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Isolation and Characterisation of Biosurfactant-producing *Pseudomonas* specie from Soil

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Abstract

Bacteria, especially members of the genera Bacillus and pseudomonads express surface-active compounds that are useful in biotechnology. Studies have shown that biosurfactant-expressing strains are rapidly isolated from both soil and water environments that are either contaminated or uncontaminated. The aim of this research is to isolate a large collection of surfactants expressing pseudomonads and to screen and characterised them for biosurfactant production. In this study, bacterial strains were isolated from Dundee Botanic Garden (United Kingdom) soil using pseudomonas selection agar supplemented with centrimide, fusidin and cephaloridine media (PSA+CFC) that select only pseudomonads. The isolates where screened for liquid surface tension reducing ability (LSTRA) using the drop-collapse assay before characterising the key strains using different metabolic and growth-based assays including their antibiogram. At least 30 key strains were identified from a collection of 58 isolated strains and further studied for diversity. A total of 27 assays were conducted to ascertain the phenotype of the 30 keys strains. All the 30 strains (100%) tested positive for catalase and glucose utilisation, while 28 (93%) tested positive for oxidase and KB broth culture acidity. Also 22 (73%), 26 (87%) and 18 (60%) were found to be positive for swarming, swimming and twitching motilities respectively, while 22 (73%) were positive for lipase, 26 (87%) for protease and 27 (90%) for gelatinase. Furthermore, 12 (40%), 2 (7%), and 9 (30%) were resistance to mercury, kanamycin and to nalidixic acid respectively. Hierarchical cluster analysis of phenotypic characterisation data confirmed that these strains were a diverse group of pseudomonads.*

INTRODUCTION

Bacteria play a significant role in the soil ecosystem by participating in nutrient cycles and microbial interactions (Agaras *et al.*, 2015). Some species help in promoting plant growth/health,while others cause diseases (Raaijmakers *et al.*, 2009). Amongst the beneficial bacteria, *Pseudomonas* spp. or pseudomonads are identified as plant probiotic due to their active root-colonising behaviour and production of compounds that stimulate plant growth/protection(Mercado-Blanco and Bakker, 2007). Examples of non-pathogenic species foundon the ground include *P. chlororaphis*, *P. fluorescens*, *P. putida* and *P. stutzeri*(Haas and Défago, 2005) and the plant pathogens *P. cicchorii*, *P. savastanoi* and *P. syringae*(Peix *et al.*, 2009). Moreover, *Pseudomonas* spp. have been found to produce compounds that aid in phosphate solubilization, phytohormone production and induced systemic resistance and production of antibiotics (Preston, 2004). This behaviour in particular makes pseudomonads attractive microorganisms for research (Walsh *et al.*, 2001).

The genus *Pseudomonas* is characterised by its intrinsic genetic and physiological diversity. They are Gram-negative, motile and oxidase-positive organism found in the air, soil and water (Peix *et al.*, 2009). Isolation and identification of bacteria belonging to the genus *Pseudomonas* can be difficult because of its intrinsic antibiotic resistance, and therefore can require antibiotic-based selective media that will allow the selection of only *Pseudomonas* spp. (Gould *et al.*, 1985). *Pseudomonas* selective agar supplemented with cetrimide, fusidin and cephaloridine is recommended for isolating *Pseudomonas* spp. from soil and other environments (Mead and Adams, 1977). This media makes it easier to select *Pseudomonas* spp. with high precision; however, grouping strains to species-level may be a challenge.

Over the years, different techniques such as growth-phenotypic assay, metabolic assay, biochemical test or 16S rDNA sequencing have been employed in differentiating and identifying bacteria belonging to pseudomonads (Andersen *et al.*, 2000).

Growth-based phenotypic assays are typically required to differentiate organisms using their nutritional requirement, for instance growth on different carbon and nitrogen sources (Sandman and Ecker, 2014). This method is cheap and does not require expensive technology (Bochner., 2009). The biochemical test also helps reveal an organism's activities, including the expression of enzymes useful for their activities (Bochner, 2009).

Bacillus and pseudomonads produce surface-active compounds called biosurfactants that help them in carbon-intake and enhanced biofilm-attachment (Raaijmakers *et al.*, 2010; Ron and Rosenberg, 2001). Other roles include heavy metal binding, quorum sensing and antimicrobial activity (Davey *et al.*, 2003, Hamouda *et al.*, 2001, Mulligan *et al.*, 2001). In the late 1960s, biosurfactants were identified as hydrocarbon-dissolving agents with the potentiality for replacing synthetic biosurfactants (sulfonates, carboxylates and esters), especially within the food and pharmaceutical industries. Synthetic biosurfactants have a toxic effect and leave behind a high residual effect that may lead to loss in biodiversity (Sáenz-Marta *et al.*, 2015). However, isolating biosurfactant expressing bacteria requires a survey of a large collection of isolates that are screen based on qualitative test and further subjected to quantitative surface activity measurement. In this research, Dundee BotanicGarden soil was used to recover and characterised *Pseudomonas* spp. capable of expressing biosurfactant.

MATERIALS AND METHODS

Sample collection

Site description:

The Dundee Botanic Garden is located at the geographical coordinates 56°27'21.7" N 3°01'09.0"W. The garden belongs to the University of Dundee and was established in 1970. It has two large glass houses for tropical and warm temperateplants, and its outside areas containan extensivecollection of plants from all around the world.

Sampling and isolation of bacteria

Soil samples were obtained during five visits to the garden and collected close to the following trees: Cornelian Cherry (*Cornus mas*), Hance (*Hemiptelea davidii*), Moroccan Cypress (*Cupressus atlantica*),Scots Pine (*Pinus sylvestris*)and White Willow (*Salix alba*), as shown in Figure1.The soil in the rhizosphere zone was obtained using grid sampling technique (grid sampling could be useful for future work where more samples are needed for further analysis).

In order to isolate *Pseudomonas* spp. from soil samples, serial dilutions were performed and an aliquots were inoculated on *Pseudomonas*selection agar (PSA+CFC) plates using the spread-plate method. At least 7 to 8 colonies were carefully selected from each plate to minimise possible biological replicates and were re-streaked on PSA+CFC. A total of 251 bacterial strains were isolated and screened for biosurfactant expression using the drop-collapse assay.

Figure 1 Soil sampling sites. Soil samples were collected from Dundee Botanic Garden for the isolation and identification of biosurfactant-producing pseudomonads. Figures **A** and **B** show the sample collection points.

Screening bacterial strains for biosurfactant expression

Previous studies have shown the use of different techniques including haemolysis test, drop-collapse assay and oil spray in the screening and identification of biosurfactant-expressing bacteria (Walter *et al.*, 2010). In the course of this research, the drop-collapse assay was used to identify strains based on the expression of biosurfactants.

To assay strains, aliquots of 18 overnight KB* (a modified form of KB media)broth cultures were placed onto the surface of a clean Petri dish. A positive result was recorded if the droplet lost its bead-like shape and spread or collapsed,

while negative was registered if the drop retained its bead-like shape (Figure **2**).

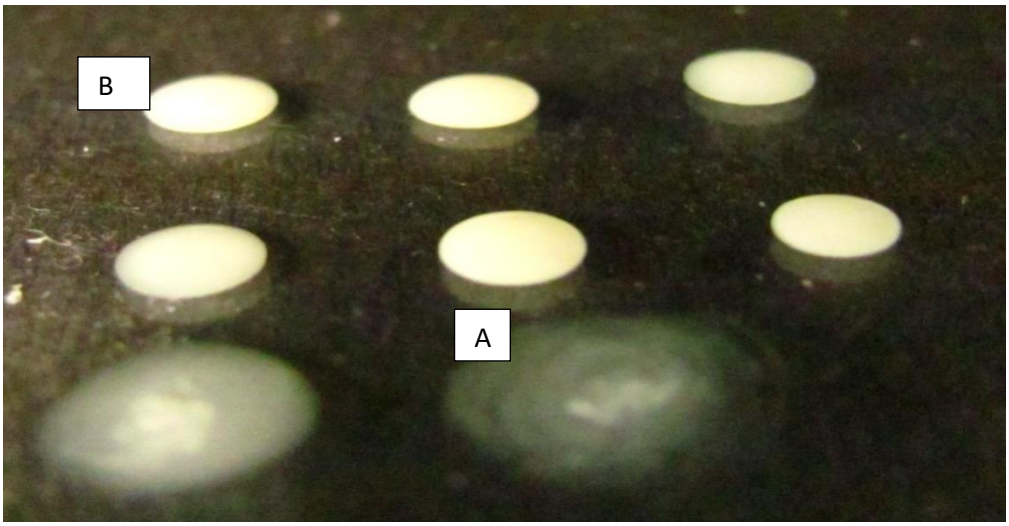

Figure 2 Screening strains for biosurfactant expression using the drop-collapse assay. Dundee Botanic Garden strains were screened using the drop-collapse assay. Aliquots of KB* broth culture were dropped onto a clean Petri dish plate and observed after 10 s. (**A**)A positive result was confirmed if the drop lost its shape and (**B**)negative if it remained static.

Characterisation of key strains

Observable properties of bacteria such as morphology, growth, appearance and biochemical characteristics are relevant parameters that can be used to differentiate between species. Since the publication of Bergey's manual in 1923, microbiologists have been using growth-based techniques to differentiate bacteria based upon their carbon, nitrogen, sulphur and other growth requirements (Bochner, 2009). Other bacterial activities including enzyme secretion and antibiotic and heavy metal resistancesare employed in characterising and differentiating strains.

In this research, a series of behaviour and growth-based assays used for pseudomonads after Robertson *et al*. (2013) were employed to characterise the 30 strains. Growth-based assays undertaken includes swimming, swarming and twitching motilities, sugar utilisation and a siderophore test. An expression such as lipase and protease secretion, the presence of catalase, gelatinase and oxidase enzymes where also tested. Resistance to antibiotic and heavy metals was tested using an antibiotic disc, kanamycin, mercury chloride and tetracycline, while the tolerance activity was tested by KB* broth culture for acidity, salt and temperature tolerences.

RESULTS

Out of the 251 strains that were tested using the drop collapsetechnique, 58 were found to be positive for biosurfactant expression (Table 1).Atleast 25 surfactant expressing strains and 5 non-surfactant expressing strains (controls) were randomly selected and further characterised using different metabolic and growth based assays and the strains are hereafter reffered to as the key strains.

Table 1: Drop-collapse positive strains recovered from Dundee Botanic Garden soil

Visit	No. of samples collected	No. of strains isolated	No. of drop-collapse positive strains
1	8	59	15
2	8	65	15
3	8	34	8
4	8	55	5
5	8	38	15
TOTAL	40	251	58

Phenotypic characteristics of key strains

A total of 27 assays were undertaken to ascertain the phenotype of the 30 keys strains. All the 30 strains (100%) tested positive for catalase and glucose utilisation, while 28 (93%) tested positive for oxidase and KB* broth culture acidity. Also 22 (73%), 26 (87%) and 18 (60%) were found to be positive for swarming, swimming and twitching motilities respectively, while 22 (73%) were positivefor lipase, 26 (87%) for protease and 27 (90%) for gelatinase. Further, 12 (40%) were resistance to mercury, 2 (7%) to kanamycin and 9 (30%) to nalidixic acid. None of the strains grew on tetracycline plates (Table 2).

Table 2: Phenotypic and biochemical tests results

Test	% positive results
Oxiadase	93
Catalase	100
Glucose Utilisation	100
Swarming	60
Swimming	60
Twitching	60
Lipase	73
Protease	87
Gelatinase	90
Marcury	40
Kanamycine	7
Nalidixic acid	30
Tetracycline	0

In order to explore diversity amongst the key strains, phenotypic data were examined for similarity using Hierarchical Clustering Analysis and visualised with a constellation dendrogram, as shown in Figure **3**. The result indicated a significant difference between the key strains with a very low level of biological replication. It was observed that the constellation tree differentiated the key strains into two major groups, with each cluster having sub-groups. Isolatessimilar to each another were placed on nearby branches, thereby indicating a significant diversity amongst the key strains (p <0.05).

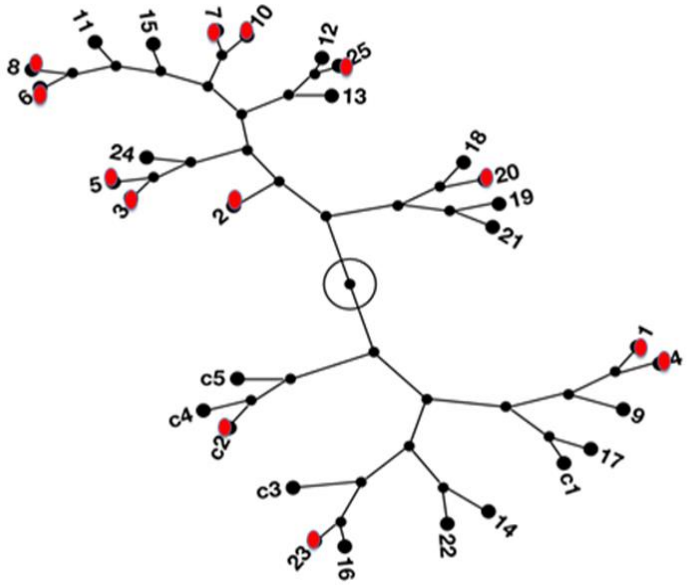

Figure 3: Key strains were a diverse collection of pseudomonads

The 30 key strains were assessed for similarity using Hierarchical Clustering Analysis (HCA) of the phenotypic data. Shown above is a constellation dendrogram in which the 30 key strains (1-25, c1-c5) show considerable diversity within the collection. The constellation is automatically rooted halfway along the longest branch (circled). API 20e identification of selected strains confirmed that these were pseudomonads (red circles).

DISCUSSION

Soil represents a conducive zone for microbial proliferation (Kumar *et al.*, 2012). Isolation and screening of pseudomonads for biosurfactant expression isrelatively straightforward (Desai and Banat, 1997). However, their identification can present challenges (Yamamoto and Harayama, 1995). In this research, a total of 251 pseudomonads were isolated from Dundee Botanic Garden soil. Strains were first screened for biosurfactant expression which lead to identifying key strains for further investigation using different phenotypic and biochemical assays.

The results showed that the 30 key strains exhibited phenotypic characteristics similar to those of genus pseudomonads. Moreover, further characterisation of selected strains using the API 20e kit and 16S rDNA gene is required to confirmed strains were pseudomonads. This corresponds to a study by Yamamoto *et al.*(2000) that indicates that *Pseudomonas* spp. identification could not be resolved using 16S rDNA sequencing alone. Similarly, a review by Janda and Abbott, (2002), shows that all methods used to classify bacteria to species level have limitations because no single method can provide results that are 100% reliable. However, this research concluded that based on phenotypic characterisation and other enzymatic expression results, that the 30 key strains were members of the genus pseudomonads.

Although phenotypic characterisation was conducted to identify strains as pseudomonads, information obtained from the assays mainly enzymatic activities provided useful information in prospecting for novel applications in biotechnology. In addition, it is noteworthy that over 80% of the key strains were positive for catalase, lipase and protease secretion. These essential qualities are useful in different biotechnological industries (Adrio and Demain, 2014); for instance, lipase secretion has been helpful in the synthesis of biopolymers and biodiesel, enantiopure pharmaceuticals, flavouring compounds and agrochemicals (Jaeger and Eggert, 2002). Protease is useful in industries such as agrochemicals, leather and pharmaceuticals, which comprise over 60% of the global market and 25% of total worldwide enzyme sale (Adrio and Demain, 2014). Catalase finds relevance in a range of different bioprocesses and chemical industries including personal care, pulp and paper (Kirk *et al.*, 2002).

Hierarchical Cluster Analysis of the 30 key strains using the phenotype data indicated strains were significantly different to one another. The phenotypic behaviour exhibited confirmed strains were a diverse collection of pseudomonads, and is in agreement with findings by Campbell *et al.* (1995) that showed variation amongst a group of *Pseudomonas* isolated from soil. However, although variationwas observed amongst phenotypic data, a study by Smits *et al.* (2006) shows that properties may vary and expression may depend on environmental conditions such as temperature, pH, and the nature of the substrate used.

CONCLUSION

In this research, a collection of 251 strains of bacteria were isolated from the Dundee BotanicGarden, which were screened for biosurfactant expression using a drop-collapse assay, with 58 strains found to express biosurfactants. Moreover, of the 58 strains, 30 key strains (25 plus five non-biosurfactant expressing strains (controls)) were selected and characterised phenotypically. The phenotypic characterisation by plate-based growth and enzymatic assays confirmed that these strains were a diverse group of pseudomonads.

REFERENCES

Adrio J. L. and Demain A. L. (2014). Microbial enzymes: Tools for biotechnological processes. *Biomolecules* 4, 117-139.

Agaras B. C., Scandiani M., Luque A., Fernández L., Farina F., Carmona M., Gally M. and Valverde C. (2015). Quantification of the potential biocontrol and direct plant growth promotion abilities based on multiple biological traits distinguish different groups of pseudomonas spp. isolates. *Biological Control* 90, 173-186.

Andersen S. M., Johnsen K., Sørensen J., Nielsen P. andJacobsen C. S. (2000). Pseudomonas frederiksbergensis sp. nov., isolated from soil at a coal gasification site. *International Journal of Syst Evol Microbiology*50, 1957-1964.

Bochner B. R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbioliogy Rev* 33, 191-205.

Bossis E., Lemanceau P., Latour X. andGardan L. (2000). The taxonomy of pseudomonas fluorescens and pseudomonas putida: Current status and need for revision. *Agronomie* 20, 51-63.

Campbell J. I., Jacobsen C. S. andSørensen J. (1995). Species variation and plasmid incidence among fluorescent pseudomonas strains isolated from agricultural and industrial soils. *FEMS Microbiol Ecol* 18, 51-62.

Davey M. E., Caiazza N. C. andO'Toole G. A. (2003). Rhamnolipid surfactant production affects biofilm architecture in pseudomonas aeruginosa PAO1. *J Bacteriol* 185, 1027-1036.

Desai J. D. and Banat I. M. (1997). Microbial production of surfactants and their commercial potential. *Microbiol Mol Biol Rev* 61, 47-64.

Fiechter A. (1992). Biosurfactants: Moving towards industrial application. *Trends in Food Science Technology*3, 286-293.

Georgiou G., Lin S. andSharma M. M. (1992). Surface-active compounds from microorganisms. *Bio/Technology* 10, 60-65.

Haas D. and Défago G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology* 3, 307-319.

Hamouda T., Myc A., Donovan B., Shih A. Y., Reuter J. D. and Baker J. R. (2001). A novel surfactant nanoemulsion with a unique non-irritant topical antimicrobial activity against bacteria, enveloped viruses and fungi. *Microbiol Res* 156, 1-7.

Janda J. M. and Abbott S. L. (2002). Bacterial identification for publication: When is enough enough? *J Clin Microbiol* 40, 1887-1891.

Kirk O., Borchert T. V. and Fuglsang C. C. (2002). Industrial enzyme applications. *Curr Opin Biotechnol* 13, 345-351.

Kumar A., Kumar A., Devi S., Patil S., Payal C. andNegi S. (2012). Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: An in vitro study. *Recent Research in Science and Technology* 4.

Mead G. and Adams B. (1977). A selective medium for the rapid isolation of pseudomonads associated with poultry meat spoilage. *Br Poult Sci* 18, 661-670.

Mercado-Blanco J. and Bakker P. A. (2007). Interactions between plants and beneficial pseudomonas spp.: Exploiting bacterial traits for crop protection. *Antonie Van Leeuwenhoek* 92, 367-389.

Moore E. R., Mau M., Arnscheidt A., Böttger E. C., Hutson R. A., Collins M. D. and Timmis K. N. (1996). The determination and comparison of the 16S rRNA gene sequences of species of the genus pseudomonas (sensu stricto and estimation of the natural intrageneric relationships. *Syst Appl Microbiol* 19, 478-492.

Mulligan C., Yong R. andGibbs B. (2001). Surfactant-enhanced remediation of contaminated soil: A review. *Eng Geol* 60, 371-380.

Peix A., Ramírez-Bahena M. andVelázquez E (2009). Historical evolution and curren status of the taxonomy of genus pseudomonas. *Infection, Genetics and Evolution* 9, 1132-1147.

Raaijmakers J. M., de Bruijn I. and de Kock M. J. (2006). Cyclic lipopeptide production by plant-associated pseudomonas spp.: Diversity, activity, biosynthesis, and regulation. *Mol Plant-Microbe Interact* 19, 699-710.

Raaijmakers J. M., Paulitz T. C., Steinberg C., Alabouvette C. and Moënne-Loccoz Y. (2009). The rhizosphere: A playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321, 341-361.

Raaijmakers J. M., De Bruijn I., Nybroe O. andOngena M. (2010). Natural functions of lipopeptides from bacillus and pseudomonas: More than surfactants and antibiotics. *FEMS Microbiol Reveiw*34, 1037-1062.

Robertson M., Hapca S. M., Moshynets O. andSpiers A. J. (2013). Air-liquid interface biofilm formation by psychrotrophic pseudomonads recovered from spoilt meat. *Antonie Van Leeuwenhoek* 103, 251-259.

Ron E. Z. and Rosenberg E. (2001a). Natural roles of biosurfactants. *Environmental Microbiol* 3, 229-236.

Sáenz-Marta C. I., Ballinas-Casarrubias M., Rivera-Chavira B. E. and Nevárez-Moorillón G. V. (2015). Biosurfactants as useful tools in bioremediation. *Advances in Bioremediation of Wastewater and Polluted Soil.2nd Ed.Tech Publish* , 94-109.

Sandman K. and Ecker C. (2014). Pseudomonas isolation and identification: An introduction to the challenges of polyphasic taxonomy. *J Microbiol Biol Educ* 15, 287-291.

Smits W. K., Kuipers O. P., andVeening J. (2006). Phenotypic variation in bacteria: The role of feedback regulation. *Nature Reviews Microbiology* 4, 259-271.

Yamamoto S., Kasai H., Arnold D. L., Jackson R. W., Vivian A. andHarayama S. (2000). Phylogeny of the genus pseudomonas: Intrageneric structure reconstructed from the nucleotide sequences of gyrB and rpoD genes. *Microbiology* 146, 2385-2394.

Yamamoto S. and Harayama S. (1995). PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of pseudomonas putida strains. *Applied Environmental Microbiology*61, 1104-1109.