

Isolation, screening and characterisation of cyanide-degrading *Serratia marcescens* strain aq07

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ABSTRACT

Biodegradation is now being considered as the best form of cyanide transformation because it is less expensive and has fewer hazards than other conventional methods. Soil and drainage water samples from the premises of Universiti Putra Malaysia (UPM) Serdang, were collected at different points and analysed for available bacteria. 28 bacteria were isolated from the samples and subjected to in-vitro cyanide remediation assay by using spectrophotometric method. Six bacterial isolates were observed to have cyanide-degrading ability. Screening was conducted in three phases that include primary, secondary and tertiary screening. In the primary phase of the screening, the six bacterial isolates were assayed for cyanide-degrading activity using 25 ppm filter-sterilised KCN in a buffer medium (pH 7.0) containing 5 g/L glucose, and 0.5% yeast extract. It was observed that three isolates have a greater potential of converting cyanide, degrading above 70%. The three isolates were further subjected to screening which is the secondary screening by incorporating 100 ppm of filter sterilised KCN. All three isolates showed good ability of degradation removing above 60% of the cyanide. The isolate tagged as AQ-007 was found to have a better degradation capability, removing 75% of the cyanide while isolate AQ-003 and AQ-004 degraded 61.5% and 64.6% respectively. The tertiary screening phase was carried out using resting cells of the bacteria. The resting cells illustrates that it is the best form of inoculums. Isolates AQ-003 removed 92.8%, AQ-004 remediated 92.9% and AQ-007 degraded 98% of the cyanide. Isolate AQ-007 was considered to be the preferred bacteria for further research because it degrades higher than the other two isolates. Bacterial identification based on 16s rRNA gene sequence analysis of this bacteria showed a 97% identity with *Serratia marcescens*. It has been registered in the GenBank as *Serratia marcescens* Strain AQ07 with accession number KP213291

Keywords: Biodegradation, Bacteria, Potassium cyanide, Resting cells.

INTRODUCTION

Cyanide has the chemical formula CN with a molecular mass of 26.007 g/mol. Potassium and sodium cyanides are alkali metal cyanides commonly used for gold recovery (Aitimbetov et al., 2005; Fatma et al., 2009). Electroplating, metal plating industries etc use cyanide in their production and as a result, cause contamination of the soil and water bodies in the environment by altering the biogeochemical cycle (Parma et al., 2012). The transformation methods for cyanide commonly used today are physical or chemical processes such as alkaline chlorination, hydrogen peroxide method, and sulphur/air oxide process, copper catalysed chemical process (Maniyam et al., 2011). These processes are considered to be more expensive than bioremediation and also generate chemical wastes that must be treated appropriately before discharging to the environment (Dash et al., 2009).

Microorganisms such as bacteria, fungi and algae have the potential of converting potassium cyanide (Barclay et al., 1998; Fatma et al., 2009). Bioremoval of cyanide has been reported to be cheaper than chemical and physical techniques and quicker than natural oxidation (Ozel et al., 2010; Dash et al., 2009). Obliteration of cyanide in wastewaters and tailing solutions by competent microorganisms has been suggested as an alternative to long-practiced chemical methods for the obliteration of cyanide. Biological processes of treating industrial effluents have been stated to have high capital expenditure but low operating expenditure. Therefore, these systems are more cost-effective than the conventional process. Biological process that could satisfy the need for removal and environmental management has now been put into practice in some countries (Dash et al., 2009). Some bacteria are able to convert cyanide as a result of the utilisation of certain pathways via the use of enzymes. These bacteria utilise the cyanidase (cyanide

dihydratase) enzyme in hydrolytic reaction to remove cyanide (Stephen, 2004) while in oxidative reactions, they utilise cyanide monooxygenase or cyanide dioxygenase to remediate it (David et al., 2006).

This study is significant because six bacterial isolates were obtained having cyanide degrading ability from a non-toxic soil environment. It signifies that cyanide degradation is an inherent character of some bacteria and that no prior introduction or adaptation is necessary to provoke this property (Patil and Parknikar, 2000). This indicates that despite the toxicity of cyanide, bacteria isolated from non-hazardous environment can be utilised to remediate it. Three phases of screening were carried out to isolate the best bacteria for further studies. It includes Primary, secondary and tertiary screening processes.

MATERIALS AND METHODS

Chemicals: Commercial grade chemicals that are used in this research were obtained from various chemical agents. Potassium cyanide was obtained from R&M Chemicals; Essex, U.K. 4-methyl pyridine (γ -Picoline) was obtained from ACROS Organics. Chloramine T sodium salt was obtained from Fisher Scientific, U.K. Barbituric acid was obtained from Sigma Aldrich CO. Phosphate buffer, nutrient agar and bacto-agar media were obtained from Merck KGaA, Germany.

Isolation of cyanide-degrading microorganisms: Soil and wastewater samples were obtained from the premises of Universiti Putra Malaysia (UPM) Serdang, Selangor. The samples were placed in a sterilised nutrient broth medium for 24 hours; 1 g or 1 ml of sample in 100 ml nutrient broth medium (Mirzadeh et al., 2014). 1 ml of nutrient broth inoculated sample was taken and inoculated on bacto-agar medium containing 25 mg/L filter sterilised potassium cyanide (KCN) and was incubated in an incubator at a temperature of 30°C. The plates were observed after 48 hours and all bacteria that were able to grow on the medium were selected for further studies. The individual colonies of the bacteria were then sub-cultured on bacto-agar containing 25 mg/L filter sterilised KCN for another 48 hrs and pure cultures were obtained using four way streaking method.

Enrichment medium was prepared containing: NaHPO₄ – 4.0 g, Na₂SO₄ – 2.13 g, K₂HPO₄ – 3.1 g, MgCl₂.6H₂O – 200 mg, - FeCl₃.6H₂O – 2.0 mg, and CaCl₂ – 1.0 mg in 1 L of distilled water and pH was adjusted to 7.2 (Potivichayanon and Kitleartpornpairat, 2010). The medium was autoclaved for 15 minutes at 121°C before use. 10 ml of each sample were inoculated into 100 ml of the enrichment medium in a 500 ml Erlenmeyer flask and incubated on a rotary shaker at 28 – 30°C for 48 hours. When adequate growth of the organisms has been obtained, in order to screen the organisms for cyanide degradation, 10 ml of each sample from the enrichment medium were transferred into 500 ml Erlenmeyer flask containing 100 ml of buffer medium containing 25 mg/L filter sterilised KCN. The buffer medium is composed of KH₂PO₄ – 7.2 g, K₂HPO₄ – 3.5 g, 10 ml of trace salts (FeSO₄.7H₂O – 300 mg/L, MgCl₂.6H₂O – 180 mg/L, Co(NO₃)₂.6H₂O – 130 mg/L, CaCl₂ – 40 mg/L, ZnSO₄ – 40 mg/L, MoO₃-20mg/L) and 0.5 g yeast extract in 1 L of distilled water. The pH is adjusted to 7.0. It was autoclaved at 121°C for 15 minutes (Potivichayanon and Kitleartpornpairat, 2010). Sterilised glucose – 5 g/L that has been autoclaved separately from the medium to avoid caramelisation was added to the medium and 25 mg/L filter sterilised KCN was added. It was incubated on a rotary shaker at 150 rpm, 28 – 30°C for 48 hours. Cyanide biodegradation assay was conducted to ascertain the capability of degrading potassium cyanide by each bacterium.

Analytical method: Modified spectrophotometric determination of cyanide with γ - picoline and barbituric acid was used for the cyanide assay as described by Shigeru (1977). The modification is based on the quantity of sample utilized. 500 μ l of diluted sample was transferred into a sterilised 1.5 ml centrifuge tube. 250 μ l of pH 5.2 buffer solution was added to the tube and 13 μ l of 1% (w/v) chloramin T sodium salt solution was added. The tube was stoppered and stirred gently. It was then incubated at room temperature for 1-2 minutes. 150 μ l of γ - picoline - barbituric acid reagent was added and mixed. The mixture was allowed to stand at 25°C for 5 minutes. Absorbance was measured at 605 nm using Shimadzu U.V. Mini 1240, spectrophotometer against reagent blank. The test was measured against an established linear standard curve ranging from 0.1 to 1 mg/L KCN. Optical density 600 nm was used for measurement of bacterial growth.

Preparation of resting cells: This was carried out by the modified method of Maniyam et al., (2011). The modification was L- proline was not used in the medium. Broth medium consisting of 8 g/L nutrient broth and 8 g/L glucose was prepared. It was autoclaved for 15 minutes at 121°C. The medium was allowed to cool at room temperature, and then 2% (v/v) of 24 hours pre-cultured bacteria were added. Aeration was provided via BB-800 aquarium air pump fitted with chrome tech MCE 0.45 μ m syringe filter. It was incubated at room temperature for 48 hours. The bacterial cells were harvested at early stationary phase by centrifuging at 10,000 x g, temperature 4°C for 10 minutes using Beckman Coulter Avanti J-26 XPI centrifuge. The harvested cells were then washed twice using 100 mM phosphate buffer and then re-suspended in the same 100 mM phosphate buffer solution. The optical density 600 nm was adjusted to the range of 0.9 to 1.0. It was stored at 8°C for further studies.

Screening of cyanide-degrading bacteria:

i. Primary screening: Six selected bacterial isolates out of the 28 previously isolated were observed to have cyanide degradation capability. The bacteria were temporarily tagged as isolate AQ-001, AQ-002, AQ-003, AQ-004, AQ-007, and AQ-020. They were screened to ascertain the favorable cyanide degrading bacteria. The isolates cultured in a nutrient broth medium were standardized to approximately 1.0 using O.D_{600nm}. 10% of the medium was centrifuged and inoculated in a 50ml buffer medium containing 25 ppm filter sterilized KCN. The buffer medium was contained in a 250 ml screw cap schott bottle and incubated in a Protech orbital shaker for at 150 rpm for 96 hours. Cyanide degradation was tested using the method described above.

ii. Secondary screening: Three isolates namely, AQ-003, AQ-004 and AQ-007 were observed to have better cyanide degradation capability in the primary screening. The same method of screening was applied as in primary screening using 100 ppm filter sterilised potassium cyanide (KCN). The buffer medium was incubated on a Protech orbital shaker at 150 rpm for 120 hours. Cyanide degradation was tested using the method mentioned above.

iii. Tertiary screening: The three selected isolates were subjected to further screening, this time using 10% (v/v) seed culture of resting cells of the bacteria. All culture conditions remained the same. It was periodically observed for a period of 72 hours.

Identification of selected bacteria: The selected bacteria for further studies, which is isolate AQ-007 was identified using 16s rDNA gene sequencing analysis. The genome was amplified using PCR universal primers; 27F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R: 5'-TAC GGT TAC CTT GTT ACG ACT T-3', which corresponds to the forward and reverse primers of 16s rRNA, respectively. The polymerase chain reaction (PCR) was carried out under the following conditions: 1 cycle of initial denaturation at 96°C for 4 minutes; 30 cycles (94°C denaturing for 1 min, 52.3°C annealing for 1 min, and 72°C extension for 1 min) and 1 cycle of final extension at 72°C for 7 minutes. Preservation was done at 10°C.

Phylogenetic tree analysis and evolutionary relationship of taxa: Twenty one (21) 16s rDNA sequences of related *Serratia* species were obtained from gene bank and aligned using Clustal W by means of Mega 6.06.

RESULTS AND DISCUSSION

Isolation of microorganisms: Twenty soil and water samples were collected at various points within UPM Selangor. The soil and water samples were obtained from different points that are not contaminated with any chemical substance such as ponds, drainages and surface soil. Most reports indicated that cyanide-degrading bacteria were found in cyanide-laden environment or environments that are contaminated with petroleum/chemical products or cassava starch industries (Maniyam, 2011; Potivichayanon and Kitleartpornpaioat, 2010; Kao, 2003). This research proves contrary to the previous reports, because cyanide-degrading bacteria were isolated from non-hazardous environments. Although the bacteria were unable to grow in medium that contains potassium cyanide as the only source of carbon and nitrogen, it is in tandem with the reports by Adjei and Ohta (1999). This could be due to the inability of bacteria to adequately synthesise the enzyme that could degrade the cyanide. It needs carbon source and growth enhancers that will provide adequate energy, such as glucose and yeast extract to be able to metabolise the highly toxic KCN (Dhillon and Shivaraman, 1999).

The samples were first placed in nutrient broth for all available bacteria in the sample to proliferate. Bacter-agar containing 25 mg/L KCN was used in order to screen any bacteria that can stand the toxicity of cyanide. 28 isolate were isolated using the process of subculture on the same type of bacter-agar to obtain pure colonies of bacteria. Each bacterium was then transferred into an enrichment medium (Potivichayanon and Kitleartpornpaioat, 2010). In order to screen bacteria that are able to degrade cyanide, buffer medium containing 25 mg/L potassium cyanide was used. All bacteria were cultured separately in triplicates and incubated on a rotary shaker at 150 rpm for 48 hours. Cyanide degradation assay was conducted using γ -picoline-barbituric acid method (Shigeru, 1977). Out of 28 isolates screened, six were found to have the ability of degrading cyanide. Interestingly, this indicated that not only bacteria that are isolated from cyanide laden environment or chemical contaminated environment can poses the ability to degrade cyanide. Furthermore, this result demonstrated that different species of bacteria can proliferate on medium containing cyanide but may not necessarily have the ability to degrade it. The fact that 28 bacterial isolates illustrates good growth ability on medium containing cyanide, but only six representing 21.4% were found to have biodegradation ability.

Cyanide biodegradation: Biodegradation of cyanide is confirmed by reduction of cyanide in the medium due to the action of microorganisms. Although depletion occurs in the control sample, it has been reported that cyanide depletes as the result of air stripping or volatilisation especially if the pH of the medium is within neutral level (Fatma et al.,

2009; Patil and Paknikar, 2000). However, degradation due to biotic activity was proved in the research to indicate high cyanide degradation within specified period of time compared to the control that has small percentage depleted. Table 1 illustrates the degradation ability of the screened bacteria.

Table.1. Degradation capabilities of screened cyanide degrading bacteria

Screening type	Cyanide concentration (ppm)	Cyanide degradation (%)						
		Isolate AQ-001	Isolate AQ-002	Isolate AQ-003	Isolate AQ-004	Isolate AQ-007	Isolate AQ-020	Control
Primary	25	30.5	40.8	76	100	100	69.6	4
Secondary	100	-	-	61.5	64.6	72.7	-	12.3
Tertiary	100	-	-	92.9	92.8	98	-	13.1

Screening of cyanide-degrading bacteria: The screening was carried out in three phases consisting of the primary, secondary and tertiary phases. This is to enable adequate screening of the best bacteria for cyanide bioremediation. The primary phase was conducted on the six bacteria identified to have the ability to degrade cyanide. Isolate AQ-007 and AQ-004 demonstrated ability to degrade 100% of 25 ppm KCN while showing good bacterial growth. Isolate AQ-003 and AQ-020 showed good degrading ability of 76 and 69.6% respectively. Isolate AQ-001 and AQ-002 only degraded less than 41% of the cyanide. Although the growth rate of all of the isolates was almost equal, some demonstrated better degrading ability than the others. About 4% of cyanide was depleted in the control as a result of abiotic activity; nevertheless, this shows that significant percentage of the degradation process is as a result of the action of bacteria. Isolates AQ-003, AQ-004 and AQ-007 were selected for further screening to ascertain the best cyanide degrading bacteria (Figure 1).

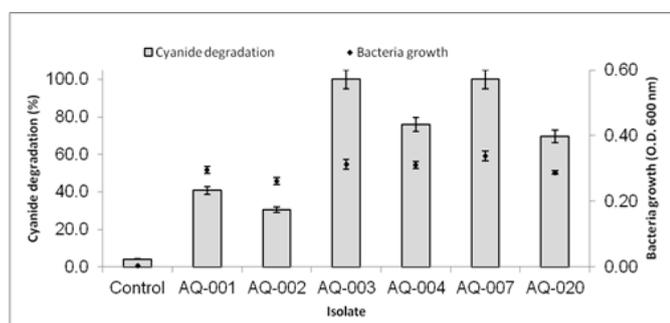


Figure.1. Primary screening using 25 ppm KCN by six different strains of bacteria for 96 hours. Data represent mean \pm STDEV, N = 3.

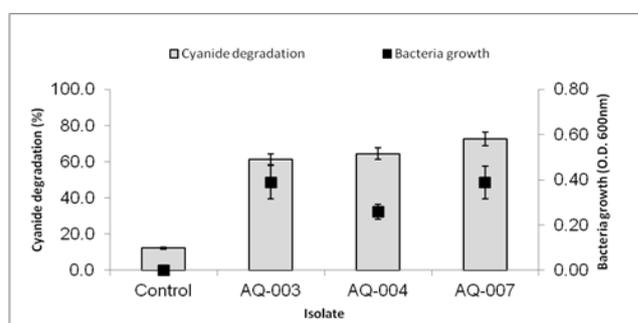


Figure.2. Secondary screening using 100 ppm KCN by three different bacterial strains for 120 hours. Data represent mean \pm STDEV, N = 3.

The secondary phase was conducted on the three bacterial isolates, eliminating three others that have less cyanide degradation ability. In this phase, 100 ppm KCN was used. All other conditions were similar with the primary phase. Degradation was observed for a period of 120 hours. Isolate AQ-007 illustrates high biodegrading ability by degrading up to 72.7% of 100 ppm cyanide. The other two isolates degraded 64.6 and 61.5%, respectively. Degradation due to abiotic activity stands at 12.3% which is higher than that of primary screening probably because of the difference in concentration and duration of incubation. This screening demonstrates the ability of the bacteria to degrade considerable percentage of 100 ppm KCN (Figure 2).

In the tertiary screening phase, resting cells of the three bacteria were used, using the same culture conditions as described above. The three isolates showed sufficient ability for cyanide degradation. Although Isolate AQ-007 degraded up to 98% of the 100 ppm KCN, Isolate AQ-003 and AQ-004 have good potential of cyanide degradation because they remove 92.8 and 92.9% respectively. The cyanide loss in this case stands at 13.1%, which is at the same level with secondary screening control sample, but differs with the primary screening. This could be as a result of the difference in potassium cyanide concentration (Figure 3). Consequently, the reduction in the degradation time from 120 to 72 hours illustrates that resting cells have a greater ability of activity than the previous inoculums used in the primary and secondary screening. The rationale might be in the preparation of the resting cells, where cells were harvested at early stationary phase after 48 hours of incubation, and are considered to have greater degrading ability than cells cultivated at exponential phase since their ability to withstand environmental stress at that phase could be higher compared to those harvested at exponential phase of growth (Adjei and Ohta, 1999). In this study, adaptation to cyanide was not determined, which has been reported (Maegala, 2013) in which successive culturing was conducted for more than four weeks to adapt the bacteria to cyanide conditions before good cyanide biodegradation ability was obtained by the bacteria.

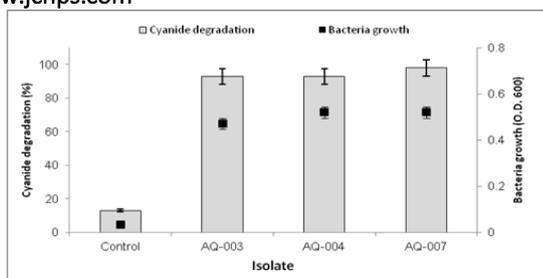


Figure.3. Tertiary screening using 100ppm KCN and resting cells of three different bacterial strains for 72 hours. Data represent mean \pm STDEV, N = 3.

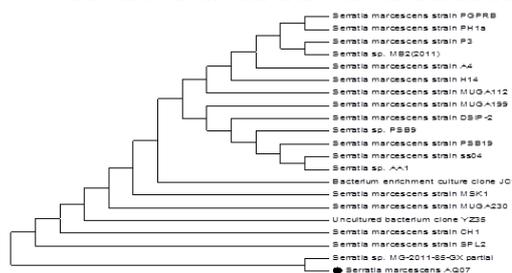


Figure.4. The cladogram was conducted based on neighbor joining method indicating the phylogenetic affiliation between strain AQ07 and other interrelated comparable bacteria based on 16s rDNA gene sequence study. The names of the species are tracked by the accession numbers of their 16S rDNA sequences.

Based on the results obtained, Isolate AQ007 was selected for identification using 16s rDNA sequence. The resultant 1319 nucleotides sequence were compared using with Gen Bank data base using NCBI BLAST (Table 2). 16s rRNA gene sequencing indicated that it has 97% identity with *Serratia marcescens*. The 16s rRNA gene sequence was deposited in the Gen Bank as *Serratia marcescens* strain AQ07 and it was assigned accession number KP213291.

Neighbour joining method was used to infer the evolutionary history (Saitau and Nei, 1987). The optimal tree, which has a total branch length of 14.64096229 is revealed (Figure 4). Maximum composite likelihood method was used to compute the evolutionary distance (Tamura et al., 2004) and is in the unit of number of base substitutions per site. 21 nucleotide sequences are involved in the analysis. Codon positions incorporated were 1st+2nd+3rd+Noncoding. All points that have gaps and lost data were disposed. The final data set contains 1258 positions. Evolutionary analyses were carried out using MEGA6.06 (Tamura, 2013).

Table.2. First ten sequences generating significant alignment with *Serratia marcescens* strain AQ07 from NCBI blast

Accession Number	Max Score	Total Score	Query Cover	E value	Identity
KJ672369.1	2,193	2,193	98%	0	97%
KJ631129.1	2,193	2,193	98%	0	97%
KJ082095.1	2,193	2,193	98%	0	97%
AP013063.1	2,193	15,307	98%	0	97%
KF647218.1	2,193	2,193	98%	0	97%
NR102509.1	2,193	2,193	98%	0	97%
CP003959.1	2,193	15,244	98%	0	97%
KC007128.1	2,193	2,193	98%	0	97%
JQ954966.1	2,193	2,193	98%	0	97%
AB698033.1	2,193	2,193	98%	0	97%

CONCLUSION

The bacteria were isolated from soil samples sampled within UPM Serdang premises in Malaysia, which is a completely nontoxic environment. This contradicts some reports that says due to the toxicity of cyanide, bacteria intended for its remediation should be isolated from cyanide laden environment or other toxic sites such as gold mine, electroplating, metal plating companies environments etc because the environment tends to be polluted with chemicals used for their activity. Hence, the microorganisms around the environment are seen to adapt the hazardous environmental condition. This research has proved that even in non-cyanide contaminated environment, cyanide-degrading bacteria can still be isolated. In this study *Serratia marcescens* strain AQ07 was selected for further research because of its higher ability to degrade potassium cyanide compared to the other strains of the bacteria.

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