Research Journal of Recent Sciences _____ ISSN 2277-2502
Vol. 4**(ISC-2014),** 14-18 **(2015)** *Res. J. Recent. Sci.*

Identification of *Pseudomonas* using Probabilistic identification of Bacteria (PIB) Software

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Available online at: www.isca.in, www.isca.me
Received 14th November 2014, revised 25th December 2014, accepted 12th January 2015

Abstract

The genus Pseudomonas is a metabolically versatile group of gram-negative, motile, rod-shaped bacteria. They are common soil-dwelling aerotactic gram-negative proteobacteria with the unique ability to utilize exotic carbon sources for energy. Some members of the genus Pseudomonas are able to metabolize chemical pollutants in the environment, and as a result can be used for bioremediation. Therefore, characterization of various species of Pseudomonas is of significant importance. In the present study four isolates HMR1, HMR4, HMR7 and HMR16 were characterized on the basis of morphological and biochemical characteristics aided with Probabilistic identification of bacteria (PIB) software. For the isolates, HMR1 and HMR16 identification threshold reached to 1.0 and for the isolates, HMR4 and HMR7 identification threshold reached to 0.98906. Isolate HMR1 and HMR16 were identified as Pseudomonas aeruginosa and isolate HMR4 and HMR7 were identified as Pseudomonas putida.

Keywords: Pseudomonas, HMR, PIB software, Biochemical characterization, Bioremediation.

Introduction

The genus *Pseudomonas* is found ubiquitously in nature with many ecological, economic functions and health-related importance. In the environment, these bacteria are involved in various metabolic activities like cycling of element and the degradation of xenobiotic pollutants. Many *Pseudomonas* species were recovered from different heavy metal contaminated sites such as soil, sewage, irrigation and agricultural drainage canals[1,2]. Under laboratory conditions, *P. aeruginosa* resist high concentrations of heavy metals like Zn, Cu, Ni, Pb, Cd and Hg[3,4,5]. The taxonomy of the *Pseudomonas* genus is complex, consisting of at least 105 recognized species. Many *Pseudomonas* species are metabolically versatile and utilize large number of organic compounds as unique carbon and energy sources[6]. This versatility allows them to survive in many extreme conditions as natural autochthonous microflora making them attractive candidates for use in bioremediation[7, 8]. An accurate and rapid system for the *Pseudomonas* identification is essential in order to determine or monitor their role in the environment.

Molecular techniques like PCR have been used for microorganism identification; however, they are not without their own limitations i.e. they are too expensive specially species specific PCR and some time it gives false results due to mutations and some other changes. The exquisite sensitivity of PCR is a double edged sword which makes pseudo positive results from even the minutest degree of contamination a serious threat [9, 10]. The DNA or RNA extraction method used can also bias diversity studies. Harsh extraction methods like bead beating can shear the nucleic acids which lead to problems in subsequent PCR detection. Various nucleic acid extraction methods will result in different yields of product[11]. There is a loss of DNA or RNA during subsequent purification steps which again potentially biasing PCR-based diversity studies. Differential amplification of target genes can also bias molecular diversity analysis. Therefore, biochemical characteristics are still the touchstone for bacterial identifications. Biochemical testing aided with probabilistic statistical software[12] provides reliable and cheaper method for the identification of bacteria.

Probabilistic identification of bacteria (PIBWin) is a windows version of a DOS program PIB (also called Bacterial Identifier), an implementation of Bayes theorem by Willcox et al.[13]. An identification score was calculated as the Willcox probability P, for identification thresholds of P $\geq$95, P$\geq$98 and P $\geq$99 for all the isolates and reference strains. Lapage[14] described identification of 1,079 reference and 516 field strains of gram-negative, rod-shaped bacteria using computer-assisted probabilistic method. Joshi and Chaudhary[15] used PIB software and identified different species of *Lactobacillus* with maximum ID score of 0.980. Ottaviani et al. [16] identified *Vibrio* using the free software probabilistic identification of bacteria with identification thresholds of P > 0.9. Rajput et al. [17] characterized and taxonomically identified various strain of *Streptomyces* using probabilistic identification of bacteria (PIB) Win software. Carson et al.[18] identified motile *Aeromonas* species with Willcox probability scores of 0.99 using an improved probability matrix. The present study was aimed to identify *Pseudomonas* bacteria by biochemical tests aided with PIB software.

Material and Methods

Source and maintenance of bacteria: Heavy metal tolerant bacterial isolates HMR1, HMR4, HMR7 and HMR16 previously isolated from heavy metal contaminated sites of Zawar, Udaipur [19] on nutrient agar medium supplemented with zinc sulphate heptahydrate were used in this study. All the isolates were routinely grown at 37°C for 24h on nutrient agar medium supplemented with 1 mM zinc sulphate heptahydrate and stored at -20°C in glycerol.

Morphological and Biochemical characterization of the isolates: Identification of pure bacterial isolates was performed by studying colony morphology, motility morphological characteristics such as Gram staining and microscopic examination and growth at different temperature. The biochemical tests such as oxidation fermentation reaction (OF) on Hugh and Leifson oxidation-fermentation medium [20], catalase test, oxidase activity, citrate utilization, arginine hydrolysis, starch hydrolysis, lipid hydrolysis, urease activity, malonate utilization, nitrate reduction, gelatin and casein hydrolysis were performed. The growth on MacConkey agar, cetrimide agar and King's Medium was also checked.

PIB software based identification: The data obtained from morphological and biochemical characterization was fed to the identification matrices of known strain. The program provides Identification scores (ID scores) and Identification modal scores (ID modal scores). A threshold of 0.95 (95%) was used as the working confidence level to establish agreement between isolate identification based on conventional methods and that generated by the probability-based method.

Results and Discussion

Unlike many environmental bacteria, *Pseudomonas* are of utmost importance and well-studied. These bacteria are of particular concern not only because of their high resistance to heavy metals and other toxic substances, but also for their simple nutritional requirements and rapid growth on standard laboratory media. In the present investigation efforts were made to identify *Pseudomonas* by series of biochemical test aided with PIB software. Four heavy metal tolerant isolates HMR1, HMR4, HMR7 and HMR16 were characterized on the basis of various morphological and biochemical characteristics (table-1). In Gram's staining, the morphology of isolated *Pseudomonas* strains showed Gram-negative, pink colored, medium rod shaped appearance. These findings agreed with the findings reported by earlier researchers[21,22]. Isolate HMR1 and HMR16 have light brown, flat, small, transparent, round colonies and isolate HMR4 and HMR7 have Off-white, Elevated, Round, small colonies. These characteristics colonies were similar with that of previous studies[23,24]. All the isolates showed motility at room temperature and 37°C. All the isolates gave negative reaction for fermentation of glucose but they gave positive reaction for oxidation of glucose when grown on Hugh and Leifson oxidation-fermentation medium. All the isolates gave positive reaction for catalase activity, oxidase activity, citrate utilization, arginine hydrolysis and negative reaction for amylase activity. Both the isolates HMR1 and HMR16 gave positive result for lipase activity, urease activity, utilized malonate, reduced nitrate and hydrolyzed gelatin and casein whereas isolates HMR4 and HMR7 gave negative results for lipase activity, urease activity, utilization of malonate, nitrate reduction, gelatin and casein hydrolysis. Biochemical characterization of the isolated *P. aureginosa and P. putida* were similar to previous studies which identified the same organisms from various sources[21,25]. Growth of these four isolates was further checked on selective and differential media like cetrimide agar and MacConkey agar respectively. All the isolates were able to grow on cetrimide agar. All of them produced colorless colonies on MacConkey agar which indicated that they were lactose non fermenter. In this study both the isolates HMR1 and HMR16 produced green pigment and fluorescence pigment on King's A medium and King's B medium respectively. Our results are consistent with Todar[26] who stated that majority of the *Pseudomonas* species produce blue-green pigment pyocyanin. The isolates HMR4 and HMR7 fail to produce any pigment when grown on King's A medium but produced fluorescence pigment on King's B medium. The results obtained for pigment production are in agreement with the earlier reports[27,25]. Growth of these four isolates was checked at different temperature like 5°C, room temperature, 37 °C and 42 °C. All of them were able to grow at room temperature, 37 °C and 42 °C, but failed to grow at 5°C .The above results obtained for morphological and biochemical characteristics were then fed to the identification matrices of known strain in PIB software. The Identification score (ID) and Identification modal score for isolate no. HMR1 and HMR16 were same i.e. 1.0 and 0.29870 respectively (figure 1). The identification score and ID Modal score for isolate no. HMR4 and HMR7 were same i.e. 0.98906 and 0.04167 respectively (figure-1). The analysis revealed that identification score reached the maximum 0.9500 therefore the isolates HMR1 and HMR16 were identified as *Pseudomonas aeruginosa* and the isolates HMR4 and HMR7 were identified as *Pseudomonas putida.* These results are parallel to previous studies which identified various species of *Pseudomonas* using PIB software and obtained identification score in the range of 0.8 to 0.99 [28, 29]. Our results were in accordance with that of Holmes et al.[30] who used computer-based probabilistic method and identified 621 strains of gram-negative, aerobic, non-fermentative bacteria including 20 species of *Pseudomonas* with identification score of 0.99. Vinitha Ramanath Pai et al.[31] identified different species of *Pseudomonas* using PIB software. They obtained maximum Identification score of 0.80411. In this regard our results are better because high Identification score was obtained for identification of *Pseudomonas* using PIB software.

Table-1
Morphological and biochemical characterization of heavy metal tolerant isolates.

Characteristics	Isolates			
	HMR1	**HMR16**	**HMR4**	**HMR7**
Motility at 37°C	+	+	+	+
Motility at RT	+	+	+	+
Growth at 5°C	-	-	-	-
Growth at RT	+	+	+	+
Growth at 37°C	+	+	+	+
Growth at 42°C	+	+	+	+
Gram reaction	-	-	-	-
Shape	rod	rod	rod	rod
(O/F) test	+/-	+/-	+/-	+/-
Catalase	+	+	+	+
Oxidase	+	+	+	+
Citrate Utilization	+	+	+	+
Arginine Hydrolysis	+	+	+	+
Amylase	-	-	-	-
Lipase	+	+	-	-
Urease	+	+	-	-
Malonate utilization	+	+	-	-
Nitrate Reduction	+	+	-	-
Gelatin Hydrolysis	+	+	-	-
Casein Hydrolysis	+	+	-	-
Growth on				
a) MacConkey agar	+	+	+	+
b) Cetrimide agar	+	+	+	+
c) King's A Medium (Pyocyanin)	+	+	No Pigment	No Pigment
d) King's B Medium (Fluorescent)	+	+	No Pigment	No Pigment
+ =positive,- =negative, RT=room temperature, O/F= oxidation/fermentation				

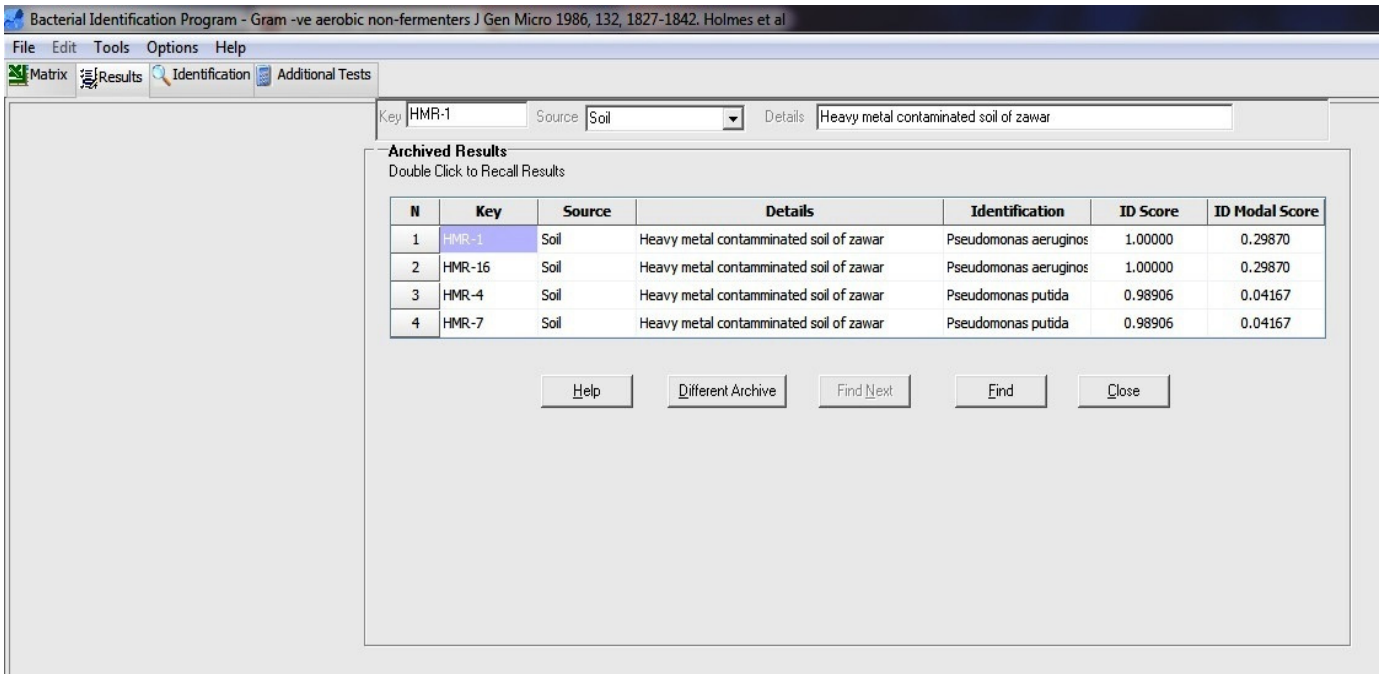

Figure-1
Identification of heavy metal tolerant bacteria by PIB software

Conclusion

The biochemical testing coupled with a robust identification matrix, provides a convenient basis for identifying unknown bacteria and re-establishes the importance of biochemical tests. Thus this identification system for identifying *Pseudomonas* will be of use as these strains could be a potential candidate for heavy metal removal from polluted sites.

Acknowledgement

Financial assistance to Ali Asger Bhojiya by the University Grants Commission, India in the form of Maulana Azad National Fellowship (MANF-SRF), and research support from the Department of Biotechnology, Mohanlal Sukhadia University, Udaipur, Rajasthan, India is acknowledged.

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