Potential Uses Of Lipopolysaccharide And Exopolysaccharide Isolated From *Agrobacterium* spp.

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Abstract

A gram-negative bacterium, *Agrobacterium*, synthesizes lipopolysaccharide and exopolysaccharide, both indispensable in establishing complex interactions with plant hosts. These polysaccharides have biocontrol applications. This study intended to determine: 1) LPS potential as insecticide; 2) EPS production capacity. LPSs obtained from *Agrobacterium* isolates were tested for insecticidal activity against test insects, damaging stored grains. Agrobacterial LPS elicits insect mortalities comparable to Permethrin (standard insecticide). Typical strains (At₂, At₃, At₅, At₆) incited 80% mortality of tested insects. EPS yield was quantified at regular intervals under varying physical parameters. Maximum EPS yield of 12.25 g/L was recorded for strain At₁. Antibacterial activity of all isolates (before and after UV light exposure) was tested against Gram-positive and Gram-negative reference strains. Agrobacteria exposed to UV light exhibited antibacterial activity against Gram-positive reference strains. Therefore, agrobacterial LPS can be future candidate for biocontrol of insect pests in grain stocks. Moreover, agrobacteria can not only be prospective candidates for industrial scale EPS production but can also potentially help in treating infections by antibiotic resistant Gram-positive bacteria. Hence, same native isolates carry numerous benefits for the community.

Keywords: Biocontrol, Bioinsecticide, Mutagenesis, Lipopolysaccharide, Exopolysaccharide

Introduction

Insect pests have been a problem for mankind since humans started agricultural practices and stored grains/crops to satisfy future food necessities. Stored grains offer nutritional support to numerous insects but in return get infested resulting in economic losses attributable to low-grade stored foods. Further, these infestations remarkably influence the consumable food quantity as well. Most affected ones are the underdeveloped or developing countries where food reserves are already scarce to fulfill the food requirements of overgrowing populations leading to malnourishment issues. However, nature has offered indigenous solutions to the problem. *Agrobacterium* is a Gram-negative plant pathogen responsible for crown-galls in plants. However, this bacterium may offer many benefits as well that need to be explored.

Microorganisms can trigger a vast array of diseases in both animals and plants (1). After settlement of primitive man to farmland, his main opponents proved to be weeds, insects and microorganisms, devastating the crops, fruits and vegetables. Pesticide applications to crops led to production of high-quality food in surplus quantities, thus eradicating hunger and pests. However, increased use of pesticides can be seen as a hazard to human health, environment and sustainable agriculture itself (2). Pest suppressing organisms and their metabolites produced in the laboratory can typically substitute the pesticides. In nature, antagonist metabolites are already present and are generally harmless to the mammals. In addition, these metabolites are degradable in nature. Thus, biocontrol agents portray a novel concept of pest control (3). Entomopathogenic bacteria, viruses, fungi, protozoans and nematodes can be used for insect pest control. Their major advantages include protection of humans and other animals (4) and decrease of residual pesticide content in the food (5).

On the other hand, there is an increased demand for natural polymers due to their numerous industrial applications in recent years. Besides the natural polymers produced by marine algae and plants, bacterial biopolymers have numerous valuable qualities (6). There are applications of microbial exopolysaccharides in food, pharmaceutical and other industries (7). The interest in biological activities of the polysaccharides is on rise. Their immunological activities are being assayed as antitumor, antioxidant, antiviral and immunostimulatory agents (8). *A. tumefaciens* produces an acidic succinoglycan exopolysaccharide (9). Although a number of bacteria, comprising *Rhizobium*, *Alcaligenes* and *Pseudomonas*, are capable of producing succinoglycan, only *Agrobacterium* is considered to be the ideal candidate for industrial production of succinoglycan due to high yield and quality of product (10).

Present study focused on extraction of LPS and EPS from *Agrobacterium* isolates, evaluation of LPS as potential insecticide, and assessment of UV-exposed bacterial isolates for antimicrobial activity.

Material and Methods

Sample Collection, Processing and Biochemical Characterization

Samples were collected from stem tumors found on *Robiana pseudoacacia* L. (Family Fabaceae), *Frexinus excelsior* L. (Family Oleaceae) and *Morus nigra* L. (Family Moraceae) located in campus area, University of Balochistan, Quetta, Pakistan. After washing with tap water to remove debris, gall samples were sterilized with 10% household bleach for 1-3 minutes. Sterile samples were three times washed with distilled water to remove residual bleach, chopped and incubated overnight in sterile distilled water at 28°C. Enriched samples were cultured on Potato Dextrose Agar at 28°C for 18-24 hours. Six morphologically different bacterial colonies, designated At₁, At₂, At₃, At₄, At₅ and At₆, were purified. Bacterial strains were gram stained by standard protocols (11) and a battery of biochemical tests, comprising catalase, oxidase, 3-ketolactose, lactose utilization and motility tests (table 1), was employed for characterization. Pathogenicity

test was performed on carrot discs adopting methodical technique (12). Carrots purchased from local market were sterilized with 10% household bleach. After three times washing with distilled water, carrots were sliced and placed in petri dishes. Sliced carrots were overlaid with 100 μ L bacterial inoculum. Petri dishes were sealed with parafilm and incubated at 30°C for 3 weeks. During incubation, carrot discs were regularly monitored for appearance of galls on disc surfaces. Growth curves of strains in modified RCV medium (13) were plotted. Bacterial inoculums were prepared from 24 hours old bacterial cultures with optical densities comparable to McFarland standard 0.5. 100 μ L of each bacterial suspension was inoculated in the respective RCV medium flask and incubated in shaking incubator at 28°C at 100 rpm. The optical densities were measured by spectrophotometer at 600 nm at regular time intervals, i.e., 1, 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, 48, 56, 72 and 84 hours. The optical densities were recorded to plot growth curves. All the experiments were run in triplicates and the mean absorbance values were calculated for growth curves.

Lipopolysaccharide Extraction and Insecticidal Assay

Hot phenol-water method established by Westphal and Jann (14) was employed for the extraction of lipopolysaccharides from bacterial isolates. Insecticidal assay was done by impregnated filter paper method at International Center for Chemical and Biological Sciences, H.E.J. Research Institute of Chemistry and Dr. Panjwani Center for Molecular Medicine and Drug Research, University of Karachi, Karachi, Pakistan. Test organisms (insects), *Tribolium castaneum*, *Rhyzopertha dominica* and *Callosobruchus analis*, were reared by standard techniques. Insects of roughly uniform age and sex were preferred for assay. Permethrin was used as a standard insecticide. Each test sample was prepared by dissolving 200 mg crude test sample (lipopolysaccharide in this case) in 3 ml PBS. The experiment was performed in triplicates for each strain.

Exopolysaccharide Extraction and Antibacterial Activity of Agrobacterial Isolates

After 48 hours incubation in RCV medium at 28°C, cultures were centrifuged at 12000 rpm at 4°C for 10 minutes. Pellet was discarded. Two volumes of 85% ethanol (cold) were added to the supernatant and then refrigerated to 4°C. After 12-16 hours, the supernatant was re-centrifuged at above-mentioned conditions. The supernatant was discarded and pellet of EPS was weighed after air-drying. The process was repeated for each bacterial isolate. Bacterial ability to synthesize exopolysaccharide was assessed at different pH, temperature and time intervals.

Antibacterial assay was done by agar well diffusion method. Gram positive bacteria used as test organisms included *Bacillus* and *Staphylococcus* while gram negative test bacteria comprised of *E. coli*, *Klebsiella* and *Pseudomonas*. Test organisms were cultured on LB agar at 37°C for 18-24 hours and were suspended in physiological saline. Agrobacterial isolates were grown on RCV medium at 28°C for 24 hours and suspensions were prepared in sterile distilled water. After mixing, turbidities of suspensions were standardized with McFarland standard 0.5. Using sterile cotton wool swab, lawn of each test organism was prepared. With a sterile Pasteur pipette, wells were prepared in each plate and were labeled. 10-20µl of agrobacterial suspension was loaded into the respective well and Petri plates were incubated at 37°C. After 24 hours, the plates were examined for zones of inhibition.

Antibacterial Activity of Agrobacteria after UV Exposure

For each agrobacterial isolate, a set of three flasks (intended for UV exposure) was used containing RCV broth, physiological saline and water respectively. Agrobacteria were cultured in RCV broth for 16-20 hours to standardize optical densities at 600 nm. Bacterial suspensions were then inoculated into the set of three flasks. The flasks were exposed to UV light in UV transilluminator for 2 minutes, 5 minutes and 10 minutes. After exposure, the material in each flask was immediately transferred to RCV broth flask. The flasks were immediately wrapped in aluminum foil and were incubated in dark at 28°C for 1 hour. After incubation, the RCV broth was centrifuged at 12000 rpm at 4°C

for 10 minutes. Supernatant was discarded and cell pellet was washed with sterile distilled water. Washed agrobacterial pellets were re-suspended in sterile distilled water and were used for antimicrobial assay.

Results

Isolation and Characterization of Agrobacterium Strains

Six isolates, At₁, At₂, At₃, At₄, At₅ and At₆ produced cream to white colonies, with gummy appearance and stained Gram negative. Optimum growth temperature was 28°C. All isolates were positive for catalase enzyme, 3-ketolactose production, motility and oxidase enzyme. Only At₁ was able to utilize lactose in MacConkey agar. Tumor forming ability on carrot disc surfaces was observed among strains At₁, At₂, At₃ and At₄ only. On the basis of colonial morphology and biochemical characteristics, all the six isolated strains were anticipated to be *Agrobacterium tumefaciens*. Lag phase was about 6-8 hours long. Exponential phase continued till 36 hours or 48 hours in some cases. Stationary phase extended from 56 to 72 hours, followed by a decline phase.

Insecticidal Assay

100% mortality was recorded for the standard insecticidal drug Permethrin (positive control) among all insect types whereas less than 10% mortality was documented for negative control (lacking either standard insecticide or sample). The concentration of each sample tested was 1019.10 µg/cm². While the concentration for standard insecticide Permethrin was 239.5µg/cm². Most of the samples incited approximately 80% insect mortality (table 2 and 3).

Estimation of Bacterial EPS at Various pH, Temperature and Time Intervals

Optimum pH for agrobacterial growth was 6.8. EPS yield was estimated at different pH (fig. 1), i.e., 5, 6, 7, 8, and 9. Most strains exhibited highest yield at alkaline pH. Likewise, five various temperatures, i.e., 20, 24, 28, 32 °C and 36°C were chosen for EPS

Table 1: Biochemical Characteristics of selected isolates

Biochemical Test	At ₁	At ₂	At ₃	At ₄	At ₅	At ₆
Gram's stain	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+
3-ketolactose test	+	+	+	+	+	+
Lactose utilization	+	-	-	-	-	-
Motility test	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+
Pathogenicity test	+	+	+	+	-	-

+ means present, - means absent

Table 2: Insecticidal activity by contact toxicity method for strain At₅

Name of test insect	% Mortality (Permethrin)	(Mean values) (No drug)	Sample (LPS)
<i>Tribolium castaneum</i>	100	3	78
<i>Rhyzopertha dominica</i>	100	2	85
<i>Callosobruchus analis</i>	100	1	79

Positive control: permethrin, Negative control: No drug

Table 3: Insecticidal activity by contact toxicity method for strain At₆

Name of test insect	% Mortality (Permethrin)	(Mean values) (No drug)	Sample (LPS)
<i>Tribolium castaneum</i>	100	4	81
<i>Rhyzopertha dominica</i>	100	3	72
<i>Callosobruchus analis</i>	100	3	89

Positive control: permethrin, Negative control: No drug

estimation (fig. 2). Unlike pH, effect of temperature was not profound. In most cases, an increase in yield was observed at 20°C with gradual decrease in production till 36°C. Others displayed maximum yield at 28°C with a steady decline in case of either increase or decrease in temperature.

At regular time intervals, bacterial capacity to produce EPS was examined i.e., 12, 24, 36, 48 and 60 hours (fig. 3). EPS yield increased till 48 hours followed by slight decrease after 60 hours. The trend was consistent with growth of bacteria.

Antibacterial Activity of Agrobacterial Isolates before UV Exposure

Agrobacterial strains did not show antimicrobial activity against either Gram-positive or Gram-negative test bacteria.

Antibacterial Activity of Agrobacterial Isolates after UV Exposure

After exposure to UV light intended for introducing mutations among bacteria, agrobacterial isolates were tested for antibacterial activity against Gram-positive and Gram-negative test bacteria (fig. 4-5). Since exposure media varied, i.e., RCV broth, physiological saline and distilled water, zones of inhibition were not produced by agrobacteria exposed in RCV medium. However, zones appeared with agrobacteria exposed in both physiological saline and distilled water. Furthermore, these zones of inhibition were observed against Gram-positive test bacteria only. Agrobacteria were unable to inhibit growth of Gram-negative bacteria. Moreover, in most cases, UV exposure time appeared to influence the diameter of zones.

Figure 1: Effect of pH on EPS synthesis

Figure 2: Effect of temperature on EPS synthesis

Figure 3: Changes in EPS synthesis with time

Figure 4: Inhibition of *Bacillus* by agrobacteria after 2, 5 and 10 minutes UV exposure in distilled water

Fig. 5: Inhibition of *Staphylococcus* by agrobacteria after 2, 5 and 10 minutes UV exposure in distilled water

Discussion

Biological control is all about balancing the pests at a non-damaging level through their natural enemies and it has been proved successful (15). Despite the risks associated with biological control, there is an increasing need to practice this strategy (16). Though there are several biological control agents available for pest control, yet the focus of this research was on the biological control of pests damaging stored grains. The more suitable term used for this type of control is 'microbial control'. The microbial agents used to control pests are also referred to as 'biopesticides'.

Many strains of agrobacteria do not grow on Luria-Bertani agar or any salt (NaCl) containing medium (9). Therefore, NaCl was excluded from all culture media used in the study to facilitate growth of desired strains. Muroid appearance of many microorganisms is due to EPSs (6). Morphology of the bacterial colonies isolated and studied was white to cream with a gummy appearance. Two of the strains (At₅ and At₆) were non-pathogenic, while rest of the strains (At₁, At₂, At₃ and At₄) seemed pathogenic ones.

Hot phenol water method of Westphal and Jann (14) was employed in extraction of lipopolysaccharides from bacterial cell walls (17). Both LPS and non-LPS acidic polysaccharides can be extracted by same extraction method (18). Virtually all the LPS preparations were found to be effective against the test insects, most showing 80% mortality. The results were suggestive of potential potency of agrobacterial lipopolysaccharide as a bioinsecticide.

Precipitation is an easy and fast way for isolation and purification of polysaccharides (6). In order to quantify the amount of agrobacterial EPS yielded, bacterial cells were removed from the medium by high speed centrifugation like a study conducted by Florian et al. (19). Two to three volumes of ethanol (cold) were used for precipitation of white to off-white exopolysaccharide comparable to a previous study (20). Amount of EPS produced varies with medium and culture conditions provided for microbial growth (21). In a study conducted on *Enterobacter cloacae*, no significant effect of pH was observed

on EPS production (22); however, in this study, 50% of the agrobacterial strains yielded high at alkaline pH range and about 30% strains were active in yielding EPS at acidic pH. A significant factor in polysaccharide biosynthesis is incubation temperature. Optimum temperature for EPS production depends on the organism producing EPS (23). Sometimes an inverse relation exists between bacterial growth temperature and EPS yield. Higher EPS yields were observed at lower growth temperatures in a study conducted by Zamfir and Grosu-Tudor (24). Temperature effect also varied among all the isolates in this study. A particular temperature could not be selected for EPS synthesis. However, a range of 20-30°C would be suitable for getting better EPS yield. Besides it was established through experiments that four out of six (At₁, At₂, At₅ and At₆) strains yielded highest at lowest growth temperatures selected. The reason that lies behind is cells might produce slime in order to protect them under critical culture conditions (25).

Chemical factors like medium composition can also have a profound effect on EPS yields (26). Agrobacteria produce abundant extracellular polysaccharide slime on sucrose-containing medium but no pigment production has been observed in the medium (27). In this study, RCV medium used for EPS synthesis was supplemented with sucrose as a carbohydrate source. Enough EPS production was noticed in the medium. Usually exopolysaccharide synthesis is ideal in a high carbon/nitrogen ratio in a culture medium (28). To attain the purpose, concentration of yeast extract (nitrogen source) was kept very low in RCV medium as compared to sucrose (carbon source). Phosphorus concentration in the medium may have a profound effect on EPS concentration (28). For better yield of EPS, phosphates of potassium were incorporated into the medium.

EPS yield can undoubtedly be linked to bacterial growth, presenting a direct relationship between the two (29). Likewise, when EPS yield was measured with time in recent experiments, there was gradual increase in yield till stationary phase of growth. When EPS quantities were analyzed at regular time intervals, it was evident that EPS yield gradually increased from 12 to 48 hours (log phase), followed by decrease at 60 hours. All bacterial isolates exhibited similar patterns. The results of present study somewhat

resembled to the study in which bacteria synthesized EPS within first 12 hours, then yield decreased with time till 48 or 72 hours (22). In contrast, another study presented EPS production starting at the end of exponential phase through stationary phase (30). A study involving *Enterobacter cloacae* showed 2.7 g/L EPS production (23). However, in this study, highest average yield was 12.25 g/L by strain At1, comparable to reports of *Agrobacterium* strains producing more than 10 g/L succinoglycan (31).

Agrobacterial isolates were checked for antibacterial activity against Gram-positive and Gram-negative test organisms in two distinct experiments. No zones of inhibition were located in case of agrobacteria before UV exposure. However, after exposure to UV light, zones of inhibition were recorded against Gram-positive bacteria; but the zones were detected with agrobacteria mutagenized in physiological saline and distilled water only. The reason might be lack of nutrients/elements needed for DNA repair in physiological saline and distilled water. The zones of inhibition varied with the time of UV exposure as well as with the bacterial strain and medium of exposure. In case of mutagenesis of At₁ in physiological saline, zones of inhibition of *Bacillus* contracted with exposure time. When At₁, At₂, At₃ and At₅ were mutagenized in physiological saline, diameter of zones of inhibition of *Staphylococcus* increased with time of UV exposure except for strain At₃. Whether At₄ and At₆ were mutated in physiological saline or distilled water, zones of *Bacillus* remained unaffected.

Conclusions

Effective procedures of biocontrol need thorough understanding of the ecology and biology of biopesticide as well as the targeted pest organism, whether plant pathogenic organism, weed or insect. Although agrobacteria are considered as non-pathogens, concerns may arise regarding health effects of agrobacteria towards humans. But human health outcomes due to contact with noninfectious microorganisms may hinge not only on their concentration but also on their species, dimension, viability as well as growing conditions (32). These lipopolysaccharide preparations can be helpful in grain storage

and preservation. Thus, agrobacteria can be a source of bioinsecticide, exopolysaccharide as well as antimicrobials.

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Conflict of interest

None

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