Nigerian food items. PCR products were separated on 2.0% agarose gel. Lane 1= 100 kb ladder; lane 2 = LmNG3, lane 3 = Lm10403S; lane 4 = LmNG2; lane 5 = LmNG1; lane 6 = Lm4; Lane 7 = Lm23074; lane 8 = negative control.

Table 4. Lineage classification of *Listeria monocytogenes* strains.

Strain	Serotype	ASO-PCR amplification			Lineage classification	Source
		actA1	*plcB2*	*actA3*		
ATCC23074	4b	+	-	-	I	UoN
Lm27	1/2c	-	+	-	II	UoN
Lm4	1/2b	+	-	-	I	UoN
Lm10403S	1/2a	-	+	-	II	UoN
LmNG1	Unknown	+	-	-	I	*G. latifolium*
LmNG2	Unknown	+	-	-	I	*G. africanum*
LmNG3	Unknown	-	+	-	II	*V. amygdalina*

1/2b and 4b strains (Laksanalamai et al., 2014) and all the serotypes are spread over at least 4 (I-IV) evolutionary lineages (Combrouse et al., 2013). Serotype 1/2a is common in the environment while serotype 1/2b and 4b are isolated from many clinical samples. Previous reports show that isolates from human listeriosis cases are common in lineage I whereas lineage II strains are prevalent in food isolates and lineage III and IV rarely occurs (Orsi et al., 2011). The occurrence of Lineage II isolate (serotype 1/2a) in sample from *V. amygdalina* is in line with predominance of serotype 1/2a in food and environmental samples but the strains recovered in samples from *G. latifolium* and *G. africanum* were least expected because they have the serotype and lineage associated more with clinical samples. However, recovery of Lineage 1 serotypes (mostly associated with clinical samples) from food is no longer unusual because the serotype 1/2b belonging to lineage 1 has now been isolated from other vegetables (Cordano and Jacquet, 2009; Sant'Ana et al., 2012).

The isolation of *L. monocytogenes* from food product of *G. latifolium* used to prepare local salads was least expected given the anecdotal antimicrobial status of *G. latifolium* in south eastern Nigeria. Hence, the serotype 1/2b isolate from *G. latifolium* was analyzed to determine its phylogeny. After using the BLAST program and comparing sequences producing significant alignment, *L. monocytogenes* strain N2306 (Accession CP011004) involved in an outbreak of listeriosis in Switzerland was the top hit among 100 strains with 99% match (denotes percent identity of sequence query alignment) and the same Expect value (E-value). The full genome sequence of strain N2306 is on the NCBI data base hence a future study to carry out genome sequencing of the new isolate (Accession LN832629) for comparison would be beneficial.

Yin et al. (2015) found that *L. monocytogenes* strains from temporally and geographically unrelated outbreaks in different countries were clustered in the same subgroup of phylogenetic tree, and suggested that they have similar virulence genes and genetic characteristics due to adaptation. Therefore, the food isolate from *G. latifolium* might be a potential causative agent for an outbreak because the closest relative of the isolate in a geographically unrelated region has been implicated in an outbreak of listeriosis. Also, the isolate is serotype 1/2b and the ability of this serotype to cause outbreaks has been reported, while this was listed among serotypes

that caused 24 listeriosis outbreaks in the United States for the period 1998-2008 (Cartwright et al., 2013).

Presence of *L. monocytogenes* in only sundried vegetables from *G. latifolium, G. africanum, V. amygdalina* might be due to the fact that sundried leafy vegetables were not ready to use when they were purchased and may still have environmental and other post-harvest debris present which were not readily visible. Also, the vegetables still needed a final wash before it could be cooked for food. Washing reduces the soil present in fresh food and may lower the level of the organisms present. Furthermore, the prevalence of *L. monocytogenes* might come from post-harvest contamination and moreover, most vegetables sold in Nigeria are not pre-packed under controlled atmosphere.

Fresh vegetables are increasingly recognized as a source of food-borne outbreaks in many parts of the world (Tromp et al., 2010) and prevalence of *L. monocytogenes* has been severally reported in vegetables. Pre-harvest prevalence of pathogenic *L. monocytogenes* in selected vegetables from agricultural farm samples has been reported in a previous study (Soni et al., 2014) and many workers have found the post-harvest occurrence of *L. monocytogenes* in vegetables. Little et al. (2007) found that 4.8%, out of 2686 samples of pre-packed mixed vegetable salads in UK were contaminated with *L. monocytogenes* and Aparecida de Oliveira et al. (2010) used immunoassay *Listeria* rapid test and 16s rRNAreal-time PCR assay to detect *L. monocytogenes* in minimally processed leafy vegetables.

Conclusions

The molecular analysis of the three strains isolated from different popular vegetables from Nigeria indicates that the major *Listeria* serotypes and evolutionary lineages associated with listeriosis could occur in popular Nigerian leafy vegetables. To the best of the knowledge of the author, this is the first time that the major *L. monocytogenes* molecular serotypes have been linked with popular Nigerian vegetables. The harsh process of obtaining food products from *I. gabonensis, M. esculanta Crantz* and *C. citrullus* and the intrinsic and extrinsic factors in play from farm to fork appears to be unfavourable to *L. monocytogenes* denoting that occurence of the organism is unlikely if good food processing and preservation practices are applied and maintained after harvest.

Popular Nigerian leaf vegetables from *I. gabonensis, M. esculanta Crantz* and *C. citrullus* could be a major vehicle for transmission of the pathogenic organism in Nigeria based on the study. Therefore, further genomic investigations and increased surveilance would be a major concern due to the finding that the closest relative of the sequenced isolate has been implicated in an outbreak of listeriosis.

Conflict of interest

The author declares that there is no conflict of interest.

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Full Length Research Paper

In vitro production of thiophenes using hairy root cultures of *Tagetes erecta* (L.)

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Marigold (*Tagetes* spp.) is a source of thiophenes, which are a group of heterocyclic sulfurous compounds possessing strong biocidal activity, thus making *Tagetes* plants very useful as natural source of agents for controlling pathogens such as nematodes, insects, fungi and bacteria. Hairy root cultures of *Tagetes erecta* L. were developed using *Agrobacterium rhizogenes* mediated transformation. The hairy root clones showed great variations in growth pattern and total thiophene content (0.31 to 0.96 mg/gfw). Four types of thiophenes that accumulated in root cultures of *Tagetes* were butenenylbithiophene (BBT), hydroxybutenenylbithiophene (BBTOH), acetoxybutenenylbithiophene (BBTOAc) and α-terthienyl (α-T). Total thiophene contents in these isolated rhizoclones were found to be four fold higher than that of wild type hairy root cultures. The developed method of producing hairy cultures of *T. erecta* can be used for producing thiophenes at large scale.

Key words: *Agrobacterium rhizogenes,* marigold, rhizoclones, biocidal.

INTRODUCTION

The biocidal properties attributed to marigold (*Tagetes* species), particularly the nematicidal property, are related to the presence of thiophenes (Chan et al., 1975; Gommers and Geerligs, 1973). Thiophenes are sulphur-heterocyclic compounds found in many plant species (Abegaz, 1991; Hudson et al., 1986) and are well represented in the *Tagetes* species (Bohlmann et al., 1973; Downum and Towers, 1983). Thiophenes act as toxins that are activated by sunlight or UV radiation (300 to 400 nm), killing pathogens such as nematodes, insects, fungi and bacteria (Champagne et al., 1984). Nematodes causes an estimated $100 billion annually in worldwide crop damage making it agriculture largest unmet pest control need (Luc et al., 1990). Nematode

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Abbreviations: BBT, Butenenylbithiophene; **BBTOH,** hydroxybutenenylbithiophene; **BBTOAc,** acetoxybutenenylbithiophene; **α-T,** α-terthienyl; **EPA,** environmental protection agency; **MeBr,** methyl bromide.

control has traditionally depended on highly toxic contact and fumigant pesticides which have now been restricted or eliminated in the United States by the Environmental Protection Agency (EPA). Similar restrictions have occurred in other countries. Chemicals such as organophosphate and carbamate contact nematicides, fenamiphos, carbofuran, aldicarb and fumigants like metam-sodium and 1,3-dichloropropene has been further restricted. Similarly, methyl bromide (MeBr) production and import ended in the U.S.A in 2005 under the Montreal Protocol (Gareau and Dupuis, 2009). Use of the remaining stockpiles requires annual critical use exemptions from the international protocol. Therefore, new classes of nematicidal compounds are constantly being sought, but there are currently no promising materials close to commercial developments. Avermectins, a microbial origin and powerful anthelmintics have been developed for veterinary use. Its efficacy against plant parasitic nematodes is well established, however, it cannot be used successfully as soil treatments because the compound is complex. It is estimated that the current market for nematicides is between $700 million and $1 billion each year worldwide (Global Nematicide Market, 2012).

Hairy root cultures have been proven to be an efficient means for producing secondary metabolites that are normally biosynthesized in roots of differentiated plants and difficult to be synthesized chemically (Hu and Du, 2006). Thiophene accumulation in Plants/calli of different species of *Tagetes* have been recorded by various groups like Croes et al. (1989), Kyo et al. (1990), Menelaou et al. (1991), Mukundan and Hjortso (1991a, b) etc. Rajasekaran et al. (2004) also reported hairy root culture of *Tagetes patula* for production of thiophene. The thiophene produced in hairy roots of *T. patula* showed larvicidal effect against mosquito larvae. Thus, it was evident that organization is a prerequisite for production of thiophene, and hence it was concluded that root cultures would be a suitable alternative to calli/cell suspension cultures. Therefore, this experiment was carried out to establish the hairy root culture of *Tagetes erecta* L. by *Agrobacterium rhizogenes* mediated transformation for *in vitro* production of thiophenes.

MATERIALS AND METHODS

Plant material

Seeds of *T. erecta* were acquired from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. They were surface sterilized with 0.1% mercuric chloride for 90 s, and washed three times with autoclaved distilled water for 10 min each. The disinfected seeds were germinated on media containing MS salts (Murashige and Skoog, 1962) and 30 gL^{-1}sucrose solidified with 0.8% agar. The cultures were incubated at 25±2°C under cool-white fluorescent lamp intensity of 40 µm m^{-2} s^{-1} with a 16 h light/8 h dark cycle (Trypsteen, 1991). The young leaves obtained from *in vitro* grown *T. erecta* plants were used as explants source.

Induction of hairy roots by *A. rhizogenes*

The wild type A4 strain of *A. rhizogenes* harboring an agropine-type pRi A4 was grown in liquid YMB media up to $O.D_{600}$ = 0.9-1.0 (Birot et al., 1987; Hooykass et al., 1977). Transformation with *A. rhizogenes* and hairy root induction was done following a procedure previously reported by Pal et al. (2013). The young leaves were infected by pricking with needle, and loaded with suspension culture of *A. rhizogenes.* The inoculated leaf sections were placed on MS basal medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose and grown in dark. The control leaves were also kept after pricking with sterile needle. Roots produced at the infected sites were excised and placed on semi solid MS medium containing cefotaxime (250 mg L^{-1}). The putative hairy roots were made bacteria free by regular sub-culturing on fresh medium containing cefotaxime. The bacteria free hairy roots (100 mg) were transferred on to liquid ½ MS medium with 20 g L^{-1} sucrose, and incubated with agitation (60 rpm) at 25°C in the dark. The cultures were maintained for 45 days, and the root samples were collected for thiophene estimation at an interval of 5 days from the 15th day onwards (that is, 15, 20, 25, 30, 35, 40 and 45 days). Non-transformed root cultures were also initiated and maintained on same medium by excising roots from aseptically grown non-transformed *T. erecta* plantlets (Figures 1 and 2).

PCR analysis

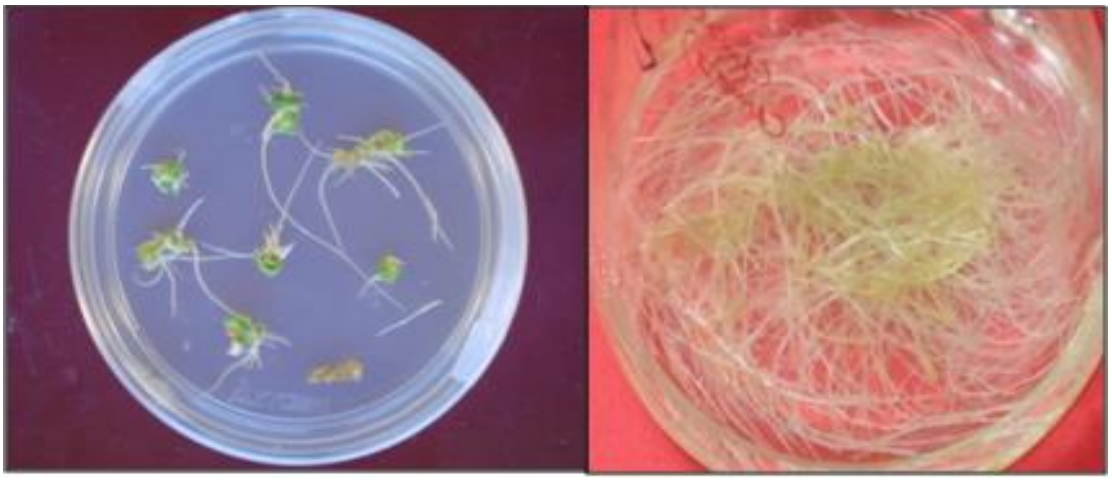
Genomic DNA was extracted from root tissue using CTAB method (Murray and Thompson, 1980), and used as template in polymerase chain reaction. The reaction was carried out using *rol C* (540 bp) gene specific primers (Bulgakov et al., 2005). Each 25 µl reaction mixture contained 1X PCR buffer, 3.5 mM $MgCl_2$, 25 p mol of each forward (5'-ATGGCTGAAGACGACCTGTT-3') and reverse (5'-TTAGCCGATTGAAAACTTGCAC-3') primers with 0.2 mM dNTPs and 1U of Taq DNA polymerase (Bangalore Genei Private Ltd. Bangalore, India), and ~10 ng of genomic DNA was added as template in respective reactions. The reaction with no template served as negative control. Amplification cycle included initial denaturation for 4 min at 94°C, followed by 30 cycles of 45 s denaturation at 94°C, annealing for 60 s at 55°C, extension at 72°C for 120 s and 10 min final extension at 72°C in a programmable peltier thermal cycler (PTC-200, M J Research, USA).

Measurement of biomass

Sampling for biomass measurement was done from the transformed as well as non-transformed root cultures at different intervals (that is, 15th days of culture initiation and thereafter at 5 days interval up to 45 days). In this ways a total of seven samples were drawn (15, 20, 25, 30, 35, 40, and 45 days culture). The fresh weight was determined after washing the roots with deionized water so as to remove the medium salts, and blotting the excess water on filter paper. The experiment was laid out in randomized complete block design and had three replications. The data of fresh weight of transformed as well as non-transformed roots at different time intervals was analyzed using the software SPSS ver. 20.0 and the same is presented as mean ± standard deviation in Figure 3.

Extraction and estimation of thiophenes

The different root samples from different time intervals were dried at 40°C in a hot-air oven. Thiophene extraction and estimation from such dried root samples were carried out according to Rajasekaran et al. (1999). A known weight of dried material was extracted with hexane for 12 h at 20°C in the dark. The crude extracts were



a b

Figure 1. a) Hairy root induction from leaf discs infected with *A. rhizogenes.* **b)** Growth of isolated rhizoclone in liquid MS media.

filtered and washed with hexane. The filtrates were evaporated to dryness and residues dissolved in hexane. The purified extracts were stored at 4°C in the dark. An aliquot of hexane extract of hairy root cultures or standard of thiophenes sample was injected to HPLC column (LC-10A HPLC, SHIMADZU JAPAN), C18 (3.9 × 300 mm) (Bondapack) and run at isocratic condition using solvent mixture of hexane-dioxan (95:5), with flow rate of 2.5 $ml.min^{-1}$ and pump pressure of 125 $kg.cm^{-1}$. Detector (Photodiode array detector) was set at 330 nm. Quantification was done by comparison of peak area with those of authentic standards, that is, 5-(3-buten-1-ynyl)-2,2-bithienyl (BBT), 5-(4-hydroxy-1-butynyl)-2,2- bithienyl (BBTOH) 5-(4-acetoxy-1-butynyl)-2,2-bithienyl (BBTOAc), and α-T (2,20:50,20- terthienyl). The experiment was conducted with three biological replicates and the data is represented as means of the standard deviation.

RESULTS

Induction and culture of transformed hairy roots

After one week of incubation on MS basal medium, roots emerged directly from the sites of infection on leaf discs (Figure 1a). The frequency of root initiation was nearly 80% from leaf explants inoculated with *A. rhizogenes*. A total of 11 hairy root lines were isolated and fast growing hairy root lines were selected for further analysis. The developed hairy roots showed rapid growth with extensive lateral branching (Figure 1b). The cultures adapted well to liquid medium. Great morphological variations were evident among the isolated rhizoclones (Figure 3 and 4). Inoculation was done with 100 mg fresh root sample of both the transformed as well as non-transformed lines. Growth of the root cultures was in exponential phase till 15 days. From 20 days onwards, cultures were in log phase. Accumulation of biomass showed a specific growth rate reaching maximum until 45 days (Figure 3).

PCR detection and opine assay for confirmation of transgenic hairy roots

Genomic DNA isolated from putative transgenic roots yielded expected size of 540 bp PCR amplification with *rol C* gene specific primers. Non template control (ntc) and wild type roots (W) did not produce any amplification (Figure 5). Thus, there was stable integration and expression of *rol* genes in transformed hairy roots.

Thiophene accumulation

Thiophene production was observed in all the rhizoclones, that is, Tr1- 4 and wild type, and NTr hairy roots. Chromatographic separation of hexane extracts showed at least four peaks representing all four structurally different thiophenes, that is, BBT, BBTOH, BBTOAc, and a-T (Figures 1 and 2). In all cases, thiophene content increased rapidly and reached to peak level in 15 day cultures, and thereafter declined gradually till 45 days (Figure 6). Thiophene accumulation pattern showed a growth associated correlation, with a maximum thiophene content on the 15^{th} day which were in exponential phase. The decline of thiophene content was found in cultures in log phase. However, total thiophene content in some of the transformed clones was nearly 4 times higher than that of wild type roots (Figure 6). The maximum thiophene content was found in line Tr-2 while the minimum thiophene content was found in line Tr-4 as compared to the non-transformed (NTr) control line.

DISCUSSION

Thiophenes found in Marigold plants make them

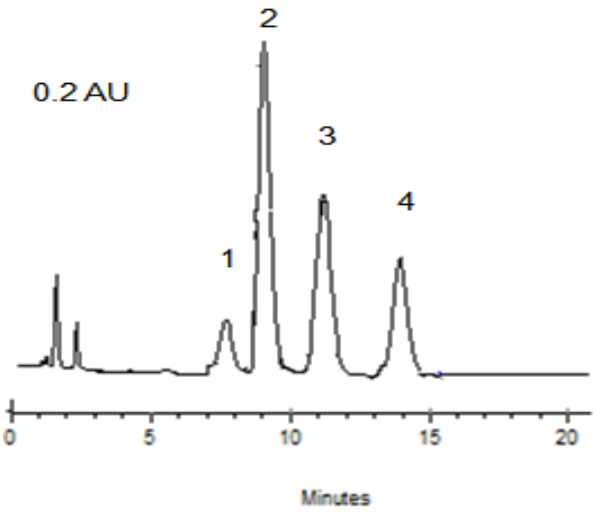


Figure 2. HPLC profile of *T. erecta* rhizoclone extracts. Peaks numbered as 1-4 corresponds to various thiophenes, that is, BBT, BBTOH, BBTOAc, and a-T respectively.

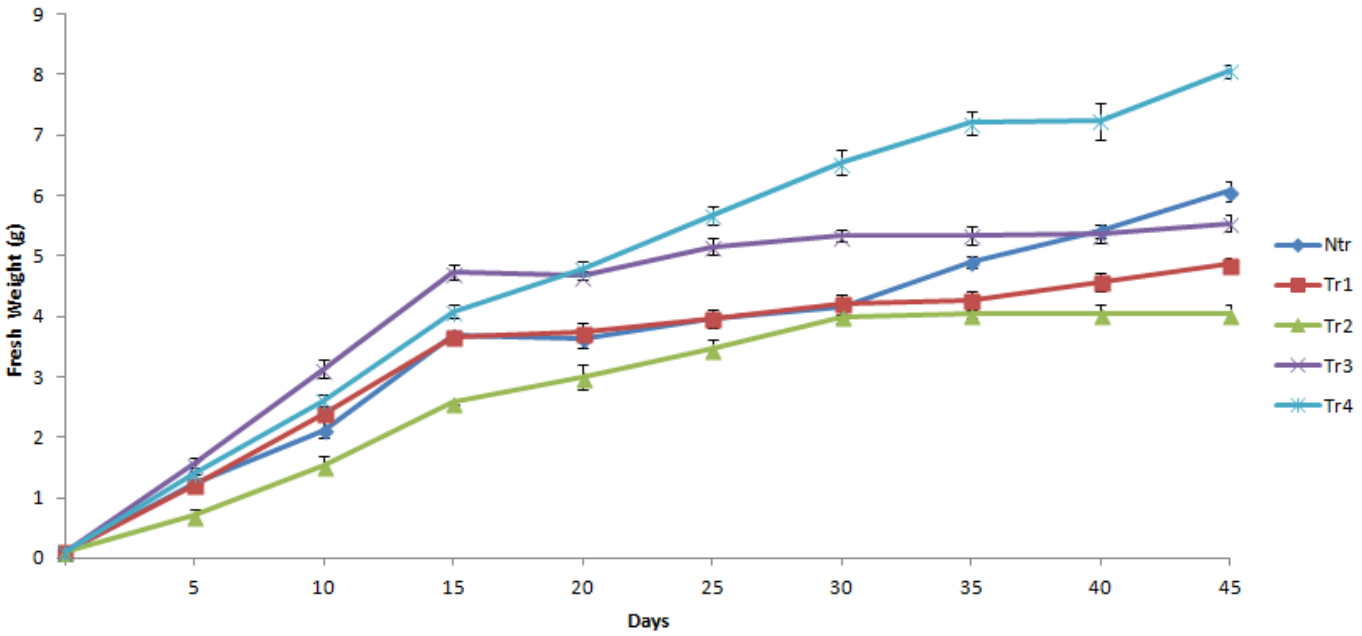


Figure 3. Growth pattern of hairy root clones of *T. erecta.* Ntr is nontransgenic /wild type root cultures; Tr1- Tr4 are transgenic hairy root clones.

attractive potential candidates for the development of plant based biopesticides. In the present work, we have attempted to establish the method of *in vitro* production of thiophenes by hairy root cultures of *T. erecta* plant. The PCR analysis (Figure 5) indicated the presence of *rol C* gene, a part of the *Agrobacterium* pRi T_L–DNA and T_R–DNA, in the host plant genome (Chilton et al., 1982; White et al., 1982). The fast growth and easy maintenance of hairy root cultures are advantageous to be used as continuous sources for the production of valuable secondary metabolites. Therefore, prove economical for commercial production purposes. All four structurally different thiophenes detected by us in *T. erecta* (Figures 1 and 2) were earlier reported in other *Tagetes* species, that is, *Tagetes laxa*, *Tagetes terniflora*, *Tagetes minuta* and *Tagetes campanulata*. (Rodriguez

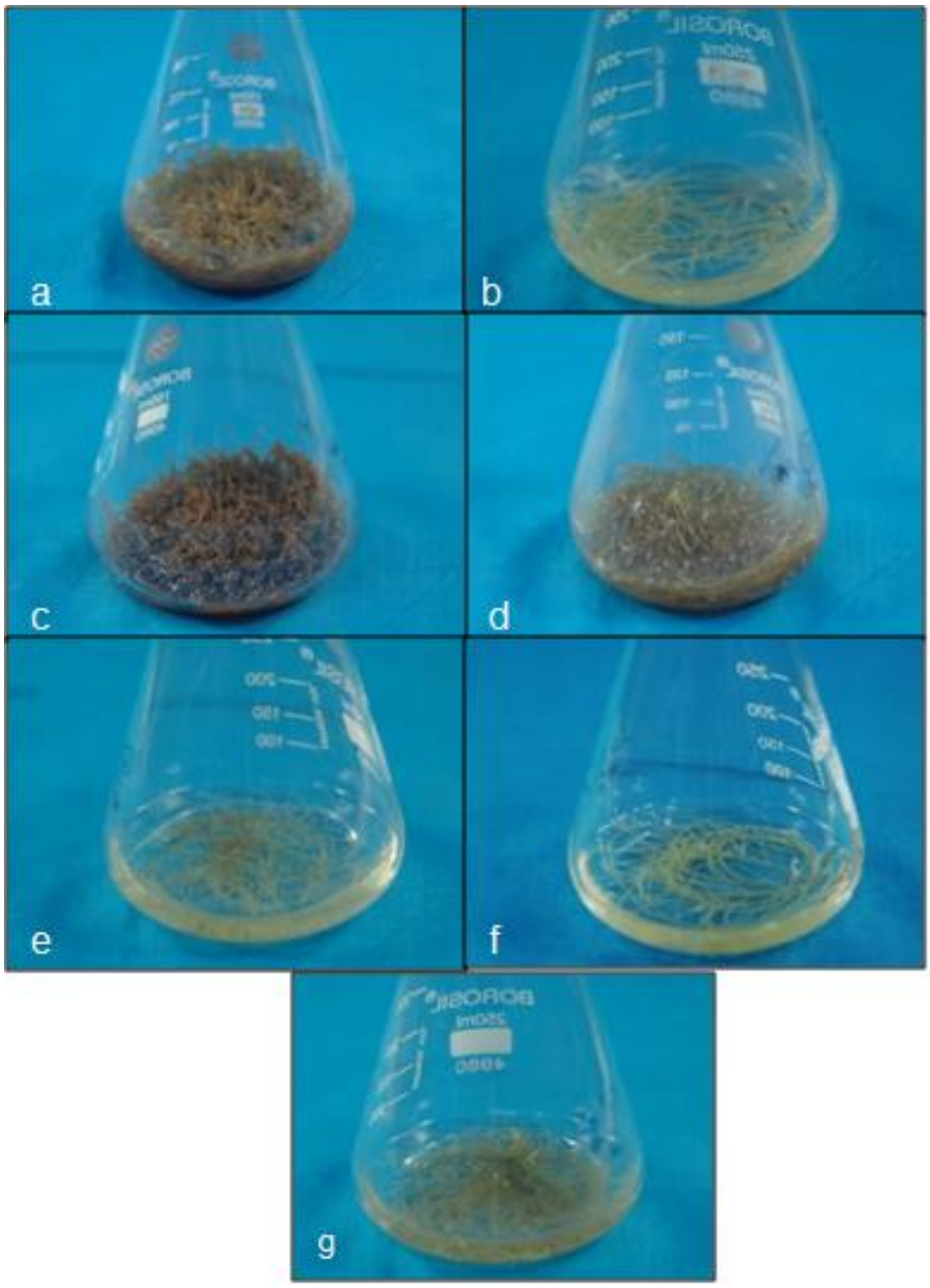


Figure 4. Various rhizoclones of *T. erecta*, that is, a-g, showing morphological variations.

Talou et al., 1994), but it differed from *Tagetes filifolia* where BBT (OAc) 2 type of thiophene was reported (Massera et al., 1998). In our studies marked differences in growth rate and root morphology were observed in root cultures (Figure 3 and 4). These morphological differences could be because of variation of auxin levels. Since *Agrobacterium* strains carry genes encoding auxins synthesis, this might be linked to variation in auxin levels which results in morphological variations (Croes et al., 1989; Arroo et al., 1995). *Agrobacterium rol* and *aux* genes are involved in rhizogenesis through the modification of plant cell growth and developmental regulation and these genes are located in the TR-DNA and TL-DNA regions of the Ri (root-inducing) plasmid of *A. rhizogenes* agropine strains. Some of the genes are also involved in auxin biosynthesis and/or auxin sensitivity, that cause differences in hairy root growth and morphology when compared to the non-transformed roots (Meyer et al., 2000; Christey, 2001). Very often noteworthy phenotypic and growth variations have been observed among hairy root clones which are derived from independent transformation events. In most cases, the

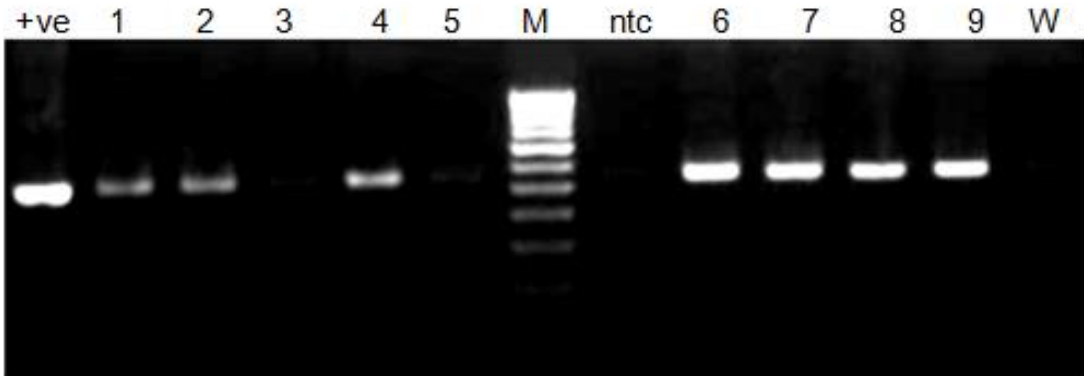


Figure 5. PCR detection of *rol C* gene from transformed roots. *+ve* is the plasmid DNA, lanes 1 to 5 and 6 to 9 are the rhizoclones, ntc is the no template control; *W* is the wild type hairy roots and *M* is the DNA marker.

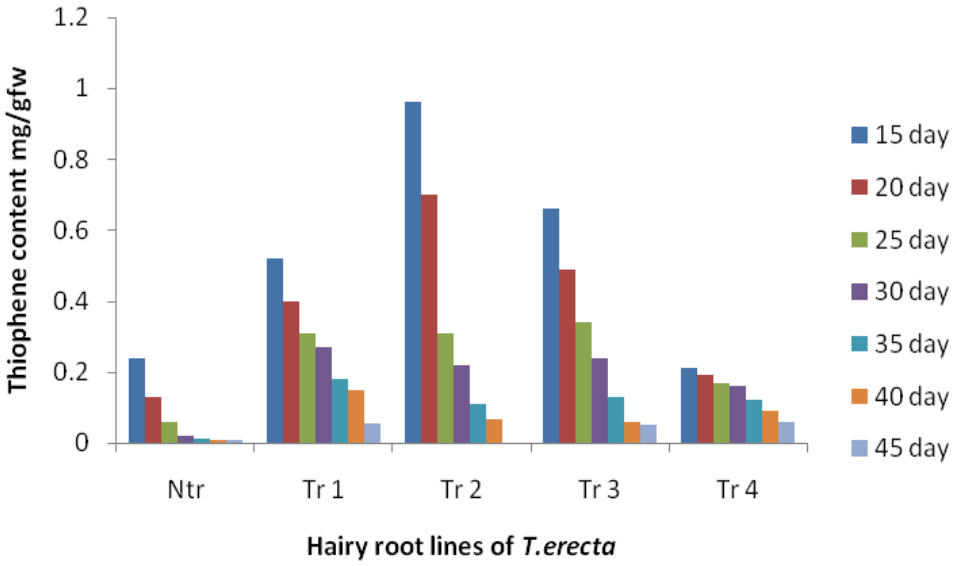


Figure 6. Accumulation of thiophene contents in *T. erecta* root cultures at various time intervals.

variability that generally affects the branching intensity, the root diameter and growth rate has been visually described. Differences in the integration of genes from the TL-DNA and TR-DNA regions of *A. rhizogenes* in the host genome are considered to be the major cause of phenotype variations (Ambros et al., 1986; Mano et al., 1986, 1989; Jouanin et al., 1987). In our results the hairy root cultures showed profuse lateral branching (Figures 3 and 4). Auxins in the presence of sucrose are known to stimulate lateral branching (Reed et al., 1998). Abundant lateral branching has very often been mentioned as one of the most typical traits within the altered phenotype of hairy roots (Tepfer, 1984; Spano et al., 1988; Guivarch et al., 1999). Our results shows that growth rate of hairy root cultures was in exponential phase till 15 days; similar results were also obtained by Mukundan and Hjortso (1991a) in *T. patula* where a well-defined exponential growth phase was identified during the initial growth phase (Bajaj, 2001). Although, the qualitative growth dynamics of the transformed *Tagetes* roots is similar to that of microbial culture or cell cultures, a quantitative description of the root growth kinetics cannot rely uncritically on models that have been developed primarily for microbial cultures. Hairy root growth data are not always so simple that single kinetic parameter, such as a specific growth rate can adequately describe them. Accumulation of biomass showed a specific growth rate reaching maximum till 45 days and thus became stationary which might be due to the depletion of limiting nutrients (Asha and Nutan, 2004). The highest level of

thiophene accumulation was found in all rhizoclones was 0.96 mg/g (Figure 6). The variations observed in thiophene accumulation in different clones could also be ascribed to plant tissue reaction to auxins. Because T-DNA integration takes place at random site into the host plant genome, the resulting rhizoclones often show different accumulation patterns of secondary metabolites (Mano et al., 1989; Rodriguez Talou et al., 1994). On the basis of the present results, 15 day hairy root cultures appear to be the best stage for the *in vitro* production of thiophenes. Further improvements might be helpful for commercial production purpose, such as screening large number of rhizoclones, optimization of various strains of *A. rhizogenus* and various media composition since the chemical synthesis of thiophenes is not economically possible (Ketel, 1987) and absence of any commercially available nematicidal compounds makes this study very useful.

Conflict of Interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Screening for attractants compatible with entomopathogenic fungus *Metarhizium anisopliae* for use in thrips management

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Several thrips attractants were screened for compatibility with *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) and a subset of these for attraction to *Megalurothrips sjostedti* Trybom (Thysanoptera: Thripidae). Conidial germination and germ tube length of *M. anisopliae* were used as indicators of its compatibility with thrips attractant. Conidial germination and germ tube length differed significantly according to volatiles of different attractants. The highest conidial germination (76.5±3.5%) and longest germ tube length (130.3±13.4 µm) were recorded in the control, followed by methyl anthranilate (63.8±3.8%; 103.8±8.4 µm), cis-jasmone (61.8±5.9%; 93.8±14.4 µm) and trans-caryophyllene (57.7±6.5%; 96.3±15.5 µm) which were found compatible with *M. anisopliae*. A Pearson correlation test indicated a significant positive correlation between conidial germination and germ tube length (r =0.6; P<0.0001). The attraction of *M. sjostedti* to selected thrips attractant also varied significantly among the attractants. Under field conditions, methyl anthranilate was equally attractive to *M. sjostedi* as Lurem-TR and could be recommended as a thrips attractant that can be combined with *M. anisopliae* in autoinoculation devices for potential control of *M. sjostedti*.

Key words: Semiochemicals, conidial germination, germ tube length, *Megalurothrips sjostedti,* attraction, persistence, field.

INTRODUCTION

In many flower dwelling thrips, host finding is linked to visual, odour and morphological (shape) cues (Rieske and Raffa, 2003; Mainali and Lim, 2011). Subsequently, semiochemical-based products such as Lurem-TR and Thripline have been developed for use in thrips monitoring and management (Sampson and Kirk, 2013;

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Teulon et al., 2014; Broughton et al., 2015). These semiochemicals can be integrated with other control strategies to improve thrips management in horticulture (Suckling et al., 2012; Sampson and Kirk, 2013).

Entomopathogenic fungi (EPF) are among the alternatives to synthetic chemical pesticides being considered for the management of thrips in horticulture (Ekesi and Maniania, 2007). EPF are generally applied through inundative spray, which requires high amount of inocula, thereby enhancing its cost (Jaronski, 2010). Further, the persistence of conidia applied on foliage is challenged by several environmental parameters such as UV light, rain, temperature (Inglis et al., 2000; Jaronski, 2010). The use of "lure and kill" strategy using autoinoculation device or spot spray application could reduce the amount of inoculum, the cost and sustain fungal persistence in the field (Dimbi et al., 2003; Nana et al., 2014; Mfuti et al., 2016). However, the success of this technology depends on the use of powerful attractants and their compatibility with the entomopathogens. For example, the tick attraction-aggregation-attachment pheromone (AAAP) could attract adult ticks from a distance of 6 m (Nchu et al., 2009) but could not be used in combination with conidia of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) because of inhibition of fungal conidia by the pheromone (Nana et al., 2012). Niassy et al. (2012a) and Mfuti et al. (2016) have also reported inhibitory effects of conidia of *M. anisopliae* by the semiochemical Lurem-TR in autoinoculation device in a screenhouse and field experiments.

Considering the growing interest in integrating attractants with EPF in thrips management (Niassy et al., 2012a; Mfuti et al., 2016), there is a need to identify compounds that are both attractive to thrips and compatible with EPF. The objective of the present study was therefore to identify thrips attractants that are compatible with *M. anisopliae* in terms of conidial germination and germ tube length since the latter plays a crucial role in fungal infection (Ortiz-Ribbing and Williams, 2006).

MATERIALS AND METHODS

Thrips attractants

Seven compounds used for thrips attraction or with potential attraction for thrips were tested for their compatibility with *M. anisopliae* isolate ICIPE 69. They were selected on the basis of structural analogies to known attractant such as methyl isonicotinate (Lurem-TR) (Teulon et al., 2007, 2010) but also based on previous studies of Koschier et al. (2000). Information on their chemical characteristics and manufacturers is presented in Table 1. The commercial attractant, Lurem-TR which was earlier reported to be toxic with the entomopathogen (Niassy et al., 2012a) was included in the study as a reference. It is a commercial product which quantity and release rate is standardized; therefore, could not be diluted. In preliminary bioassays, no significant effect of different concentrations (0.1, 10 and 100%) of attractants was observed on conidial germination and subsequently only the recommended concentration of 10% of the pure product was used in the screening bioassays. The pure concentration of all attractants was diluted in paraffin oil.

Crop

Cowpea, *Vigna unguiculata* L. Walp variety Ken-Kunde1, was planted in 80 m^2 plots with an inter- and intra- row spacing of 10 and 45 cm, respectively, in Mbita Thomas Odhiambo Campus (ITOC) (0° 26' 06.19" S, 34° 12' 53.13" E; 1,137 above sea level) earlier during rainy season (March 2014). The size of the cowpea farm was about 94 × 22 m. The field experiment of selected attractants was conducted during flowering stage of the crop (45 days after planting). No fertilizers, organic matter or synthetic chemical insecticides were applied during the experiment.

Fungal culture

M. anisopliae isolate ICIPE 69 was obtained from the Arthropod Germplasm Centre of *icipe*. It is currently commercialized as Campaign® by the Real IPM Ltd, Kenya, for the control of thrips, papaw mealy bug and fruit flies (http://www.realipm.com). It was cultured on Sabouraud Dextrose Agar (SDA) in 9 cm Petri dishes and incubated at 25 ± 2°C in complete darkness. Conidia were harvested from three week-old culture by scraping the surface using a spatula. Conidia were suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing glass beads. Conidial suspensions were vortexed for 5 min to produce a homogeneous suspension. Spore concentrations were determined using a haemocytometer.

Effect of thrips attractants on conidial viability and germ tube length of *M. anisopliae*

The conidial suspension was prepared as described earlier and titrated to 1×10^7 conidia ml^{-1}. The spores were retained on a nitrocellulose filter membrane (diameter 47 mm, pore size 0.45 µm, Sigma Chemicals) by pouring 10 ml suspension through a filter holder unit (MFS) under aspirator vacuum (Maniania, 1994). The nitrocellulose filter membranes were dried for 30 min under a laminar flow cabinet and transferred to glass desiccators (2.5 L) for exposure to the attractant volatile. Cotton wicks were soaked in 0.5 ml suspensions of each attractant diluted in paraffin oil and placed in desiccators to allow volatile diffusion. Cotton wicks were used as dispenser (Sidahmed et al., 2014). Fungus-treated nitrocellulose membranes were exposed to different thrips attractants and sampled for viability observation at different time intervals of 1, 2, 3, 6 and 8 days. An untreated control without thrips attractant was included. The commercial thrips attractant, Lurem-TR was included as a check. Treatments were randomized and the experiment repeated three times over time.

To determine conidial germination, nitrocellulose filter membranes containing conidia were removed from the desiccators and transferred into 10 ml sterile distilled water containing 0.05% Triton X-100 and vortexed for 3 min to dislodge conidia. Suspension (0.1 ml) titrated to 3×10^6 conidia ml^{-1} was spread-plated on SDA plates. Plates were incubated at 26 ± 2°C, L12: D12 photoperiod and examined after 18 to 24 h for conidial germination and germ tube length thereafter. Samples that could not be processed the same day were fixed by pouring a drop of lactophenol cotton blue onto the plate to stop further growth. Percentage germination was determined by counting approx. 100 spores per plate under a microscope Leica DMLB at 40 X magnification. The length of germ tubes was measured using a

Table 1. General information of the tested thrips attractant compounds.

Label Name	Chemical formula	CAS number	Chemical group	Company	Purity (%)	Dilution range for thrips attraction and species attracted
4-anisaldehyde	$C_8H_8O_2$	19486-71-6	Aldehyde	Sigma-Aldrich Chemicals GmbH, Germany	98	0.1-10% (applied in 1microliter paraffin oil) (Koschier et al.,2000) *Frankliniella occidentalis*
Ethyl benzoate	$C_9H_{10}O_2$	93-89-0	Ester of benzoic acid and ethanol	Sigma-Aldrich Chemicals GmbH, Germany	99	*Thrips obscuratus*; *Thrips tabaci (*Koschier et al., 2000*)*
Cis-jasmone	$C_{11}H_{16}O$	488-10-8	Jasmonate (organic compound)	Sigma-Aldrich Chemicals GmbH, Germany	≥ 99	10mg/200 microliters hexane *T. obscuratus*, *T. tabaci* (El-Sayed et al., 2009)
Linalool	$C_{10}H_{18}O$	78-70-6	monoterpene	Sigma-Aldrich Chemicals GmbH, Germany	97	1-10% (in 1 microliter paraffin oil) *F. occidentalis, T.tabaci* (Koschier et al., 2000)
Methyl anthranilate	$C_8H_9NO_2$	134-20-3	Ester of anthranilic	Sigma-Aldrich Chemicals GmbH, Germany	98	*T. coloratus*, *T. hawaiiensis* (Murai et al., 2000; Imai et al., 2001)
trans caryophyllene	$C_{15}H_{24}$	87-44-5	Sesquiterpene	Sigma-Aldrich Chemicals GmbH, Germany	≥98.5	1-10% (in 1 microliter paraffin oil) (Koschier et al., 2000)
Phenylethanol	$C_8H_{10}O$	60-12-8	Alcohol	Sigma-Aldrich Chemicals GmbH, Germany	≥ 99	*T. tabaci* (Teulon et al., 2007)
Methyl-isonicotinate	$C_7H_7NO_2$	2459-09-8	Pyridine	Pherobank Wageningen, The Netherlands.	-	Several thrips species such as *F. occidentalis*, *T. tabaci* (Davidson et al., 2007) *F. schultzei, Hydatothrips adolfifriderici* and *Megalurothrips sjostedti* (Muvea et al., 2014)

Leica Application Suite (LAS EZ V1.5.0). Average germ tube lengths were obtained from 5 spores taken at random in each cover slip (22 x 22 mm) and replicated three times.

Effect of selected thrips attractants on the attraction of *M. sjostedti*

Attractants that were found compatible with *M. anisopliae* from the screening experiment were selected for field experiment to evaluate the attraction of Bean Flower Thrips (BFT), *M. sjostedti* (Trybom) (Thysanoptera: Thripidae) on cowpea. Lurem-TR was included as reference. Attractants were diluted in Paraffin oil as indicated above. Each attractant suspension was poured in 5 ml Eppendorf tube and suspended in the middle surface of the blue sticky card (10 × 25 cm) (Plate 1). The two items were placed at 30 cm above ground level. Blue sticky cards were separated 10 m from one another to avoid interference. An untreated blue sticky card with no attractant was used as a control. The experiment was conducted during flowering and podding stages of cowpea when BFT populations are high (Ezueh, 1981; Nyasani et al., 2013). Cards were replaced every three days. Numbers of adult BFT were recorded on each card and the experiment was replicated four times over time.

Statistical analysis

Data on conidial germination of *M. anisopliae* were normalized by arcsine transformation before subjecting them to linear mixed model. Data on *M. anisopliae* conidial germ tube length and *M. sjostedti* catches were also analyzed using linear mixed model. Means were separated using Student–Newman–Keuls (SNK) test. A Pearson correlation analysis was carried out to relate conidial viability with the germ tube length. All data analyses were performed using R (R Development Core Team, 2014). The level of significance was maintained at 95%.

RESULTS

Effect of thrips attractants on conidial viability and germ tube length of *M. anisopliae*

Overall, the effects of thrips attractants on germination of conidia of *M. anisopliae* varied significantly between the attractants ($F_{9,268}$ = 22.1; P<0.0001) (Table 2). The interaction day × attractant was statistically significant ($F_{9,268}$ = 3.8;

Plate 1. *M. sjostedti* attracted in the surface of the blue sticky card baited with attractant suspension poured in 5 ml Eppendorf tube.

Table 2. Conidial germination and germ tube length of *Metarhizium anisopliae* after exposition to thrips attractants (temperature: 26 ± 2°C; photoperiod: L12: D12; time of incubation: 18-24 h)

Treatments	Mean % germination ±SE (transformed)	Mean length ±SE
Control	62.4±3.5^{a}	130.5±10.0^{a}
Methyl anthranilate	53.7±3.5^{ab}	103.8±10.0^{ab}
Trans-Caryophyllene	51.2±3.5^{ab}	96.3±10.0^{ab}
Cis-jasmone	50.9±3.5^{ab}	93.8±10.0^{ab}
Solvent (paraffin oil)	48.4±3.5^{b}	90.9±10.0^{b}
Linalool	33.3±3.5^{bc}	67.1±10.0^{c}
Phenylethanol	32.4±3.5^{c}	50.9±10.0^{c}
4-Anisaldehyde	30.8±3.5^{c}	45.5±10.0^{c}
Lurem-TR	24.4±3.5^{c}	37.1±11.7^{c}
Ethyl benzoate	20.1±4.0^{c}	36.1±10.0^{c}

Means bearing the same letters are not significantly different by the Student–Newman–Keuls test (SNK).

P<0.0001). The time of exposure had significant effects on conidial germination, except at 1 day post-exposure when no significant effect was observed ($F_{9,45}$ = 1.5; P = 0.2) (Table 3). Significant reduction in conidial germination was observed in all the treatments from day 2 ($F_{9,45}$ = 6.1; P<0.0001), day 3 ($F_{9,45}$ = 6.8; P<0.0001), Day 6 ($F_{9,45}$ = 8.3; P<0.0001) and day 8 post-exposure ($F_{9,45}$ = 8.7; P<0.0001) (Table 3). The conidial germination was significantly higher in the control (62.5 ± 10.0%), followed by cis-jasmone (44.8 ± 16.6%), Solvent (paraffin oil) (42.8 ± 11.0%), methyl anthranilate (36.6 ± 8.0%) and trans-caryophyllene (31.3 ± 16.8%) treatments after 8 days of exposure and was significantly different (Table 3). No conidial germination was observed in Lurem-TR treatment at day 8 post-exposure (Table 3).

The effect of thrips attractants on germ tube length followed the same trend as with conidial germination where treatments differed significantly ($F_{9,268}$ = 12.6; P<0.0001) (Table 2). The interaction day × attractant was not statistically significant ($F_{9,268}$ = 1.0; P=0.5). Exposure time had significant effects on length of the germ tube of *M. anisopliae* at day 1 ($F_{9,45}$ = 4.3; P = 0.003), Day 2

Table 3. Effect of thrips attractants on conidial germination of *M. anisopliae* over time.

Treatment (Thrips attractants)	Day after exposure					
	1	2	3	6	8	ANOVA
Control	91.3±3.0^{aA}	85.4±3.7^{aAB}	78.3±4.0^{aB}	65.5±10.0^{aC}	62.5±10.0^{aC}	$F_{4,22}$=14.65; P<0.0001
4-anisaldehye	85.7±2.0^{aA}	48.7±14.3^{bcdB}	9.2±7.9^{dC}	0.1±0.1^{cC}	0.1±0.1^{cC}	$F_{4,22}$=38.2, P<0.0001
Ethyl Benzoate	79.8±6.8^{aA}	44.1±14.1^{abcB}	17.5±6.1^{cdBC}	11.9±4.4^{bcBC}	7.5±3.4^{bcC}	$F_{4,22}$=35.8, P<0.0001
Jasmone	83.8±4.0^{aA}	74.6±7.5^{abcA}	61.1±12.9^{abAB}	44.6±15.5^{abB}	44.8±16.6^{aB}	$F_{4,22}$=6.1; P=0.001
Linalool	75.5±4.3^{aA}	63±3^{abcB}	50.6±6.2^{abcC}	2.5±1.4^{cD}	0.5±0.5^{cD}	$F_{4,22}$=195.5; P<0001
Methyl anthranilate	85.8±3.6^{aA}	78.8±2.7^{abA}	60.7±2.3^{abBC}	56.8±4.6^{aB}	36.6±8.0^{aC}	$F_{4,22}$=28.2; P<0.0001
Phenylethanol	76.1±8.5^{aA}	50.1±14.6^{bcdB}	36.5±15.3^{bcdB}	18.2±11.5^{bcC}	5.5±4.1^{bcC}	$F_{4,22}$=21.5; P<0.0001
Trans caryophyllene	80.0±5.0^{aA}	74.5±^{abc}7.0^{A}	53.8±16.6^{abcAB}	49.3±16.4^{abAB}	31.3±16.8^{abB}	$F_{4,22}$=6.2; P=0.001
Lurem-TR	75.7±4.2^{aA}	20.6±3.7^{dB}	13.6±3.1^{cdC}	0.03±0.0^{cD}	0.0±0.0^{D}	$F_{4,13}$=199.8; P<0.0001
Solvent (paraffin oil)	82.0±3.0^{aA}	72.7±5.2^{abcAB}	58.6±7.1^{abB}	43.9±11.0^{abC}	42.8±11.0^{aC}	$F_{4,22}$=14.7; P<0.0001
ANOVA	$F_{9,45}$=1.5;P=0.2	$F_{9,45}$=6.1;P<0.0001	$F_{9,45}$=6.8;P<0.0001	$F_{9,46}$=8.3;P<0.0001	$F_{9,46}$=8.7;P<0.0001	

Within column, means (±SE) followed by the same small letters are not significantly different Student–Newman–Keuls test (SNK), Within rows, means (±SE) followed by the same capital letters are not significantly different Student–Newman–Keuls test (SNK).

($F_{9,45}$ = 6.7; P<0.0001), day 3 ($F_{9,45}$ = 6.9; P<0.0001), day 6 ($F_{9,45}$ = 6.5; P<0.0001) and day 8 ($F_{9,45}$ = 5.6; P<0.0001) post-exposure (Table 4). The highest germ tube length was observed in the control treatment (89.1±32.4 µm) followed by methyl anthranilate (69.6±12.9 µm), solvent (paraffin oil) (54.7 ± 16.4 µm), trans-caryophyllene (42.1 ± 13.9 µm) and cis-jasmone (41.9 ± 16.7 µm) and was not significantly different at day 8 post-exposure (Table 4). No germ tube developed in Lurem-TR treatment at day 6 and 8 post-exposure (Table 4). A significant correlation was found between conidial germination and germ tube length of *M. anisopliae* (r = 0.6; P<0.0001) (Figure 1).

Effect of selected thrips attractants on the attraction of *M. sjostedti*

The number of adult BFT caught on the baited sticky cards varied significantly ($F_{3,28}$ = 7.9; P< 0.0001) between the treatments. More adult BFT were caught on sticky cards treated with Lurem-TR and methyl anthranilate than cards treated with cis-jasmone (Figure 2). No significant difference in thrips catches was found between Lurem-TR and methyl anthranilate. Similarly, there was no significant difference between cis-jasmone and control treatments (Figure 2).

DISCUSSION

Most studies on compatibility of *M. anisopliae* have focused on agrochemicals and botanicals (Nana et al., 2012; Niassy et al., 2012b) overlooking the potential of semiochemical attractants in insect pest management (IPM). However, a study on compatibility between attractants and EPF is required before their integration in an IPM strategy.

Thrips attractants tested in the present study affected conidial germination and germ tube length differently, with time of exposure being the determining factor. Three out of eight attractants tested namely methyl anthranilate, cis-jasmone and trans-caryophyllene, did not have deleterious effects on conidial germination and germ tube length of *M. anisopliae* at day 8 post-exposure (Tables 3 and 4). Interestingly, these three attractants have been reported elsewhere to have effects on fungal pathogens. For instance, methyl anthranilate has been reported to significantly reduce the growth of strawberry pathogens such as *Botrytis cinerea* (Helotiales: Scerotiniaceae), *Colletotrichum gloeosporioides* and *C. acutatum* (Glomerellale: Glomerellaceae). In addition, medium supplemented with methyl anthranilate resulted in complete cessation of growth in those pathogens (Chambers et al., 2013).

Recent evidence suggests that jasmonic acid is involved in the induction of genes that act primarily in defense against plant pathogens rather than insects (Halim et al., 2006). Jasmonic acid is part of the plant's alarm system and

Table 4. Effect of thrips attractants on conidial germ tube length (µm) of *M. anisopliae* over time.

Treatment (Thrips attractants)	Day after exposure					
	1	2	3	6	8	ANOVA
Control	190.7±2.0^{aA}	146.9±25.4^{aB}	123.2±28.2^{aBC}	102.4±28.9^{aBC}	89.1±32.4^{aC}	$F_{4,22}$=8.3; P=0.003
4-anisaldehye	102.5±19.0^{bcA}	74.6±12.4^{bcA}	27.7±16.2^{cB}	11.5±0.0^{cB}	11.5±0.0^{cB}	$F_{4,22}$=12.6; P<0.0001
Ethyl benzoate	77.8±20.4^{cA}	40.9±13.4^{cB}	26.8±5.4^{cB}	20.5±4.6^{cB}	14.4±2.0^{cB}	$F_{4,22}$=7.2; P=0.0007
Jasmone	185.4±42.6^{aA}	103.8±22.1^{abB}	90.6±21.9^{abBC}	47.3±16.3^{bcC}	41.9±16.7^{bcC}	$F_{4,22}$=13.5; P<0.0001
Linalool	122±26.4^{abcA}	94.3±15.5^{abA}	83.9±12.8^{abA}	22.2±5.7^{cB}	13.4±1.9^{cB}	$F_{4,22}$=17.58; P<0.0001
Methly anthranilate	134.4±22.6^{abcA}	120.5±20.4^{abA}	108.6±17^{aAB}	86.1±11.4^{abBC}	69.6±12.9^{abC}	$F_{4,22}$=8.4; P=0.0002
Phenylethanol	102.9±25.7^{bcA}	71.9±21.6^{bcAB}	45.9±18.2^{bcBC}	20.4±5.9^{cC}	13.4±1.9^{cC}	$F_{4,22}$=7.1; P=0.0007
Transcaryophyllene	179.3±49.1^{abA}	112.9±34.1^{abB}	81.0±21.6^{abBC}	66.2±21.8^{abcBC}	42.1±13.9^{bcC}	$F_{4,22}$=9.9; P<0.0001
Lurem-TR	75.7±22.6^{bcA}	20.6±6.1^{cB}	13.6±3.3^{cB}	15.4±7.9 ^{cB}	11.5±0.0 ^{cB}	$F_{4,13}$=14.3; P=0.0001
Solvent (paraffin oil)	139.3±19.9^{abcA}	107.9±21.4^{abB}	87.7±19.0^{abBC}	64.7±18.0^{abcBC}	54.7±16.4^{abC}	$F_{4,22}$=8.3; P=0.0003
ANOVA	$F_{9,45}$=4.3;P=0.003	$F_{9,45}$=6.7;P<0.0001	$F_{9,45}$=6.9;P<0.0001	$F_{9,45}$=6.5; P<0.0001	$F_{9,45}$=5.6;P<0.0001	

Within column, means (±SE) followed by the same small letters are not significantly different by the Student–Newman–Keuls test (SNK); Within rows, means (±SE) followed by the same capital letters are not significantly different by the Student–Newman–Keuls test (SNK).

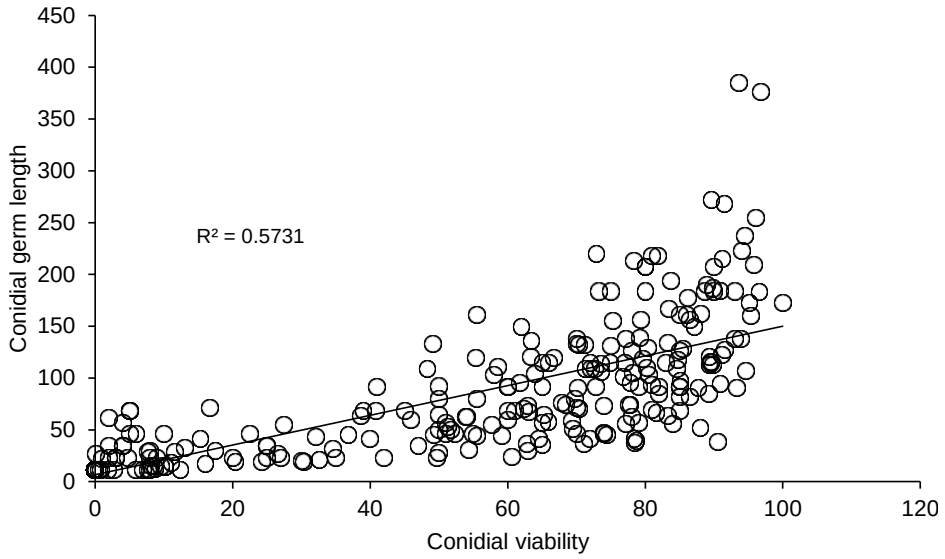

Figure 1. Scattergram showing correlation between *Metarhizium anisopliae* conidial germination and germ tube length using the Pearson method.

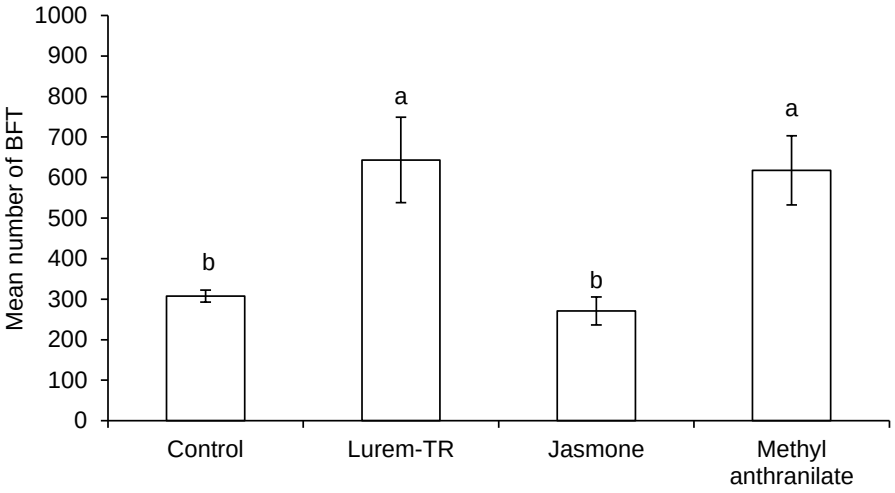

Figure 2. Mean (± SE) number of *M. sjostedti* attracted to blue sticky card baited with Methyl anthranilate, Cis-jasmone, Lurem-TR and control. Means bearing the same small letters are not significantly different by the Student–Newman–Keuls test (SNK).

defense mechanism. It is a volatile (gas phase of Cis-jasmone) which is released during insect attack and controls the response to damage (Menzel et al., 2014).

Essential oil from *Perovskia atriplicifolia* Benth (Lamiales: Liamiaceae) containing 9.30% of trans-caryophyllene are reported to have antimicrobial activity against fungal strains (Erdemgil et al., 2007). The difference between these results and our results could be explained by the fact that attractants were used as volatiles in our study while they were used as oil supplements in culture media.

This study also confirmed previous findings on the antifungal effect of Lurem-TR on conidial germination (Niassy et al., 2012a). More recently, it was demonstrated that direct exposure of fungus without separation from Lurem-TR recorded the lowest conidial germination as compared with the other treatments where separation was made. However, fungal persistence increased with distance of separation of Lurem-TR (Mfuti et al., 2016).

The strong correlation observed between conidial germination and germ tube length suggests that fungal inoculum would still cause infection in the insects. The role of germ tube formation in the pathogenesis is well established (Ortiz-Ribbing and Williams, 2006). For instance, comparing four different growth stages of *Isaria fumosorosea* (*Paecilomyces fumosoroseus*) (Eurotiales: Trichocomaceae) (conidia, germinated conidia with either one or two germ tubes and hyphal bodies), Fargues et al. (1994) found that germinated conidia and hyphal bodies were more aggressive than ungerminated conidia against first-instar larvae of *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae).

The catches of BFT were significantly higher on blue sticky cards baited with methyl anthranilate and Lurem-TR than the control and cis-jasmone baited cards. The increased attraction of BFT to Lurem-TR and blue sticky traps was reported by (Muvea et al., 2014). No difference in BFT attraction was found between the two compounds. Methyl anthrinalate has been reported to be attractive to four species of flower thrips, *Thrips hawaiiensis*, *Thrips coloratus*, *Thrips flavus*, and *Megalurothrips distalis*, irrespective of sex (Murai et al., 2000; Imai et al., 2001). However, this study is the first report on BFT response to methtyl anthranilate. This study has identified methyl anthranilate as an attractant effective for BFT and also compatible with conidia of *M. anisopliae* and hence can be considered for a “lure and kill” management strategy for BFT. The “lure and kill” strategy could be adopted either as an autoinoculation device or spot spray. Further studies need to be carried out to validate this proof of concept.

Conflict of Interests

The authors have not declared any conflict of interests.

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