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Amazing Abiotic Stress Response of a Novel Strain *Bacillus alvei*NRC-14Isolated from Egyptian Soil

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ABSTRACT

A variety of amazing extracellular metabolites produced by the novel strain *Bacillus alvei* NRC-14 have been studied. During a long-term of experiments with the strain, it was found that it, interestingly, produces extracellular metabolites whether under normal or stress environments. Exopolysaccharides synthesized by *B. alvei* NRC-14 under stress growth conditions were found to protect the bacterium from extreme pH values, elevated temperature, drying, and water activity-reducing components. Strain *B.alvei* NRC-14 was examined for anexopolysaccharide (EPS)production when exposed to abiotic stress shock. A tentative bioassay showed that the stress increased the EPS formation which was associated with about 10-fold encrease in the viscosity.It was clearly shown that the biosynthesis of EPS biofilm from strain NRC-14is growth-associated and hence displays primary metabolite kinetics. The production kinetics and EPS yields were strongly dependent on the abiotic stress conditions. Physical factors such as temperature, pH and oxygen tension as well as chemical factors such as medium composition, element concentration, and carbon source were of utmost importance. It is observed that, cells treated with ethanol or exposed to heat elevation displayed irregular and wrinkled shapes on agar surfaces. The adapted cells to each stress under sub-lethal conditions exhibited increased resistance to the same stress of lethal conditions. For example, cells adapted to 42°C exhibited markedly increased resistance to the lethal stresses of 45°C as well as to 20% ethanol.

INTRODUCTION

When thrive in an extreme environment, microorganisms develop certain adaptation mechanisms which may be useful for their defense, and the resultant products, i.e., secondary metabolites of these adaptations may be useful for human beings in many forms such as antibiotics (Ira and Kim 2010). Microorganisms, including certain bacteria, fungi and algae, produce secondary metabolites which have some degrees of bioactivity, either against another microorganism or acting against certain physiological states of a diseased body. These metabolites, otherwise known as bioactive compounds, may be inhibitor substances, antibiotics as well as enzymes, or even exopolysaccharides. Recently, it was reported that some polysaccharides are characterized to exert broad-spectrum biofilm inhibition activity (Peng *et al*. 2011). Nowadays, several arguments support the hypothesis that secondary metabolites improve the survival of the producer in competition with other living species. These arguments are as follows: 1) secondary metabolites act as an alternative defense mechanism; 2) they have sophisticated structures, mechanisms of action, and complex and energetically expensive pathways; 3) secondary metabolites act in the competition between microorganisms, plant and animals; and 4) the production of secondary metabolites with antibiotic activities is temporarily related with sporulation when the cells are particularly sensitivity to competitors and requiring special protection when a nutrient runs out (Demain and Fang 2000). Furthermore, the wide diversity of secondary metabolites suggests a broad range of functions. Nevertheless, these functions could depend on the conditions, optimal or not, surrounding the producer microorganism.

When exposed to environmental stress, bacteria increase synthesis of heat-shock proteins, acid-shock proteins, and other general stress proteins, usually in response to the accumulation of misfolded and denatured proteins (Herman and D'Ari 1998, Jarolim *et al.* 2013,Patrick *et al.* 2013), which allows them to contend with the adverse environment. Many of these stress-induced proteins are chaperones and proteases (Schlesinger 1986, Vorob'eva2004). Their biological role is to protect cellular proteins against the toxic effects generated by exposure to stress. The chaperones function to eliminate misfolded proteins by:1) unfolding these proteins and subsequently promoting proper folding; 2) targeting unfolded proteins for proteolysis (Hirshfield 2011, Paramita and Irvin 2011).The variety of microbial aggregates such as biofilms, flocs and sludge are kept together by extracellular polymeric substances (EPS). They represent the construction material which allows the cells to maintain stable micro consortia and to establish synergistic relationships. The EPS play a key role for the understanding of structure, function, properties and development of microbial aggregates. It seems that the EPS matrix may serve as a multipurpose functional element of microbial communities, including adhesion, structure, protection, recognition and physiology. Due to the metabolic activity of the cells,

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gradients develop and create different habitats within small distances, allowing a wide variety of organisms to settle and grow in the aggregate (Nwodo*et al*. 2012, Lehman and Long 2013).

Biofilms are surface associated communities embedded within an extracellular matrix. Biofilm communities exhibit enhanced antibiotic tolerance. As a result, biofilm infections tend to be chronic and difficult to eradicate. A focus of research has been to identify biofilm-associated factors that contribute to their antibiotic tolerance (Mah and O'Toole 2001). In pathogenic bacteria, the opportunistic pathogen, *Pseudomonas aeruginosa*, is used as a model organism in biofilm research (Kostakioti *et al*. 2013). *P. aeruginosa* is well known for the chronic infections it causes in individuals with the genetic disease, cystic fibrosis (Wagner and Iglewski 2008). Biofilm formation within the cystic fibrosis airways is believed to facilitate the infection, helping the bacteria to withstand aggressive antimicrobial treatment and host defenses. The extracellular matrix is a distinguishing feature of biofilms, capable of functioning as both a structural scaffold and protective barrier to antimicrobials (Mah and O'Toole 2001). A key component of the matrix is extracellular polysaccharides (Ophir and Gutnick 1994). Exopolysaccharidescarry out a wide range of functions involving surface and cell-cellinteractions, as well as protecting against antimicrobials and host defenses.

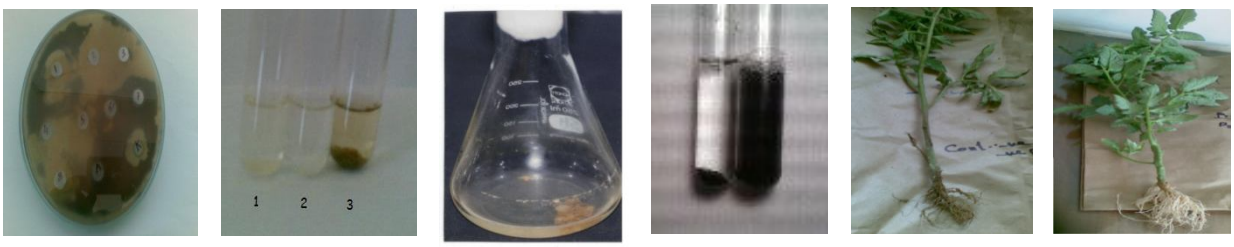

Fig. 1:Action of the secondary metabolites produced by the novel strain *Bacillusalvei* NRC-14:inhibitory effect of bioactive compounds against growth of some bacterial and fungal pathogens (Left, 1and 2 images), the flocculating properties of an exopolysaccharidebioflocculant (Middle images; precipitation of charcoal particles), and effect of the crude metabolites in biological control against plant pathogens of tomato (Right, 5 and 6 images; control and healthy plants).

Temperature, pH, and desiccation are the major stresses that all the living organisms have to face. Heat-shock response from bacteria to human has been extensively studied, while cold-shock response has caught attention of researchers relatively recently. A major reason why heat shock is extensively studied is because it causes well-defined damage to the cells, i.e. unfolding or denaturation of proteins. Heat shock-induced proteins and chaperones, assist in protein folding (Jarolim *et al*. 2013,Patrick *et al*. 2013). In contrast, cold shock does not cause such well-defined cellular damage. Cold-shock response is classically exhibited when an exponentially growing culture is shifted from its optimum growth temperature to a lower temperature. In case of majority of bacteria such as *Escherichia coli*, upon temperature downshift, there is a transient arrest of cell growth, during which general protein synthesis is severely inhibited. Secondary metabolites produced by some microorganisms such as *Streptomyces*, *Bacillus*, and *Cyanobacteria* have a broad spectrum of biological activities such as antibacterial, antifungal, antiviral, antiparasitic, immunosuppressive, anticancer, enzyme inhibitory and diabetogenic (Arasu*et al*. 2012, Parthasarathi *et al*. 2012). Secondary metabolite production in microbes is strongly influenced by nutritional factors and growth conditions. The strain *B. alvei* NRC-14, a soil isolate, possesses amazing biological properties and secrets a large variety of extracellular metabolites. We extensively examined this strain for production of biologically active compounds. The strain has never been reported previously in literatures. Extracellular metabolites produced by the novel strain *B. alvei*NRC-14 include: 1) enzymes; 2) metabolites that show inhibitory effect against growth of some bacterial and fungal pathogens; 3) exopolysaccharidebioflocculant; and 4)inhibitory substances and plant-growth stimulating factors (Fig.1). In a previous work, it was noticed that strainNRC-14 secreted an inhibitory substance when exposed to a heat shock (Abdel-Aziz *et al*. 2012)which showed an antimicrobial effects (Figs. 1-4).Thus far, most abiotic stress studies have focused on *E. coli* and *Pseudomonas* sp. strains with respect to stress adaptation and protection from heat shock, starvation, hydrogen peroxide, and acid stress(Bayer *et al*. 1990,Park *et al*. 2001, Paramita and Irvin 2011).The present study is focused on the response and reactions of strain *B. alvei* NRC-14 when exposed to some abiotic stressors and biological properties of the resultant metabolites.

MATERIALS AND METHODS

1. Bacterial strain and Cell growth

A bacterial strain isolated from Egyptian soil as a potent chitosanase producer and identified as *Bacillus alvei* (Abdel-Aziz 1999), was used in the present study. The strain was maintained onnutrient agar slants at 4°C, with monthly transfers using chitosan-containing slants to retain viability. Prior to use, cultures of the strain was grown in

nutrient broth to the log phase and repeated for 3 rounds to enrich for determination of heat- and acid-resistance as well as other stresses. In all experiments, unless otherwise, cells were grown in nutrient broth (NB) medium to the log phase at 30°C in a shaker incubator rotating at 130 rpm (Paramita and Irvin 2011). Growth of the cells was monitored by spectrophotometer at 660_{nm}. The NB medium was adjusted to pH 3 (pH shock) by adding 6N HCl to the medium.

2. Stress conditions

The strain was exposed to stress condition such as oxygen tension, osmolarity, extremes of pH values (3 and 10), elevated temperature (40 – 60^{o}C), drying at room temperature for 2 months, or freezing at refrigerator conditions (Pirog *et al*. 1997, Petronella *et al.* 1999). To investigate the effects of oxygen tension on cell growth and EPS production, oxygen volume was controlled at three different levels of 10, 20, and 40 % of air-saturation by manipulating agitation speed in a series of cultures of strain *B. alvei*NRC-14.For test of osmolarity, 1-3% each of ethanol or NaCl was added to the culture after 24h of incubation (Nakata *et al.* 1999).To study the protective function of the EPS against the extremes of pH, the cultures was alkalized to pH 10.0 with a 6% NaOH solution or acidified to 3.5 with a 6% HCl solution and then the cultures were observed and estimated for viability during the growth phase. To elucidate the role of EPS biofilm in protection of cells from the effect of high temperature, the cultures was heated in a water bath to 50, 60, or 70^{o}C for 15 min, then cell viability was determined during growth of the cells under shaking conditions (Pirog*et al.* 1997). The role of the EPS biofilm in protection of cells against drying was studied by keeping 5 ml of aculture in sterile petri dish at room temperature for2-monthsor until it completely dried out, after which the dried cells were suspended in 3-ml sterilized distilled water, cultured under shaking conditions(130 rpm), and then cell was determined. To study the ability of the EPS biofilm to protect the cells against osmolarity, each of NaCl or ethanol was used at different concentrations (Petronella *et al*.1999).

3. Preparation of heat-shocked and heat-adapted cells

Aliquots (100 ml) of fresh cultures (10^{8}CFU/ml) were heat shocked by immersion (3 cm above medium level in bottle) into 42°C and 45°Cin controlled water baths for 15 min. Heat-adapted cells were prepared by incubating fresh cultures at 42°C for 20h, followed by an additional 6 h at 45°C (Yuk and Marshall 2003). Cultures grown in NB medium for 20 h at 37°C were used as controls.

4. Survival assay of the strain

One ml inoculum (A580=0.25-0.3, equivalent to about $1x10^{8}$ cells/ml) of log phase cells at 30^{o}C, prior exposure to high temperature, was transferred into a flask containing 99 ml of nutrient broth in a water bath pre-warmed to 50°C that had 3 cm water above the medium level in the flask. Heat treatment was carried out for 30 min, with 5 ml sample withdrawn at 0, 5, 10, 15, 20, 25, 30 min, and immediately cooled in an ice bath. Growth was monitored directly by plating on nutrient agar after 24h of incubation at 30°C or indirectly by measuring the OD_{660}(Yuk and Marshall 2003).

5. IR-spectra

The exopolysaccharide product was analyzed by infrared using a FT-IR-FT Raman (Nexus 670, Nicolet-Madison-WI-USA). The spectrum of the sample was recorded on the spectrophotometer over a wave number range 4000-400 cm^{-1}.

RESULTS AND DISCUSSION

1. Growth of the strain under normal culture conditions

1.1 Medium content

Growth parameters of strain NRC-14 grown on different types of media were recorded. When the growth medium contained flaked chitin, chitosan, fungal mycelium, or cellulose (filter paper)as sole carbon source at 30^{o}C, formation of an exopolysaccharidebioflocculant (EPB) was occurred, resulting in aggregation of cells and other particles, and the medium seemed clear; the flocculating activity reached, approximately, 98% (Fig. 1E).Production of the EPB was in parallel with cell growth curve up to 40 h, after which the cells aggregated to the mycelium particles and cell growth was sharply decreased (Fig. 2A). On other hand, the culture broth was found to contain a battery of enzymes (after 48h of growth) such as chitinase, chitosanase, chitobiase, and detectable amounts of *N*-acetylglucosaminidase. High levels of glucosamine were detected in the culture broth after 72h of growth (Fig. 2B).Thus, using flaked chitosan under normal incubation (pH6/30^{o}C), the culture broth exhibits EPB production before 48h of growth, and the product (EPB)has a chitosan-like structure (Abdel-Aziz *et al*. 2011), whereas high levels of glucosamine was occurred after 72 h of growth.

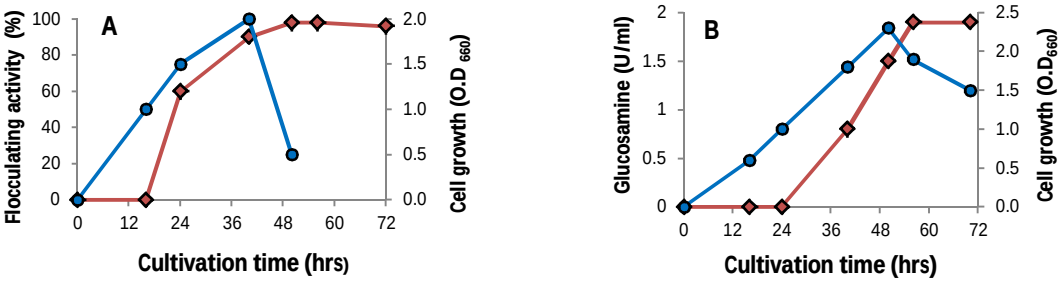

Fig. 2.Growthof strain NRC-14 under normal conditions (pH 6 and 30°C).Production of (♦) an exopolysaccharidebioflocculant (A) and glucosamine (B) during cell growth (●) of strain NRC-14, with flaked chitosan as a sole carbon source.

1.2 Effect of monovalencations

During normal growth of strain NRC-14 for the production of EPB, effect of potassium (K+) and magnesium (Mg^+) concentration in the medium was tested. As shown in Fig. 3A, growth of the strain increased when potassium (K+) concentration in the medium was raised from 0.05 - 0.10 M (Pirog*et al*. 1995).At 0.05 and 0.1Mof Mg^+ growth of cells was occurred, but affected by 0.2 m Mconcentration (Fig. 3B). It is noteworthy that, growth of the strain NRC-14 depends on the concentration of monovalent cationsin the growth medium but does not depend on whether such anions are added as Cl-, SO_4-, or PO_4- for production of the EPB by strain NRC-14 (Pirog 1996).

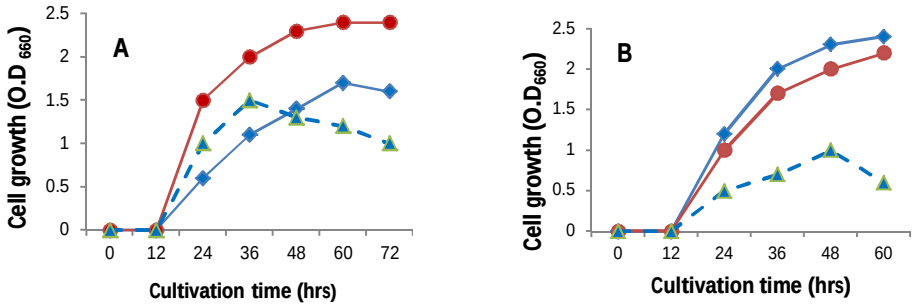

Fig. 3.Effect of cations on growth of strain NRC-14 under normal conditions (pH 6 and 30°C),using K^+ (A) or Mg^+(B) at concentrations of 0.05 (♦), 0.1 (●), or 0.2(▲) on cell growth of strain NRC-14, with flaked chitosan as a sole carbon source.

2. Growth of the strain under abiotic stress conditions

2.1 Exposure to oxygen tension

key parameter in submerged cultures is oxygen tension (OT) which is significantly influences cell growth and the fermentation process. Experiments for studying the effect of OT on formation of the EPS were performed in 250-ml conical flasks containing different volumes of culture broth. Results revealed that, reduction of oxygen increased the lag phase, decreased the growth rate, but did not enhance formation of the EPS (data not shown).These results are in accordance with those reported by Petronella *et al.* (1999) who found that, exopolysaccharide production by *Lactococcuslactis* subsp. *Cremoris* NIZO B40 could not be identified as a stress response. Increased oxygen tension and reduced water activity negatively affected both growth and EPS synthesis. Reducing the growth rate resulted indeed in an increase of the specific EPS production but the polymer formation decreased again at even lower growth rates (Petronella *et al*. 1999). On contrast, however, Bayer *et al.* (1990) reported that oxygentension represent one of the trigger mechanisms for the up-regulation of an exopolysaccharide production by *P. aeruginosa*. Kim *et al*. (2006) reported also that, dissolved oxygen and sufficient level of agitation are both important for EPS production.

2.2 Exposure to osmolarity

Addition of water activity-reducing components, such as ethanol (Fig. 4A) and NaCl (Fig. 4B) affected both the growth of cells but enhanced formation of the EPS (Jenkins *et al.* 1990, Nakata *et al.* 1999) which was associated with about 10-fold increase in viscosity.

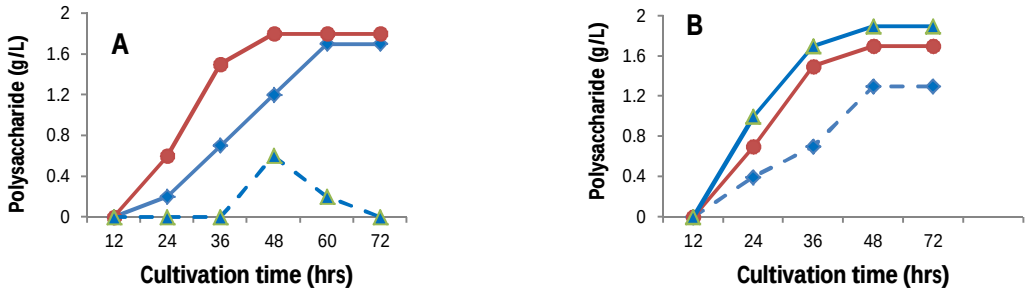

Fig. 4.Effect of abiotic stress shock by ethanol (A) orNaCl (B) during growth of strain NRC-14.Ethanol concentrations: 10% (▲), 15% (♦), and 20% (●). NaCl were used at concentrations of: 1% (♦), 2% (●), and 3% (▲).

2.3 Exposure to pH and temperature shock

Normal growth of strain NRC-14was observed at pH values of 5.0-7.5 and 25-32^{o}C. When the strain was exposed to a shock stress at pH 3.0/30^{o}C, formation of an EPS biofilm was occurred (Table 1),associated with low observed cell growth. Formation of an antimicrobial compound was determined at 40^{o}C, regardless of the pH value (Table 1).On other hand, cell viability (Table 1)was sharply affected by temperature elevation (40^{o}C) and extreme pH values (pH 3 and 10).

Table 1.Influence of abiotic stress conditions on growth of strain *B. alvei* NRC-14 and exopolysaccharide biofilm production.

Carbon source	pH	Temperature^{o}C	Product	Cell viability (%)	Time ofproduction
Flaked chitosan	6	30	-EPB*	100	48h of growth
Flaked chitin	6	40	-Antimicrobial compound	65	Before 48h
Flaked chitosan	3	30	-EPS biofilm	75	48h of growth
Flaked chitosan	3	40	-Antimicrobial compound	52	Before 48h
Flaked chitin	10	40	-Antimicrobial compound	55	Before 48h

*EPB=ExopolysaccharideBioflocculant (produced under normal growth conditions and exhibits flocculating properties, Fig. 1F). EPS= Exopolysaccharide.

It is noteworthy that, exopolysaccharides and antimicrobial compounds produced by strain NRC-14 havepolysaccharides nature rather than a proteinaceous substance. Moreover, structure of these polysaccharides is similar to that of aminoglycans. It could be suggested that, degradation of chitin- or chitin-related substrates by enzyme(s) secreted by the strain may probably result in accumulation of aminosugars, e.g. glucosamine which may polymerize to form a polysaccharide. The structure of these polysaccharides which is extremely similar to that of chitosan would provide evidence that a monomer such as glucosamine may be an important precursor for the synthesis of the polysaccharide by the strain. Of interest is that, during the twenty-year history usage of strain NRC-14 under laboratory conditions, no cell lyses was observed. Moreover, the products produced by the strain are fairly stable polymers and may have a protective function for the cells of the strain.

Over the last 15 years, much more reports have focused on defining the structure and function of so-called heat shock or stress proteins (HSPs), which normally present at modest levels in cells maintained under normal growth conditions. However, the HSPs are expressed at very high levels in cells subjected to heat shock treatment or a variety of other metabolic conditions (Heyde and Portallier 1990, Vorob'eva2004). Many of the HSPs function as molecular chaperones, essential participants in the pathway by which cellular proteins are synthesized and folded into their final biologically active state(Vorob'eva2004). Basic studies for examining the pathway of protein folding are designed to understand specific folding defects associated with a number of diseased states. Examples include cystic fibrosis and prion disease, both of which involve the abnormal folding of a particular polypeptide. New strategies are designed by which to correct abnormal protein folding defects and therefore successfully treat a variety of different diseases (Welch 1993, Nwodo*et al.* 2012).

Under normal, unstressed conditions, the constitutively stress proteins are essential for cell viability, they participate in protein folding and assembling, metabolic processes, and cell growth and development. Cytoprotective functions have been attributed to HSPs (Welch 1993). A major reason why heat shock is extensively studied is because it causes well-defined damage to the cells, i.e. unfolding or denaturation of proteins. In the present study, it was found that, elevation of temperature to 40^{o}C enhance formation of the EPS biofilm. Heating a culture in any growth phase to

40°C increased the EPS production. On other hand, cells treated with ethanol or heat elevation displayed irregular rod shapes with wrinkled surfaces on agar slants. The relation between cell growth and EPS synthesis was studied. Interestingly, EPS production was not strictly coupled to growth. Significant levels for synthesis of EPS were observed despite low growing cultures. Worthy mention is that, growth of the strain at 40°C and pH 6.0 enhanced the formation of an antimicrobial substance, which showed an inhibitory effect (Abdel-Aziz *et al.* 2013,accepted), whereas at low pH (3.0) and 30°C the strain synthesized anexopolysaccharidebiofilm, in which the IR-spectra (Fig. 6) showed a structure similar to that of polysaccharides (Aminoglycans).

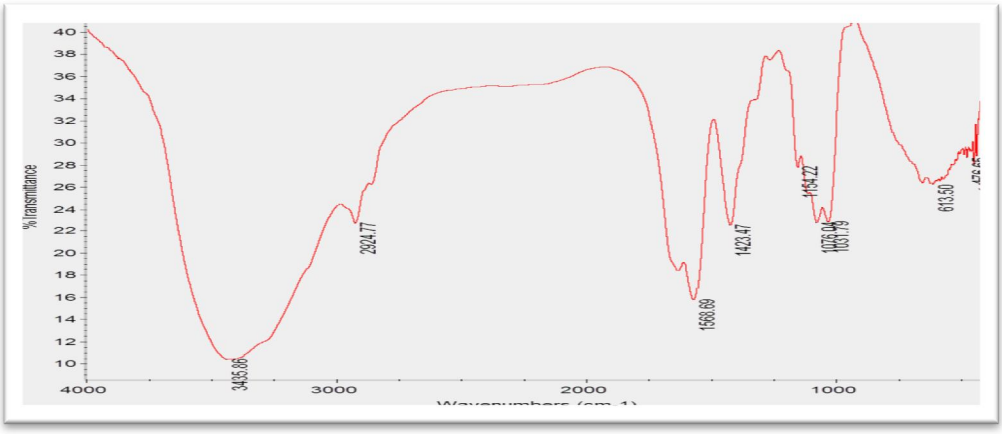

Fig. 6.IR-spectrum of the exopolysaccharide biofilm produced during a pH shock stress to the strain NRC-14.

Stress shock proteins enable microorganisms to tolerate various environmental stresses. The synthesis of such proteins is induced very rapidly to protect the cells against toxicity caused by the stresses, thereby enabling the organisms to survive in harmful environments (Flahaut *et al*. 1997, Lou and Yousef 1997).In some cases, proteins associated with one stimulus can be induced during exposure to other stresses. Forinstance, various heat shock proteins were synthesized in *E. coli* when the cells were exposed to hydrogen peroxide(Jenkins *et al.* 1988), ethanol (Nakata *et al*. 1999), UV light (Van Bogelen *et al*. 1987), or chemical agents (Mason *et al.* 1999).

2.5 Exposure to heavy metals

Exopolysaccharide and metabolites produced by strain NRC-14 were found to be capable of protecting its cells against heavy metal ions (Cu^{2+}, Pb^{2+}, $Zn^{2+}$and Hg^{2+}). In the presence of EPS, exponential cells were more metal-resistant than stationary cells. Protection of cells against heavy metal ions may probably be due to the chelating properties of the EPS produced by strain NRC-14. Our results are in accordance with previously reported studies (Pirog*et al.* 1997*a*, Yousef *et al.* 2011).

Production of the EPS bioflocculant from strain NRC-14 is growth-associated biosynthesis. De Vuyst *et al.* (1997) have reported that, the exopolysaccharide production when occurred at low growth rates, making more precursor molecules available for exopolysaccharide biosynthesis. On other hand, however, under up-normal conditions, production of EPS biofilm by strain NRC-14 is growth non-associated and exopolysaccharide biosynthesis is rapidly occurred during the log-phase for protection of cells when exposed to abiotic stress conditions. Moreover, the product (EPS bioflocculant) under normal conditions has chemical structure (IR-spectra) similar to that of chitosan (Abdel-Aziz *et al*. 2011), which, to some extent, differed under up-normal abiotic stress conditions (Fig. 6).Although some authors are convinced that the nature of the substrate cannot influence the composition of the exopolysaccharides produced (Manca*et al.* 1985; Van den Berg *et al.* 1995), others found that the chemical composition of the environment causes variation in the exopolymer composition (Cerning *et al.* 1994; Grobben*et al.* 1996). The carbon/nitrogen ratio would also play an important role in the production of exopolysaccharides as reported by De Vuyst and Vermeire (1994). It could be shown by our results that abiotic stress conditions such as temperature elevation and extreme pH values obviously influence exopolysacchari deformation and characteristics.

3. Survival of the strain

To assess the survival of cells under lethal heat stress and pH shock, changes in cell growth and viability of strain NRC-14 was observed and detected during heat treatment at 50°C and pH shock (pH 3.0) over a 15-min period. As shown in Fig. 7A and B, cells pretreated by exposure to heat and pH stress were more persist than untreated cells. Abiotic stress response by strain NRC-14 was associated with high levels of EPS biofilm as a form of self-protection especially under heat shock stress and despite of relatively a lower cell growth (Fig. 7A). Patrick *et al.* (2013) studied

exposure of *Saccharomyces cerevisiae* cells to 30–50°C and 37–50°C,and reported that, linear regression was calculated for the strain and a slope associated with strain abundance was observed, and hereafter referred to as *death rate* and that the distribution of death rates centers near zero, suggesting that most genes do not play a role in modulating sensitivity to lethal heat stress(Lou and Yousef 1997).

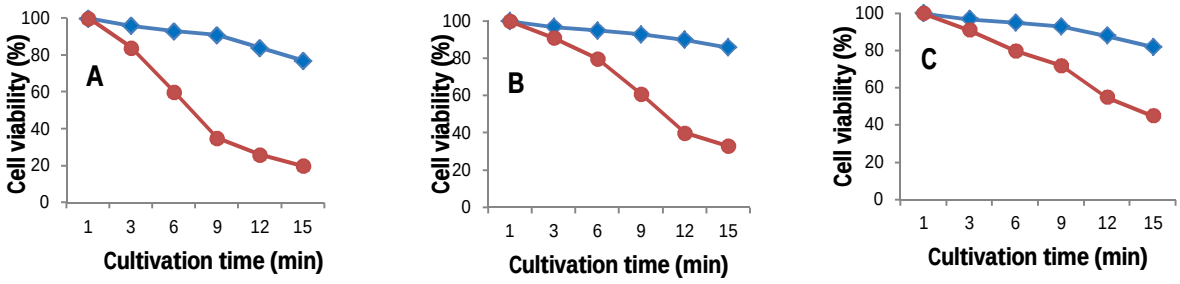

Fig. 7.Experimental approach: cells were grown to early log phase in minimal medium and then pretreated for 1h at 37°C, or pH 6, or 10% ethanol. Cells then were heat shocked at 50°C (A), or shocked at pH 3 (B), or with 20% ethanol shock (C),for 3, 6, 9, 12, or 15 min, and dilutions were plated to calculate CFU at each time point. Symbols: (♦)treated or not treated (●) cells.

Synthesis of HSPs is induced very rapidly to protect the cells against toxicity caused by the stresses, thereby enabling the organisms to survive in harmful environments. Induction of the proteins by exposure to sub-lethal levels (an adaptive dose) of stress agents was shown to confer protection against subsequent exposure to lethal levels (a challenge dose) of the same stress agent (Flahaut *et al*. 1997). Lou *et al*. (1997) reported that *Listeria monocytogenes* adapted to sub-lethal doses of ethanol, hydrogen peroxide, starvation, and acid and significantly enhanced the resistance to lethal levels of these stresses. The adapted cells to each stress under sub-lethal conditions exhibited increased resistance to the same stress under lethal conditions; i.e., cells adapted with low pH showed greater protection for survival than those adapted by other stresses such low pH and high temperature or high concentration of ethanol. In addition, those adapted cells showed increased resistance to other stresses. Cells adapted to 42°C exhibited markedly increased resistance to the lethal stresses of 45°C as well as to 20% ethanol (Lou and Yousef 1997, Park *et al*. 2001).When the cells pretreated under sub-lethal conditions of each stress were challenged with lethal conditions of various other stresses, the cells adapted by each sub-lethal stress exhibited increased resistance to lethal conditions of the stresses than non-pretreated control cells (Fig. 7).

4. Cell growth during abiotic stress

Growth of cells during abiotic stress response was determined. As shown in Fig. 8, stationary phase was the most affected by abiotic stress shock; viability of the cells ranged between 34-55%, whereas it reached a maximum during the log growth phase (Pirog et al. 1997).Viability of cells were, however, most affected by the pH and NaCl stress, and most resistant to the heat shoch stress and ethanol stress (Fig. 8); where tentative bioassay showed that the stress increased the EPS formation which was associated with about 10-fold encrease in the viscosity.

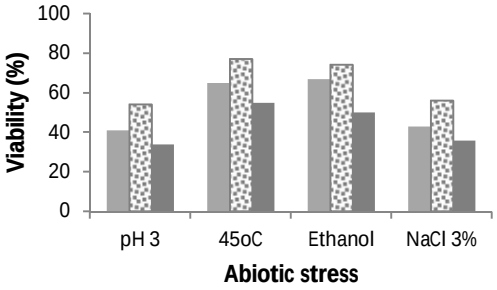

Fig. 8. Abiotic stress response during: lag phase, (*left column*), log phase (*middle column*); and stationary phase (*right column*) growth phases of strain NRC-14 exposed to low pH (3), high temperature (45°C), ethanol (20%), or NaCl (3%).

5. Antagonistic properties

Being a soil isolate, strain NRC-14 exhibits a variety of amazing extracellular metabolites, enzymes, as well as inhibitory substances. Moreover, the strain adapted with hard abiotic stress conditions and exhibited a broad-spectrum of antimicrobial properties. Inhibitory effect of the antimicrobial compound produced by the strain is shown in Fig. 9.

Mechanisms of abiotic stress adaptation by microorganisms are extensively studied (Mah and O'Toole 2001, Park *et al*. 2001). In the present study, one mechanism may be related to the formation of an EPS biofilm. Heat acclimation is the result of the organism's ability to tolerate heat more effectively by developing and secreting an EPS. The subsequent release of an antimicrobial compound may be another mechanism for complete protection by the strain. Formation of HSPs, that may also play an important role in cell protection, is also not excluded. When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to hard conditions (Mah and O'Toole 2001).

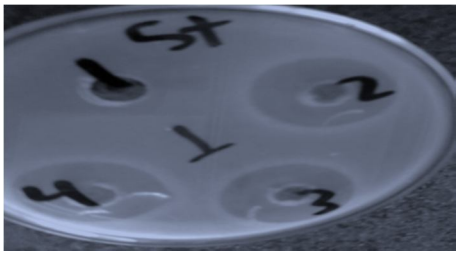

Fig. 9.Inhibitory effect of the antimicrobial compound produced by strain *B. alvei* NRC-14, under abiotic stress conditions (pH 3 at 40^{o}C), against *Staphylococcus aureus* at different concentrations, as indicated by obvious zone of inhibition.

Worthy mention is that, during more than twenty years of experimental work using the novel strain *Bacillus alvei*NRC-14, a spontaneous mutation has been occurred for this strain after about ten years of propagation. Spontaneous mutation is a mutation that arises naturally and not as a result of exposure to mutagens. Spontaneous mutations arise from a variety of sources, including errors in DNA replication, spontaneous lesions, and transposable genetic elements. Strain NRC-14 was found to adapt abiotic stress and hard environmental conditions. The stress response is a mechanism used by microorganisms to adapt to and overcome a stress stimulus in order to survive. Whilst facilitating survival, the stress response may generate a weakly mutant. However, the mutant of strain NRC-14 was found to be strong and different from the wild-type. This strain was, firstly, reported to produce two chitosanases with low activity towards other substrates such as chitin, CM-cellulose, and starch. However, after long term of work, the strain was found to be an excellent producer of carbohydrate-active enzymes, bioflocculant, aminosugars, as well as bioactive compound(s). Under well aerated conditions during long-term work with this strain, the cells were observed to form three types of colonies (occurred from one colony) on nutrient agar or chitosan-based medium: 1) circular, flat, glistening, and smooth; 2) circular, very small and convex; and 3) circular and edges irregular, wrinkled and glistening. The first and second types were mainly dominant after isolation of the strain from soil, while the third type was developed after a long period. Of interest is that, exactly similar results are reported by Chung *et al*. (2000). The long-term storage viability of the strain either being on agar slants or in liquid culture is high; this may be due to the formation of an exopolysaccharide biofilm and adaptation to hard abiotic stresses by the strain.

In nature, bacteria exists in colonies accumulating at interfaces to form poly-bacterial aggregates such as mats, flocs, sludge or biofilms and not planktonic dispersed single cells as will be seen in laboratory pure cultures (Flemming andWingender2010). Exopolysaccharides play crucial roles, and the term biofilm means microbial aggregates that accumulate at a solid-liquid interface and are encased in a matrix of highly hydrated extracellular biopolymers(Nwodo*et al*. 2012), although this description does not take into account groups of free floating microbial aggregates (flocs).A summary of the roles played by exopolysaccharides in bacterial biofilms is articulated in Table 1.

Some human diseases related to formation of biofilm include: *Pseudomonas aeruginosa*, causal of Cystic fibrosis pneumonia, has extensively been referred to in biofilm research. *Staphylococci*, causal of Musculoskeletal infections; *E. coli*, causal of urinary catheter cystitis; *E. coli* and other enteric bacteria, causal of biliary tract infection, and *Haemophilus influenzae*, causal of otitis media, are all potentially form biofilm matrix (Nwodo *et al*. 2012). Mechanisms of resistance in the biofilm include increased cell density and physical exclusion of the antibiotic. The individual bacteria in a biofilm can also undergo physiological changes that improve resistance to biocides (Sutherland 2001, Nwodo *et al*. 2012). Various authors have speculated that the following changes can occur in biofilm-grown bacteria: 1) induction of the general stress response (an rpoS dependent process in Gram-negative bacteria); 2) increasing expression of multiple drug resistance; (3) activating sensing systems; and (4) changing profiles of outer membrane proteins (Mah and O'Toole 2001).

Table 2.Some of the roles ascribed to exopolysaccharides in biofilms (Nwodo*et al*. 2012).

Process	Functional Relevance of Exopolysaccharides to Biofilms
Adhesion	Exopolysaccharides make provision for the initial steps in the colonization of surfaces and long-term attachment of biofilms

Bacterial cell Aggregation	The bridging between cells is enabled by exopolysaccharides, thus temporarily immobilizing bacterial population thus, the subsequent development of high cell densities and cell–cell recognition
Water retention	Hydrophilic exopolysaccharides have high water retention ability thus maintaining a hydrated microenvironment around biofilm and this leading to the survival of desiccation in water-deficient environments
Cohesion of Biofilms	Neutral and charged exopolysaccharides forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalentcations), determining biofilm architecture, as well as allowing cell-cellcommunication.
Nutrient source	Exopolysaccharides serves as source of carbon, nitrogen and phosphorus containing compounds for utilization by microbes inhabit the biofilm community.
Protective barrier	Exopolysaccharides(of pathogens) confers resistance to non-specific and specific host defences duringinfection, confers tolerance to various antimicrobial agents, protects cyanobacterialnitrogenase from the harmful effects of oxygen and offers protection against somephagocytic protozoa.
Binding of enzymes	Non glycolytic extracellular enzyme interaction with exopolysaccharides leads toretention stabilization and accumulation.
Sink for excess energy	Exopolysaccharides stores excess carbon under unbalanced carbon to nitrogen ratios.

Tomanek (2010) reported that, the preferential synthesis of HSPs in response to thermal stress, i.e., the heat shock response (HSR) has been shown to vary in species that occupy different thermal environments. Studies on the HSR in organisms from environments that differ in absolute temperature and thermal range suggest that the response may be : i) absent in organisms that occupy thermally stable environments, ii) employed at maximal or near maximal levels in species from highly variable thermal environments, and iii) a widely “expandable option” in organisms from moderately variable thermal environments. Organisms from moderately variable environments can modify (acclimate and acclimatize) their constitutive levels of HSPs and shift their response to a higher onset temperature. This suggests that species from the extreme ends of the thermal spectrum, either from very stable or highly variable thermal environments, live closer to their thermal limits and that they will be more affected by global climate change than species from moderately variable thermal environments (Tomanek2010). However, increasing the HSR due to increasing temperatures in organisms from moderately variable thermal environments may become detrimental to the long-term fitness of the species and restrictive of the thermal niches in which the organisms can occur. Bacteria encounter stresses in their natural environments, including, for pathogens, their hosts. These stresses elicit a variety of specific and highly regulated adaptive responses that not only protect bacteria from the offending stress but also manifest changes in the cell that impact natural antimicrobial susceptibility. Thus, exposure to nutrient starvation/limitation (nutrient stress), reactive oxygen and nitrogen species (oxidative/nitrosative stress), membrane damage (envelope stress), elevated temperature (heat stress) and ribosome disruption (ribosomal stress) all impact bacterial susceptibility to a variety of antimicrobials through their initiation of stress responses that positively induce resistance determinants or promote physiological changes that compromise antimicrobial activity(Poole 2012).

Conclusion

Based on data obtained, the following conclusion could be drawn for the strain: 1)*Bacillus alvei* NRC-14 produces extracellular metabolites whether under normal or stress environments, 2) it can adapt with extremely hard conditions as a form of self-protection, 3) under normal environmental conditions, the strain produces a biopolymer flocculant that possesses high flocculating properties due to its similarity to chitosan-like structure, an extracellular polysaccharide biofilm was found to be synthesized by the strain due to exposure to abiotic stress; acidic pH-shock, 4) it produces antimicrobial compounds, when exposed to a high temperature, which exhibits a broad spectrum activity against some human pathogens and plant-fungal pathogens. The IR-spectrum of the partially purified EPS from strain NRC-14 showed structure similar to that of aminoglycans. The produced biopolymer flocculant possesses good flocculating activities and highly stable against biodegradation by enzymes. It may find possible application as a biopolymer for biotechnological processes.

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