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Extracellular Metabolites Produced by a Novel Strain *Bacillus alvei*NRC-14: 6. Heat-Shock Stress Induces Production of Antimicrobial Metabolites

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ABSTRACT

Heat shock and other proteotoxic stresses cause accumulation of nonnative proteins that trigger activation of heat shock protein genes. The strain *Bacillus alvei*NRC-14 was found to produce antimicrobial metabolites when exposed to a heat shock.The present study investigated the influence of cultural and environmental parameters affecting growth and antimicrobial metabolites produced by the novel strain *Bacillus alvei*NRC-14. The antimicrobial metabolites exhibited a broad spectrum of *in vitro* antimicrobial activity against some human pathogens. Antimicrobial metabolites by the strain are highly produced with chitin, chitosan or related substrates as compared to other tested carbon sources. Fungal mycelium (1%) and ammonium sulfate (0.2%) were found to be the most suitable carbon and nitrogen sources for optimum growth and production, at pH 6.0 and 40^{o}Cfor 48h, under shaking conditions. The antimicrobial metabolites inhibited the growth of some human bacterial and fungal pathogens. The metabolites are heat-stable and remained active after sterilization at 121°C for 15 min. The antimicrobial metabolite activity is stable over a wide range of pH values (4–10) with a maximum between 5 and 8. This is the first report on production of antimicrobial metabolites by the novel strain *Bacillus alvei*NRC-14.

INTRODUCTION

Employment of synthetic antibiotics in medicine for management of infectious diseases had led to the emergence of multiple-resistant patterns (resistance to more than one of antimicrobial drugs) to the antibiotic among microorganisms. Emergence of antibiotic resistance among bacteria and fungi has obviously increased worldwide over the past two decades due to enhancement use of antimicrobial drugs. This has led to many clinical problems in treatment of infectious diseases, and the antibiotics used are commonly associated with adverse effects such as hypersensitivity, allergic reaction, and immunosuppression in the host (Jean *et al.* 2011). To abolish deleterious effects of antibiotics, an alternative control is urgently required. Development of novel naturally antimicrobial metabolites and antibiotics is found to be a current trend (Manilal and Akbar 2014).In contrary to the synthetic drugs, antimicrobials from natural origin are not associated with negative effects and have a broad-spectrum therapeutic potential to heal many of animal and human infectious diseases(Maria *et al.* 2013, Suja*et al.* 2013, Manilal and Akbar 2014).

Pathogenic strain, *S. aureus*, can cause skin infections, such as pimples, impetigo, boils, cellulitis folliculitis, carbuncles, scald skin syndrome, and abscesses. It can also cause life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. It is still one of the five most common causes of nosocomial infections (Munarol *et al.* 2011). Strains of*E. coli* are most frequent causes of many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection, traveler's diarrhoea, and other clinical infections such as neonatal meningitis and pneumonia (Sahoo*et al.* 2012). *P. aeruginosa* are opportunistic pathogens often associated with infections of the urinary tract, respiratory system, soft tissue, bone and joint, gastrointestinal infections, dermatitis, bacteremia, and a variety of systemic infections, particularly in patients with severe burns, cancer and AIDS (Ozumba 2003). *Aspergillusniger* is one of the most common causes of human otomycosis (fungal ear infections) which can cause pain and severe cases, damage to the ear canal and tympanic membrane. Some strains of *A.niger* have been reported to produce potent my cotoxins called ochratoxins (Schuster *et al.* 2002). *Aspergillus flavus*is also well known to cause human diseases; it is the second most common agent of aspergillosis, the first being *Aspergillus fumigates*. *A. flavus*may invade arteries of the lung or brain and cause infarction. *A.flavus*also produces anaflatoxin which is one of the aetiological agents for hepatocellular carcinoma (Klich 2007).Frequency infections by such microorganisms are partially attributable to the fact that these bacteria exhibit significant genetic versatility in the acquisition of drug resistance and have the ability to survive in the hospital environment (Karen*et al.* 2010,Suja*et al.* 2013).

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Antimicrobial substances are widely produced among fungi. These substances have received increasing interest because of their potential efficiency against pathogenic microorganisms (Manilal and Akbar 2014). Searching for novelstrains to improve and allow rapid discovery of natural products for modern drugs is increased. Little informationare available regarding isolation of antimicrobial compounds and antibiotics from the genus *Bacilli*(Nalisha*et al*. 2006, Chen *et al*. 2008, Okulate 2009, Fickers 2012, Mondol*et al*. 2013) comparing to the genus *Streptomyces* which has extensively reported (Sarigullu*et al*. 2013).*Bacillus* spp. isolates produce structurally diverse classes of secondary metabolites that exhibit a wide range of biological activities(Mandol*et al*. 2013). Due to having genetic capability to adapt extreme conditions, *Bacillus* strains isolated from unique niches of environments (e.g., hydrothermal vent, deep sea, pH > 9.0 and salt lakes) may produce useful bioactive compounds. Moreover, new natural products may be produced by culturing the strains under varying stressful conditions (e.g., nutrient, pH, salinity or temperature stresses).

When exposed to elevated temperature, some microorganisms produce antibiotics as a form of self-protection against such abiotic stress (Karen *et al*. 2010).The heat-shock response is the mechanism by which cells react to increases in temperature to prevent damage, and it involves the expression of the almost universally conserved heat-shock genes (Maria *et al*. 2004, Karen *et al*. 2010). Many heat-shock proteins are molecular chaperones or proteases and function by facilitating refolding of damaged proteins or eliminating proteins that can't be repaired (Karen *et al*. 2010). An Egyptian soil isolate, identified as*B.alvei* NRC-14 (Abdel-Aziz 1999), was found to produce vital extracellular metabolites whether under normal conditions or when exposed to hard abiotic stress conditions. The properties of a resultant metabolite depend on the ambient environment.The ability of strains to form antimicrobial substances can be significantly influenced by different conditions of nutrition and cultivation. Therefore, the medium constitution together with the metabolic capacity of the producing microorganism greatly affects synthesis of bioactive metabolites. Several environmental factors, such as temperature, pH, and incubation period, play a major role in the production of antimicrobial agents (Kiranmayi*et al*. 2011).

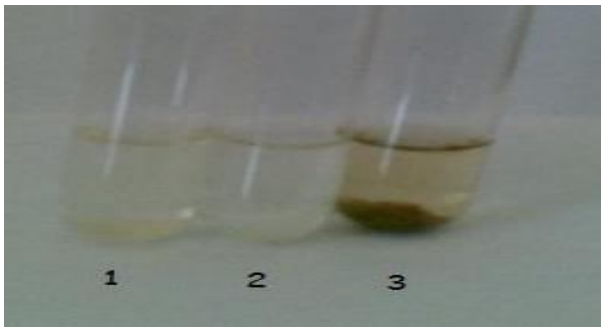

Fig. 1.Inhibitory effect of antimicrobial metabolites produced by *B. alvei* NRC-14, during growth at 40^{o}C, against: 1) *E.coli*; 2) *Staphylococcus aureus*; and 3) *Aspergillusflavus*. To a prepared cell suspension of the strains, 0.2 ml of the antimicrobial metabolites was added and incubated at room temperature for 2 hrs.

During a previous study for production of chitinase from strain NRC-14, using flaked chitin, it was noticed that when the temperature was shifted from 30^{o}C to 40^{o}C after 24h of growth, the culture broth (after 48h) was found to be completely free of enzymes but it exhibited antimicrobial effects (Fig. 1) when tested against some pathogens (Abdel-Aziz *et al*. 2012).We extensively examined this strain for production of biologically active compounds. The strain, *B. alvei*NRC-14, has never been reported previously in literatures despite of its: 1) efficiency for production of carbohydrate-active enzymes using a minimal medium; 2) capability for production of bioactive compounds; and 3) capability for adaptation with various abiotic stress conditions as well as secretion of a variety of extracellular metabolites and lytic enzymes important for biological control of phytopathogens (Abdel-Aziz 2012*a*).In our previous work, it was reported that, when strain NRC-14 exposed to a heat-shock stress (during growth with flaked chitin as a sole carbon source), the culture supernatant was found to be completely free of enzymes but the cells secreted an antimicrobial compound, probably, as a form of self-protection (Abdel-Aziz *et al*. 2012).The present study was undertaken to optimize the nutritional andculture conditions for production of antimicrobial metabolite produced by strainNRC-14, when exposed to a heat-shock. Evaluation of its potential antimicrobial properties against some human and fungal pathogens was also estimated.

MATERIALS AND METHODS

Bacterial strain and growth medium

The strain, used in the present study, was isolated from Egyptian soil as a potent chitosanase producer and identified *Bacillus alvei* (Abdel-Aziz 1999) as described in *Bergey's Manual of Systematic Bacteriology* (Juni

1986).It was maintained on nutrient agar slants at 4°C. The strain was routinely propagated and kept as a stock culture in nutrient broth and transferred to nutrient agar plates (at 30°C/24 h) for short term usage. To achieve high yield of the inhibitory metabolites, different carbon and nitrogen sources, salts, pH values as well as different temperatures were tested (Suja *et al*. 2013). Experiments for growth parameters were achieved in Erlenmeyer-flasks each contained 25 ml of media containing: 1.0 % (w/v) of a carbon source,0.2 % (w/v) nitrogen source, 0.1% K_2HPO_4, 0.05 % (w/v) $MgSO_4.7H_2O$ as well as trace elements and minerals, before being autoclaved at 121°C for 15 minutes.

Optimization of culture conditions

Effect of cultural medium

Four different media were prepared for the antimicrobial metabolites production (Uddin *et al*. 2013): Medium A: glucose (0.3%), Beef extract (0.3 %), Peptone (0.5 %), NaCl (0.1 %); Medium B: Starch (1%), ammonium sulfate (0.2%), $MgSo_4.7H_2O$ (0.05 % (w/v), K_2HPO_4(0.1%), NaCl (0.5%) Medium C: cellulose (1.0%), ammonium sulfate (0.2%), K_2HPO_4(0.1%), NaCl (0.5%); Medium D: flaked chitosan (1.0%), ammonium sulfate (0.2%), $MgSo_4.7H_2O$ (0.05 % (w/v), K_2HPO_4(0.1%); Medium E: flaked chitosan (0.5%), ammonium sulfate (0.2%), $MgSo_4.7H_2O$ (0.05 % (w/v), K_2HPO_4(0.1%), NaCl (0.5%), $FeSO_4$(0.002%),$ZnCl_2$ (0.001%); Medium F: Fungal mycelium (1.0%), K_2HPO_4(0.1%), $MgSo_4.7H_2o$ (0.05 % (w/v),ammonium sulfate (0.2%); Medium G: Fungal mycelium (1.0%), ammonium sulfate (0.25%),$MgSo_4.7H_2O$ (0.05 % (w/v), KH_2PO_4 (0.1%), $FeSO_4$(0.002%), $ZnCl_2$ (0.001%). The pH of all media was adjusted at 6.0. Sterile media were inoculated with strain NRC-14 at a 4% (v/v) and incubated at 40°C. Samples were withdrawn every 24 h, centrifuged (4000 x g for 15 min), and kept at 4°C for further estimations. The inhibitory effect of the produced metabolites was expressed as a zone of inhibition by agar diffusion method for 42h.In all tests, cell growth (%) was determined in relation to the growth in fungal-mycelium medium as a control.

Effect of carbon and nitrogen sources

Effect of carbon and nitrogen sources to enhance inhibitory metabolites production was investigated. To study the effect of different carbon sources, chitin, chitosan, and cellulose were tested as polysaccharides, whereas glucose and sucrose were used as mono- and di-saccharides. Carbon sources were added individually at a concentration of 1% to a medium supplemented with $(NH_4)_2SO_4$(0.2%) as nitrogen source. Similarly, effect of various nitrogen sources such as $(NH_4)_2SO_4$, $NaNO_3$, KNO_3, NH_4Cl, yeast extract, peptone, casein, and beef extract was studied by adding the nitrogen source (0.2%) to Medium F, containing fungal mycelium (1%). Final pH of the medium adjusted to 6 and antibacterial activity was checked by the disc diffusion method. The medium in which the strain exhibited maximum inhibitory metabolites production expressed in terms of zone of inhibition was used as an optimized medium for further tests (Kiranmayi *et al*. 2011).

Effects of minerals on metabolites production

To evaluate the effects of minerals on the growth and inhibitory metabolite production, the optimized medium with the best carbon and nitrogen sources was treated with different minerals (Kiranmayi *et al*. 2011), such as K_2HPO_4, KH_2PO_4,$MgSO_4$, $FeSO_4$, and $ZnSO_4$, each at a concentration of 0.05% (w/v).

Effect of pH and temperature on metabolites production

The effect of pH on growth and inhibitory metabolites production of strain NRC-14 was tested using liquid cultures each containing 25 ml of F media with the best nitrogen source. Cultures were adjusted to different pH values (pH 2-8)by adding NaOH or HCl (Kiranmayi *et al*. 2011). Similarly, the optimum temperature for cell growth and bioactive metabolite yield was measured by incubating the production medium at temperatures ranging from 15°C to 45°C, while maintaining all other conditions at optimum levels. Flasks were autoclaved, inoculated, and incubated as mentioned above. Growth of strain NRC-14 and metabolites production were recorded.

Production and Extraction of the antimicrobial metabolite

For production:apre-culture,24h old (A_{660}=0.25-0.3, equivalent to about $1x10^7$ cells/ml) of strain NRC-14, was inoculated (4%, v/v) into a conical flask (1L) contained an optimized growth medium (400 ml/pH 6) and incubated at 40°C for 48h at 130 rpm. For extraction of the antimicrobial compound: the supernatant was treated as crude extracellular extract and used for solvent extraction and salt precipitation. These two procedures were used because the nature of the metabolite was uncertain. After preliminary tests, cell-free supernatant, of 48h old culture, was fractionated with ammonium sulfate which was gradually added to the supernatant to achieve 30 to 70% saturation (w/v). The mixture was slowly stirred at 5°C for 1 h and left to stand overnight at 5°C (Okulate 2009). This resulted in the formation of a precipitate which was removed after centrifugation at 7000 x g for 20 min at 5°C and dissolved in10 m Mcitrate phosphate buffer pH 6.0. Traces of ammonium sulfate were removed from the solution by dialysis against the same buffer. Finally, the solution was freeze-dried and kept at 4°C for further studies. Fractions positive

for antimicrobial activity were dialyzed against the same buffer. The resultant solutions, designated as the partially purified antimicrobial metabolites were tested for inhibitory activity, against some bacterial and fungal pathogens (Uddin *et al*. 2013).

Antimicrobial assay

In performing the sensitivity spectrum analysis by agar diffusion method, nutrient agar plates were heavily seeded uniformly with the test organisms. Then a hole was made in media by a cutter in sterile condition. Then one drop of melted agar was poured into hole and allowed to solidify to make a base layer, after which, specific amount of culture supernatant (0.1 ml) was poured into the hole. Then plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion (Barry 1976). The plates were then incubated at 30^{o}C for 24 hours. The antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter.

Test organisms

The antimicrobial efficiency of the metabolites produced under optimized conditions, was tested by the agar-diffusion assay against several bacteria and fungi (Kiranmayi *et al*. 2011).The inhibitory efficiency of the metabolites was expressed as a zone of inhibition against test organisms. Test organisms included Gram- positive bacteria: *Staphylococcus aureus*, *Bacillus cereus* and Gram-negative bacteria: *Escherichia coli*, *Seratiamarscecens*, and *Pseudomonas aeruginosa* as well as fungal pathogens such as, *Aspergillusniger*, *Aspergillusflavus*, *Fusariumoxysporum*, *Fusariumsolani*, *Rhizoctoniasolani*, *Pythiumultimum* and *Sclorotiumrolfsii*. All tested strains were got from the Dairy Microbiology Lab., and Microbial Biochemistry Lab., National Research Center, Egypt. The strains were sub-cultured on fresh appropriate agar plate 24 h prior to any antimicrobial test.

Enzymes assay

Activity of chitinase and chitosanase was determined as described in our previous work (Abdel-Aziz *et al*. 2012). Activity ofβ-1,3-glucanasewas determined by a colorimetric method (Burner 1964). The amount of reducing sugars released from hydrolyses of laminarin (Sigma Chemical Co.) was measured by a standard assay reaction contained l.0ml of the crude enzyme solution and 0.5 ml of 5 mg/ml laminarin, and 0.2M sodium acetate buffer pH 5.0to a final volume of 3.0 ml. After incubation at 40^{o}C with gentle shaking for 10 min, the reaction was stopped by cooling. One ml of the mixture was added to 0.5 ml of 1% dinitrosalicylic acid (DNS) and boiled for 10min, placed in an ice bath, and followed by addition of 1.0 ml distilled H_2O. The optical absorption was measured at 540 nm (Wichitra *et al*. 2006). The amount of reducing sugar released was calculated from a standard curve prepared with glucose, and the glucanase activity was expressed in units (µmol glucose equivalent/min).

pH and thermal stability of the antimicrobial metabolite

A solution of antimicrobial metabolites was dissolved in buffers at final pH values of 3- 10. Solutions were kept at 30°C for 3 h, and tested for residual antimicrobial activity against the tested microorganisms. The thermal stability of the antimicrobial metabolite was determined by autoclaving a solution of metabolites at 121°C for 15 min (Munimbazi and Bullerman 1998). The autoclaved solution was quickly cooled under running tap water and tested for residual antimicrobial activity of the metabolites evaluated against growth of the tested microorganisms.

Infra-Red Spectroscopy

The IR-spectra of the partially purified extract was determined (Cordero *et al*. 2011, Parthasarathi *et al*. 2012).The spectrum of the sample was recorded on the spectrophotometer over a wave number range 4000-400 cm^{-1}using a Fourier Transform Infrared Spectrum(FT-IR) - Raman (Nexus 670, Nicolet-Madison-WI-USA).

RESULTS AND DISCUSSION

Optimization of culture conditions

Antibiotics and inhibitory substances are metabolic by-products of complex biosynthetic pathways in microorganisms. They are usually produced by aerobic spore-forming bacteria in the genera *Bacillus* and *Streptomyces*. The yield of antimicrobial compounds can be increased by optimization of physical (such as temperature, salinity, pH, etc.) and chemical (media components, stimulators, precursors, etc.) factors for optimal growth of microbes(Motta *et al*. 2007). Thus, optimization of culture conditions is essential to get high yields of secondary metabolites. Production of the inhibitory metabolites from strain NRC-14 was carried out by optimizing culture conditions. Among different media tested, medium D and F contained flaked chitosan and fungal mycelium were found to be the most favorite for growth and antimicrobial metabolites production (Fig. 2).Worthy mention is that, growth of the strain in a minimal medium was favorable, comparing with other synthetic growth media supplemented with metal ions and stimulant growth factors (Medium E and G). Antimicrobial metabolite production

for other studies was optimally produced with glucose or sucrose in the medium (Ripa *et al*. 2009, Zidan *et al*. 2012), whereas potato dextrose broth medium was the best for maximum growth and metabolite production by *Aspergillusterreus* KC 582297 (Suja*et al*. 2013), and starch for optimum production by *Streptomyces fulvoviridis* (Uddin *et al*. 2010). Following the appropriate medium, antimicrobial tests were achieved. Secondary metabolites from strain NRC-14 exhibited a wide range of antimicrobial activity against all tested microorganisms after less than 24h of incubation (zone of inhibition), in contrary to other reports described maximum antimicrobial activity at 5th and 6th days of incubation (Ripa*et al*. 2009). Moreover, in our previous study, the inhibitory effect of antimicrobial metabolites from strain NRC-14,in a liquid culture, was occurred after 2h of incubation at room temperature (Fig.1).

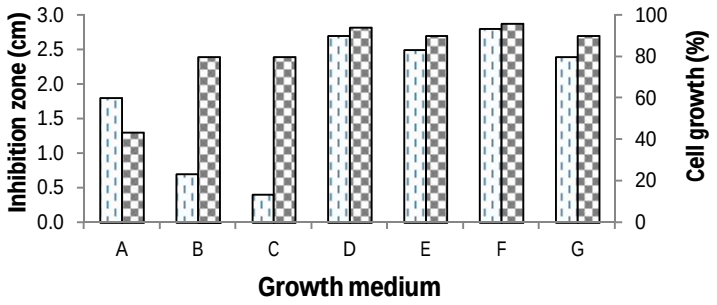

Fig. 2. Growth (right column) and antimicrobial metabolites production (left column) of strain NRC-14 grown in different media contained various carbon and nitrogen sources as well as minerals and trace elements. Strain NRC-14 was grown in seven medium (A-G) at40^{o}C and pH 6.0 for 48h.

Effect of different carbon and nitrogen sources on growth and inhibitory metabolites production by the strain is shown in Fig. 3 (A and B). Among the carbon sources, flaked chitosan and fungal mycelium were found to be the best carbon sources for both cell growth and highest levels of the inhibitory effect; maximum antimicrobial activities were obtained during 48h of incubation at 40^{o}C.Cell growth was weakly produced when the medium was supplemented with glucose as a sole carbon source, in contrary to other reported studies (Suja *et al*. 2013, Ripa *et al*. 2009), however, secondary metabolites (inhibitory effect) was relatively produced with glucose as a sole carbon source (Fig. 3A). Effect of nitrogen sources on growth and inhibitory metabolites production is given in Fig. 3B. Highest activities were obtained with $(NH_4)_2SO_4$, $NaNO_3$ and yeast extract, as indicated by zone of inhibition. Medium supplemented with casein and peptone showed lower results (Fig. 3 B). Suja *et al*. (2013) reported that peptone, beef extract, and yeast extract were the best nitrogen sources, whereas yeast extract was the favorite as a nitrogen source for inhibitory metabolites production by *Streptomyces* sp. RUPA-08PR (Ripa *et al*. 2009), *Streptomyces* sp. PT1(Zidan *et al*. 2012), and *Aspergillusterreus* KC 582297 (Suja*et al*. 2013).

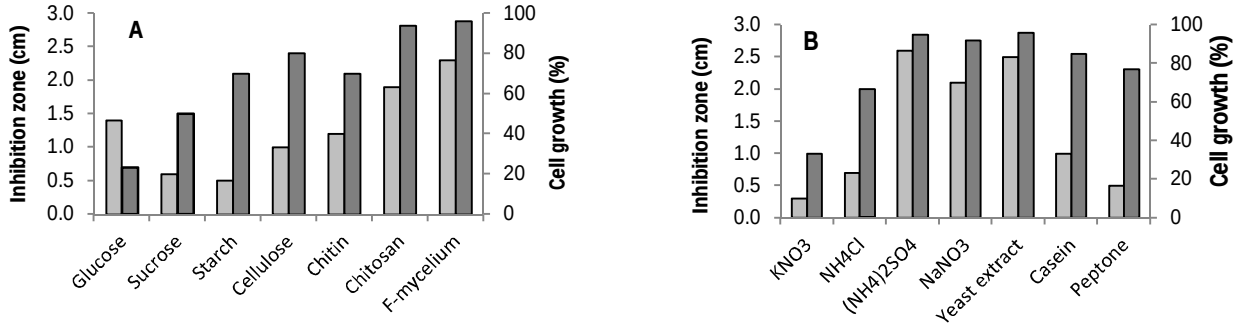

Fig. 3. Growth (■) and antimicrobial metabolites production (■) by strain NRC-14 in media contained different carbon (A)and nitrogen (B) sources at40^{o}C and pH 6.0.

Effect of pH and temperature on inhibitory metabolites production by the strain is shown in Fig. 4 (A and B). The strain produced high levels of inhibitory metabolites when cultures exposed to 40^{o}C (Fig. 4 A). The optimum pH for growth and inhibitory metabolites production is5.0-7.0 with a maximum at pH 6.0 (Fig. 4 B). The correlation between pH and temperature during the metabolism of strain NRC-14 is amazing. Of interest is that, when the strain was grown at 40^{o}C and pH 6.0, the culture broth (metabolites) exhibits inhibitory effect, whereas at 30^{o}C and pH

6.0, production of the inhibitory metabolites was retarded while formation of an exopolysaccharidebioflocculant was occurred (Abdel-Aziz *et al*. 2011).In fact, during a long term period of work by strain NRC-14, it was found that the strain adapts with different conditions and persists with high temperatures and extreme pH values. Moreover, strain NRC-14 is not exigent; the strain can grow well in low-cost medium containing raw and waste materials with high production of valuable products. Furthermore, the strain grow well with inorganic nitrogen sources such as $(NH_4)_2SO_4$instead of organic sources (yeast extract, for exp.), which economically means use of inexpensive materials. When the initial temperature was 40^oC, the strain quickly adapts for hard stress by increased secretion of the inhibitory metabolites during 48h of growth as indicated by a clear zone of inhibition. Effect of different minerals on inhibitory metabolites production was also determined. Among different minerals tested $(NH_4)_2SO_4$, $CaCl_2$, MgSO4, and $FeCl_2$ had positive effects on both growth and antimicrobial metabolites production. However, ZnCl2 and KCl showed negative effect on secondary metabolites production (data not shown). These results are in accordance with other reported studies (Ripa *et al*. 2009, Uddin *et al.* 2010,Suja*et al.* 2013).

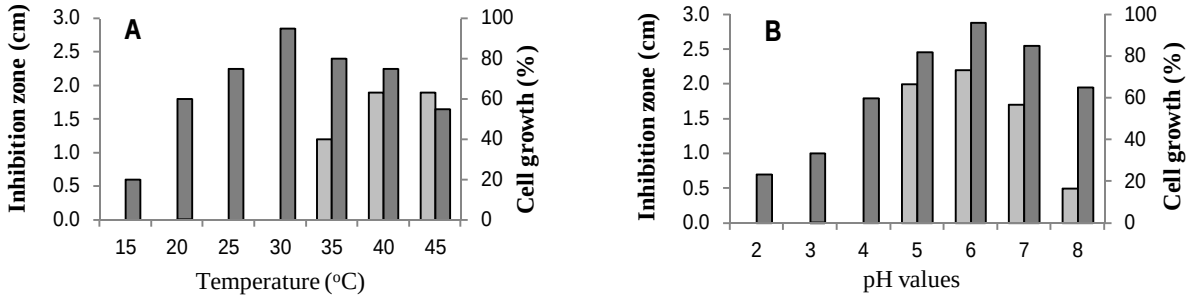

Fig. 4. Influence of different temperatures (A) and pH values (B) on growth (■) and antimicrobial metabolites production (■) by strain NRC-14, using fungal mycelium as a carbon source.

Growth of strain NRC-14 with various carbon sources at different temperatures for production of inhibitory metabolites was also studied. As shown in Table 1, inhibitory metabolites production is induced by polysaccharide polymers such as chitin, chitosan, or related substrates with a maximum when fungal mycelium (*Asp.niger*) was used as a carbon source at 40^oC, followed by cellulose (Avicel). Mono- and Di-saccharides such as glucose and sucrose exhibit, relatively, low extent of metabolites production at 40^oC (Table 1).Other studies revealed that, maximum antimicrobial metabolite production was found at 35°C (Kiranmayi *et al.* 2011,Uddin *et al*. 2013), whereas 25^oC was reported for *Aspergillusterreus* KC 582297(Suja*et al.* 2013),and 39^oC for *Streptomyces*sp. RUPA-08PR (Ripa *et al.* 2009).

Of interest is that, high levels of chitosanase, β-1,3-glucanase, and detectable amounts of chitobiase were determined in cultures grown at 40^oC with chitosan and fungal mycelium as carbon sources, which means a thermostable property of these enzymes. These lytic enzymes, synergistically with the inhibitory metabolite resulted in rapid degradation of cell wall for the tested fungi. The mode of action of the antifungal compounds produced by *B. avlei*NRC-214 against the pathogenic fungi is probably due to fungal cell wall degradation (Wichitra *et al*. 2006). Fungal cell wall composed of chitin, β-1,3-glucans and other oligosaccharide compounds. Chitosanase, chitinase, andβ-1,3-glucanase produced by this strain play a crucial role in the degradation of the fungal cell walls, as indicated by obvious zone of inhibition (Fig. 5). A rapid increase in enzymes production during growth with fungal mycelium as carbon source was observed, which reflects a rapid activity in fungal cell wall degradation during growth. It is reasonable to suggest that, chitosanase and β-1,3-glucanase along with the inhibitory metabolite produced by the strain are responsible for the efficacy of culture broth which targets the cell wall of fungi at the hyphae (Wichitra *et al*. 2006). If these antimicrobial agents are produced by the strain for *in vivo* treatments they could function as key fungicides to control diseases caused by plant pathogens.

The mode of action of antimicrobial agents against bacteria is, however, widely believed to be due to interacting with and disrupting the wall/membrane structure (Helander *et al*. 2003). In Gram-positive bacteria, the cell membrane is covered by a cell wall made up of 30–40 layers of peptidoglycans, which contain *N*acetyl glucosamine, *N*acetyl muramicacid as well as D- and L-amino acids including isoglutamate and teichoic acid, to which the antimicrobial agents can bind, resulting in distortion, disruption, and exposure of cell membrane to osmotic shock and exudation of the cytoplasmic contents (Helander *et al*. 2003). Gram-negative bacteria, on other hand, contain an outer membrane wherein lipopolysaccharide and proteins are held together by electrostatic interactions with bivalent metal ions, one to two layers of peptidoglycans, and a cell membrane (containing lipid bilayer, trans-membrane proteins and inner/outer membrane proteins). The negatively charged O-specific antigenic

oligosaccharide-repeating units of the *E. coli* lipopolysaccharide, for example, form ionic-type of binding with the antimicrobial agents, thus blocking the nutrient flow, resulting in bacterial death due to depletion of the nutrients (Kumar *et al.* 2005).

Table 1. Enzymes and inhibitory metabolites produced by *Bacillus alvei*NRC-14 during growth with various carbon Sources at different temperature.

Carbon Source/°C	Enzymes produced*	Cell growth(%)	Zone of inhibition (mm)**
Flaked chitin/30°C	Chitinase, chitosanase, chitobiase	+++	-
/35°C	Chitosanaseand chitobiase	++	10
/40°C	Free of enzymes	++	12
Flaked chitosan			
/30°C	Chitinase, chitosanase and chitobiase	++++	-
/35°C	Chitosanase and chitobiase	+++	12
/40°C	Chitosanase	++	18
Fungal mycelium			
/30°C	Chitinase, chitosanase, β-1,3 glucanase	+++++	-
/35°C	Chitinase, chitosanase, β-1,3 glucanase	++++	15
/40°C	Chitosanase and β-1,3 glucanase	+++	22
Sucrose /30°C	Chitosanase	++	-
/35°C	Chitosanase	+	-
/40°C	Free of enzymes	-	8
Glucose /30°C	Chitosanase	++	-
/35°C	Chitosanase	+	-
/40°C	Chitosanase	-	14

*****Enzymes were produced using minimal growth medium as indicated in text with different carbon sources (0.5%); ** Production of inhibitory metabolites was expressed by measuring the diameter of inhibition zone against the test microorganisms. Symbols; -,++,+++,+++++ are indicated according to the zone of cell growth, ranging from negative; - , moderate; ++/+++, and numerous; +++++.

Bacteria react to a sudden change in their environment by expressing or repressing the expression of a whole lost of genes(Kumar *et al.* 2005). This response changes the properties of both the interior of the organism and its surface chemistry. A well-known example of this adaptation is the so-called heat shock response of *E. coli*. The name derives from the fact that the response was first observed in bacteria suddenly shifted to a higher growth temperature(Kumar *et al.* 2005).One of the adaptations in the surface chemistry of Gram-negative bacteria is the alteration of a molecule called lipopolysaccharide. Depending on the growth conditions or whether the bacteria are growing on an artificial growth medium or inside a human, as examples, the lipopolysaccharide chemistry can become more or less water-repellent. These changes can profoundly affect the ability of antibacterial agents or immune components to kill the bacteria. In our previous study, it was found that strain NRC-14 adapted with elevated temperature (shift-up temperature from 30°C to 40°C) by formation of an inhibitory substance, probably, as a form of self-protection (Abdel-Aziz *et al.* 2012).The stress response is a mechanism used by organisms to adapt to and overcome a stress stimulus. In considering the physiological process and its contribution to survival, it could be illustrated that, stress response can't be considered a single response to a single system (Lavey and Taylor 1985).

***Invitro* inhibitory spectrum**

Results of the present study showed that there were differences between the antimicrobial activities of crude supernatant and those of fractions. Fractionation (ammonium sulfate saturationfrom30 to 70%) of the crude supernatant decreased its antimicrobial activity. This indicates that the active principles might be more concentrated in the crude supernatant rather than one fraction and be more absent in other fractions due to the fractionation. However, all the isolated compounds from strain NRC-14 showed antimicrobial activities on at least three microorganisms. The crude inhibitory metabolites produced by *B.alvei*NRC-14 were found to be strongly active against wide number of bacterial and fungal pathogens (Table 2). Plates of sensitive bacterial and fungal species were kept for more than one week at room temperature after inhibition and observed for changes, no bacterial growth or spore germination was occurred. These results suggest that the activity of the antimicrobial metabolites is long lasting. Our results are in accordance with other reported work (Philippe *et al.* 2009, Bashir *et al.* 2012,Uddin *et al.* 2013).

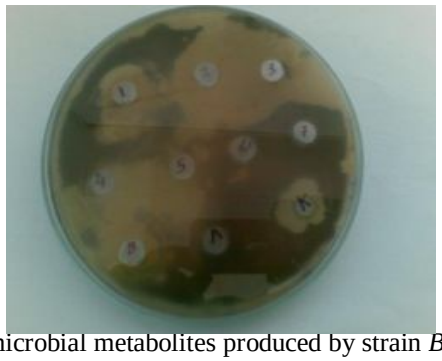

Fig. 5. Efficacy of the crude antimicrobial metabolites produced by strain *Bacillus alvei*NRC-14 against *Fusarium oxysporum*, causal of root-rot for many economical plants.

On other hand, antimicrobial potency of the crude supernatant emphasize efficiency of the synergistic effect between produced enzymes and the inhibitory metabolite compared to the fractions and enzymes alone. Results of the antimicrobial activity of the produced metabolites showed that the culture supernatant, fractions, and all the isolated compounds produced by strain NRC-14 showed both antifungal and antibacterial activities and that the effect is varied among the microbial strains. As shown in Tables 2,the antimicrobial activity of the culture supernatant is highly active against fungal strains rather than the metabolic extract, most probably due to the synergistic effect of enzymes (Chen *et al*. 2008).In contrast, metabolic extract exhibited strong effect against G+ and G- bacteria, probably due to the differed nature of cell wall structure. Antimicrobial substance produced by strain NRC-14 were shown to be broadly active; as it inhibited the growth of some human pathogens as well as phytopathogenic fungi (Table 2). On other hand, antimicrobial metabolites from strain NRC-14 is suggested to be produced under complex genetic regulation due to the heat shock and, probably, the formation of heat shock proteins (Yuk and Marshall 2003).

Secondary metabolites are a source of biologically active natural products with various functions, including antibacterial, antifungal, antiviral, anticancer, anticoagulant, cytotoxic, hypocholesterolemic, insecticide, and neurotoxic activities, acting also as inhibitors and plant growth promoters (Cordero *et al*. 2011). This wide range of properties allows these compounds to have great potential for industrial application. In addition, chemical research related to the isolation, biosynthesis and structural elucidation of new natural compounds has contributed to pharmaceutical and agricultural progress (Chen *et al*. 2008, Jean*et al*. 2011, Kiranmayi*et al*. 2011).

Table 2. Antimicrobial activity spectrum of metabolites produced by the strain *Bacillsalvei* NRC-14*

Organism	Culture supernatant Inhibition zone (mm)	Metabolic extract Inhibition zone (cm)
Gram-positive bacteria		
Bacillus cereus	11	20
Bacillus subtilis	11	19
Staphylococcusaureus	13	21
Gram-negative bacteria		
E. coli	18	21
Pseudomonas aerogenosa	15	23
Seratiamarscecens	13	21
Fungi		
Aspergilusniger	22	15
Aspergilusflavus	22	21
Fusariumsolani	20	13
Fusariumoxysporum	22	17
Scloretiumrolfsii	22	12
Pythiumultimum	17	11
Rhizoctoniasolani	25	19

*****A culture supernatant was used at a 0.1 ml concentration/disc, and 0.05 ml of metabolic extract. Nutrient agar plates were used for bacteria and incubated at 30^{o}C/24h. For fungi, PDA plates were used and incubated at 30^{o}C for 48h.

Stability of the antimicrobial substance

Effect of some enzymes, chemical substances, and protein-denaturing agents on the antimicrobial activity of the partially purified antimicrobial metabolite was evaluated. Residual activity was measured by agar plate disc assay against *E. coli* as an indicator. The antimicrobial substance showed a complete activity after autoclaving at 121^{o}C for 15 min, and was stable at pH range between 4 and 10, where it remained 100% of initial activity. When treated with enzymes and detergents, the antimicrobial substance remained all activity after treatment (Table3), whereas acetone and trichloroacetic acids lightly affected the activity of the antimicrobial substance produced by strain NRC-14(Table3). Considering the properties of the antimicrobial substance produced by strain NRC-14, it is a

polysaccharide compound has heat-stability, rather than a substance has a proteinaceous nature. Our results are in accordance with antimicrobial substance that has a polysaccharide nature (Munimbasi and Bullerman1998, Nalisha *et al*. 2006, Motta *et al*. 2007, Uddin *et al*. 2013). In contrast, other inhibitory substances are peptides (Motta and Brandelli 2002), while others are lipopeptides (Chen *et al*. 2008).

Table 3.Effect of some chemical substances and protein-denaturing agents on activity of the partially purified antimicrobial metabolite* produced by strain *B. alvei* NRC-14.

Treatment	Concentration	Residual activity (%)
Control	-	100
Enzymes		
Protease, lipase, amylase	2U/ml	100
Solvents		
Ethanol	50% (v/v)	100
Methanol	50% (v/v)	100
Acetone	50% (v/v)	95
EDTA	10 mM	100
Trichloroacetic acid	100 mg/ml	92
protein-denaturing agents		
Tween-20	0.1 ml/ml	100
Tween-80	0.1 ml/ml	100

*0.1 ml concentration of metabolite per disc in agar diffusion assay.

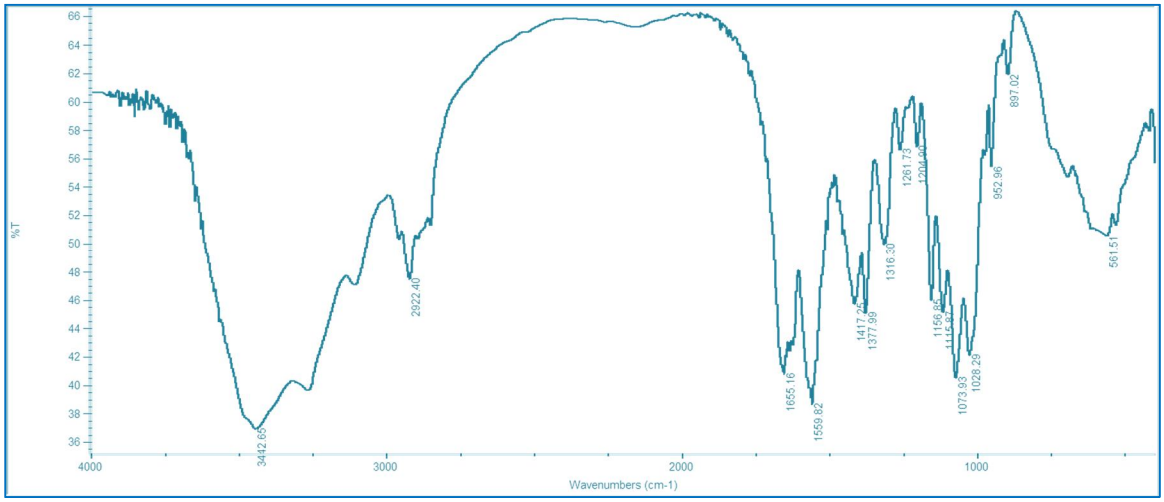

Fig. 6.Infrared spectrum of the inhibitory metabolite produced by *B. alvei*NRC-14.

IR-spectra

The IR spectrum of the partially purified metabolite (Fig. 6) indicated that it possesses characteristic absorption peaks for polysaccharides. The strong peak at 3442 cm^{-1} is the absorption of the O-H bond. The observed band at2922cm^{-1} is the absorption of the C-H bond. The band at 1655cm^{-1} is the hydrated water in the polysaccharide(Adebayo *et al*. 2012). The peaks at 1200-1000cm^{-1} are the absorption of C-O bond. There was no absorption at 1730cm^{-1} nearby (Fig. 6), indicating that metabolite contains no uronic acid (Adebayo *et al*. 2012). The band close to 1559 - 1655 cm^{-1}represents a characteristic band due to conjugated carbonyl C=O stretching and alkenes (C=C) group (Pavia *et al*. 2009, Cordero *et al*. 2011, Parthasarathi *et al*. 2012).The absence of carboxylic acid (COOH) and ester (COOR) alkynes was confirmed by the lack of bands in the region of 1670-1674 and 1700-750 cm^{-1} (Parthasarathi *et al*. 2012).

In general, metabolite biosynthesis in microbes is tightly controlled by regulatory mechanisms to achieve over production and avoid undesirably low levels. Production of inhibitory metabolites by strain NRC-14 is suggested to be occurred under complex genetic regulation due to exposure to elevated temperature (Motta *et al*.2007). It has been observed that modification of the culture conditions, like addition of minerals and trace elements did not enhance production of the inhibitory metabolites indicating that strain NRC-14 is not an exigent microorganism; it can grow and adapt under minimal growth conditions. However, most of the products secreted by strain NRC-14 are highly stable against proteases which refer to the un-proteinaceous nature of these products. The potential use of inhibitory compounds as bactericide and fungicide requires more investigations, including inexpensive materials, large-scale production, purification and characterization as well as evaluation for safety and degradation in the environment (Motta *et al*. 2007).Antimicrobial substances produced by different microorganisms seem to play an important role in antagonism in aquatic eco-systems as well as in soil. The broad inhibitory

spectrum of strain NRC-14, as a soil isolate, may indicate an ecological advantage, since it would be capable to inhibit several competing bacteria. While many studies on antimicrobial substances have shown their importance as food preservatives, few attention have been paid to their possible application as antimicrobial substances in clinical studies, as an alternative for disease control (Motta *et al*. 2007). The rapid rise and spread of multiple-resistant pathogens have forced the consideration of alternative methods of combating infections. Thus, there is a need for new substances that exhibit efficient antimicrobial activity against multiple-resistant pathogens. Therefore, research for new products with antimicrobial activity is a very important field.

Conclusion

The study showed that, strain *B. alvei*NRC-14 secretes antimicrobial metabolites when exposed to a heatshock. Optimum environmental conditions for maximum production of antimicrobial metabolites by the strain were examined. Chitin, chitosan, and related materials were the most appropriate carbon source for production of the secondary metabolites. Yeast extract and ammonium sulfate $(NH_4)_2SO_4$ were the best nitrogen sources. No significant factors such as trace elements or growth stimulators were required to enhance the antimicrobial activity of secondary metabolites produced by strain *B. alvei*NRC-14.The IR-spectra of the product is extremely similar to that of polysaccharides rather than a protenaceous nature; the inhibitory substance is heat-stable and active over a wide range of pH values. We concluded that, the nature of the inhibitory substance as a polysaccharide may be related to the carbon source; degradation of chitin or its related substrates, i. e, chitosan and fungal mycelium, by enzymes secreted during growth of the strain may probably result in accumulation of aminosugars which may polymerize to form the polysaccharide inhibitory substance. The IR-spectrum structure of the product as a polysaccharidemy support this assumption.

Acknowledgment

The authors declare that they have no conflicts of interest in this research.

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