ISOLATION OF ANTIBIOTIC PRODUCING BACTERIA FROM SOIL AND DEMONSTRATING THEIR PATTERN OF ANTIBIOTIC PRODUCTION DURING BATCH CULTURE

K. Ellepola[1] and M. Gunawardane[1]

[1]*Department of Microbiology, University of Kelaniya*

INTRODUCTION

Competition, which is a negative interaction among microorganisms, arises when different microorganisms within a population or community try to acquire the same resource, whether this is a physical location or a particular limiting nutrient. 'Amensalism' is the unidirectional process based on the release of a specific compound by one organism which has a negative effect on another organism during competition. A classic example of amensalism is the production of antibiotics that can inhibit or kill a susceptible microorganism (Prescott, 2002). Antibiotics are classes of chemical compounds which are derived mainly from microorganisms (Van Epps, 2006). Based on the chemical structure of naturally produced antibiotics, similar substances are produced wholly or partly by chemical synthesis; which in low concentrations inhibit the growth of one or more other species of microorganisms (Kelmani, 1997). Soils and habitats lacking organic matter stimulate microorganisms to produce antibiotics to eliminate competitors (Waksman and Woodruff, 1940). During the current study, antibiotic producing bacteria were isolated from different soil environments. Primary screening was done using the spot test and the cross streaking method. Agar well diffusion technique was used for secondary screening to confirm the results from the primary screening. Identification of antibiotic producing bacteria and characterizing them up to the species level was performed using several morphological and biochemical tests. The growth curves of the isolates were constructed to study the relationship between the growth phase and antibiotic production. The underlying principle of antibiotic production as a result of depletion in nutrients or space when bacterial growth reaches the stationary phase was studied.

METHODOLOGY

Screening for antibiotic producing isolates

Samples from different soil environments were tested for the presence of antibiotic producing bacteria by screening soil bacterial isolates for antibiotic production using standard cultures (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Bacillus subtilis*). A dilution series was prepared using soil and each dilution was plated out using nutrient agar. After incubation, well isolated bacterial colonies were used for the spot test where they were spotted on a lawn of standard organisms spread on an agar surface using the spread plate technique. The cross streak method was also performed for primary screening where a perpendicular streak was performed using the isolate across a streak made using the standard culture. The agar well diffusion method was used for secondary screening. A solution of antibiotic containing cell free suspension was obtained by growing the isolate in nutrient

[1] Tel: +94779948450 , e-mail: kassapa.ellepola@gmail.com

broth and filtration using the Hemming's filtration technique. The antibiotic solution was poured into metal wells placed on an agar surface spread with a standard culture. The primary and secondary screenings were done in triplicate for accuracy. Antibiotic producing *Bacillus subtilis* was used as the positive control while a culture of *Escherichia coli,* which is a non-antibiotic producer, was used as the negative control during primary and secondary screening.

Identification of the isolates

Morphological studies and biochemical tests were performed to identify the antibiotic producing isolates into their genus and species levels. (Motility of the isolates, Grams' stain of the isolates, Endospore staining, Catalase test, Oxidase test, Glucose fermentation test, Oxidative/ fermentative test (Hugh and Leifson's' test), Test for anaerobic growth, Test for acid production from carbohydrates, Hydrolysis of casein and gelatin, Hydrolysis of starch, Utilization of citrate, Nitrate reduction test, Indole test, Urease test, Temperature tests, Growth in *Pseudomonas* selective media, Florescence test, Growth on MacConkey Agar, Arginine dihydrolase, Pigment production and Egg yolk reaction)

Construction of the growth curve

A small amount of inoculum from a selected isolate was transferred to 10 ml of sterilized nutrient broth in a test tube. It was incubated for 24 hours. Nutrient broth (90 mL) was prepared in a 500 mL screw capped flask and was autoclaved. Incubated 10 mL broth was aseptically transferred to the 500 mL screw capped flask containing 90 mL of nutrient broth and the added time was recorded as zero. It was shaken well and a dilution series was prepared up to 10^{-8} using 1 mL of the original sample. Each dilution (1 mL) was plated out using nutrient agar. The flask was continuously shaken at a constant rate of 250 r.p.m. At constant time periods i.e. every 30 minutes for 14 hours, 1 mL of the original broth was pipetted out and using a dilution series, pour plating was performed. The plates were incubated for 24 hours in an inverted position using the 30 ^{o}C incubator. The number of colony forming units were counted and recorded.

Determination of antibacterial activity

An overnight broth culture of a particular test organism was inoculated on to the surface of a solidified Mueller Hinton agar plate using the spread plate technique. Stainless steel autoclaved antibiotic wells (6 mm in diameter) were placed on Mueller Hinton Agar. A Cell free suspension of the antibiotic producing isolate was obtained using Hemming's filtration. Using a micropipette, 200 μL of the cell free suspension was aseptically and carefully added into the well. The plates were incubated at a face-up position for 24 hours in a 30 ^{o}C incubator.

Statistical analysis of data

Data was analyzed using the MINITAB 14 software package. Correlation between the diameter of the inhibition zone and the Log CFU/mL was tested with Pearson correlation analysis. A linear regression analysis was performed to determine the relationship between the log CFU/ml and time (min), during the log phase of the growth curves.

RESULTS AND DISCUSSION

A zone of inhibition was observed around isolates spotted on a bacterial lawn grown on nutrient agar during primary screening. The diameters of the zones of inhibition vary with the type of isolate and the type of bacterial lawn. The observation of a clear zone was recorded. (Table 1) When the tested organism is streaked over by the isolate, growth suppression in the region streaked, indicates a positive result. The growth of each isolate against the tested organisms is indicated in Table 1.

Table 1: Results of primary screening for antibiotic production (Spot test and Cross streak method) and secondary screening (Agar well diffusion method).

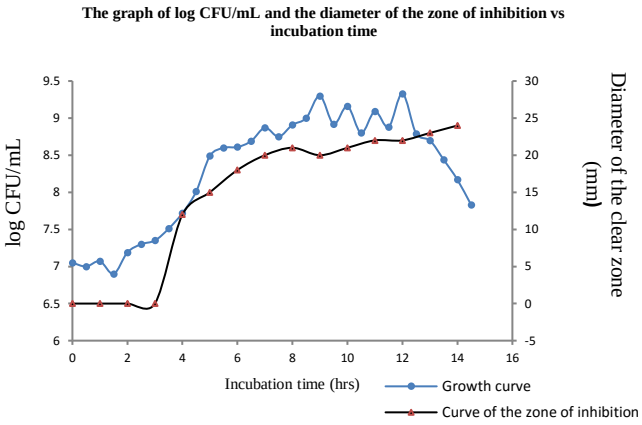
Isolate	Formation of a clear zone and the mean diameter of the clear zones (mm)			
	B.subtilis	*E.coli*	*P. aeruginosa*	*S.aureus*
$MS_2$4	-	-	-	+ (15mm)
KD1	+ (19 mm)	-	-	+ (23mm)
KD6	+ (21 mm)	-	-	+ (24mm)
Positive Control *B. subtilis*	-	+(19mm)	+(18mm)	-
Negative Control *E. coli*	-	-	-	-

\+ (Positive result) - (Negative result)

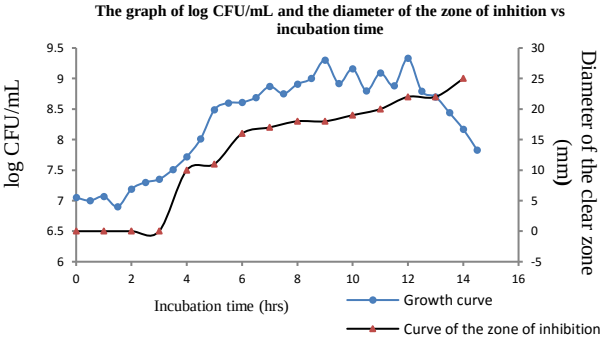
According to the results of the microscopic characteristics and the biochemical tests, the isolates were identified using the 'first stage table for Gram negative bacteria' in the Cowan and Steels' manual (Cowan, 1989), the 'second stage table for identifying Pseudomonads' in the Cowan and Steels' manual and using the Bergy's Manual of Systematic Bacteriology. $MS_2$4 isolate was identified as *Pseudomonas putida* and both KD1 and KD6 isolates were identified as *Pseudomonas aeruginosa.*

Physical parameters such as temperature, design of the shaker flask, shaking speed, volume of the broth, agitation and aeration plays a key role. Therefore during the design of the laboratory shaker flask incubator setup, a presumed set of physical parameters for the incubation of Pseudomonads were employed and the same parameters were continuously followed during repeated incubation processes.

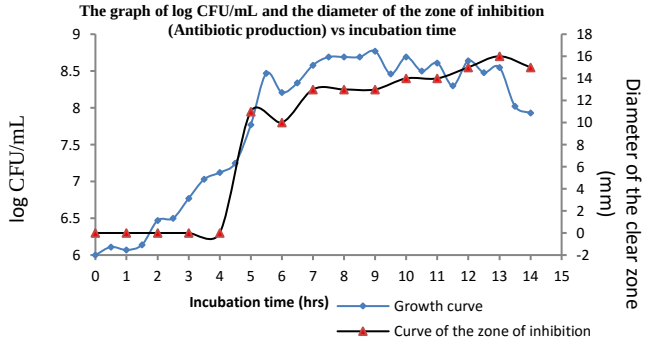
According to graph 1 and graph 2, cultures KD1 and KD6 took six hours to reach the stationary phase. The stationary phase persists for around 6hours till the death phase. The time taken for the isolate $MS_2$4 to reach each phase of growth was quite similar to that of KD1 and KD6.



Graph 1: Growth curve of KD1 and the zone of inhibition against *Staphylococcus aureus*



Graph 2: Growth curve of KD1 and the zone of inhibition against *Bacillus subtilis*



Graph 3: Growth curve of $MS_2$4 and the zone of inhibition against *Staphylococcus aureus*

Bacteria in the lawn and an isolate spotted on it will compete for nutrients and space in the nutrient agar plate. This will lead to the production of inhibitory substances that would inhibit the competitor (Atlas, 1995).

Primary and secondary screening revealed that the growth of Gram negative *Pseudomonas aeruginosa* has the ability to inhibit the growth of Gram positive *Staphylococcus aureus* and *Bacillus subtilis*. Gram negative *Pseudomonas putida* can inhibit the growth of *Staphylococcus aureus* but not *Bacillus subtilis*. This might be due to the antibiotic produced by *Pseudomonas putida* being specific only for the inhibition of *Staphylococcus aureus* or as the concentration of the antibiotic is deficient than the minimum inhibitory concentration needed to inhibit *Bacillus subtilis.*

Growth curves were constructed for all three isolates. The basic focus during the construction of the growth curve was on the time taken for the culture to reach the stationary phase which is important for the antibiotic production. The growth was measured in terms of colony forming units per milliliter (CFU/mL). The production of antibiotics is believed to take place in the late logarithmic phase or in the stationary phase. (Atlas, 1995)

The production of antibiotics was tested alongside the construction of the growth curve. Agar well diffusion technique was performed at time intervals for samples withdrawn from the shaker flask culture which were filtered using Hemming's filtration. After the initial few hours clear zones started to appear and the diameter of the clear zones kept increasing as the antibiotic production increased with time during the stationary phase. The amount of antibiotic produced did not decrease with the death phase of growth due to the accumulation of it in the broth.

Statistical analysis of data explains that there is a correlation between log CFU/mL and the diameter of the zone of inhibition for all three isolates (R-Sq = 82.3%, $P<0.05$). However, based on the assumption that the diameter of the clear zone is directly proportional to the

amount of inhibitory compound/antibiotic produced, it can be stated that there is a correlation between the growth of bacteria and antibiotic production.

CONCLUSIONS/ RECOMMENDATIONS

During the screening process, one of the notable features found was that some isolates, those that displayed strong antagonism against many other bacteria of the same habitat failed to show such strong activity against test organisms. This may be because they have evolved to gain the capacity to fight against competitors in their own habitat, but not against the limited number of laboratory strains chosen by us. Among the isolates, those belong to the Genus *Pseudomonas* were found to have the strongest antagonistic activity against test organisms. The degree of growth inhibition was found to be dependent on the isolate, test organisms, phase of the growth and changing environmental/ growth parameters. The growth curves constructed using the results of the particular isolates ($MS_2$4, KD1 and KD6) confirmed the established principle which says that the antibiotics are usually produced starting from the end of the log phase and during the stationary phase of growth.

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