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Research Article

Evaluation of API 20E system in fluorescent *Pseudomonas* identification from button mushroom *Agaricus bisporus* cultivation casing soil

Anwar O. MOHAMMAD[1]*, Abdulkareem E.S. ALKURTANY[2], Abdullah A. HASSAN[3]

[1]*University of Sulaimani, Natural Resources Department, College of Agricultural Engineering Sciences, Al-Sulaymaniyah, Iraq; anwar.mhamad@univsul.edu.iq (*corresponding author)*
[2]*Tikrit University, Department of Soil and Water Science, College of Agriculture, Tikrit, Iraq; alkurtany@tu.edu.iq*
[3]*Tikrit University, Department of Plant Protection, College of Agriculture, Tikrit, Iraq; drabdullah.has67@tu.edu.iq*

Abstract

Bacterial activity, mainly *Pseudomonas spp.* plays a vital role in the fruiting process of white button mushroom, hence a rapid procedure to identify these bacteria is crucial. In the current study, the validity of commercial identification system, Analytical profile index API 20E to identify *Pseudomonas* isolates from mushroom casing soil were assessed. Using API strips fifty bacterial isolates from a selective medium (King B medium) were examined, all isolates were belonged to the genus *Pseudomonas* according to API 20E identification systems. However, only 74% of *Pseudomonas* bacteria were identified to species level. The molecular identification using 16S rRNA gene was used as a reference tool to identify bacteria at the species level. The results show that the accuracy of the system to classify florescent *Pseudomonas* to species level was 60%. This was species dependant, and the system accuracy were 100%, 87.5%, 81.3% and 63% in identifying *P. aeruginosa*, *P. putida*, *P. fluorescens* and *P. tolaasii* respectively. Our finding indicates that although the classification of the *Pseudomonas* genus with API 20E system is useful, but it is not enough to distinguish these bacteria to species level, genomic studies are necessary to confirm the exact taxonomic position of *Pseudomonas* spp.

Keywords: bacteria; biochemical tests; classification; fluorescent *Pseudomonas*; PCR

Introduction

The fungus white button mushroom (*Agaricus bisporus*) is world's most cultivated mushroom species. The formation of mushroom fruit body requires the application of 3-5 cm casing layer which is made from material such as peat, soil, and granulated bark to mycelium colonized compost (Long and Jacobs, 1974). The casing layer supports beneficial microbial populations that release growth stimulants, which are reportedly involved in stimulating the initiation of mushroom. Fluorescent *Pseudomonas* represents up to 50% of the

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bacterial population of the casing layer, of which the *Pseudomonas putida* is the most crucial (Noble *et al.*, 2003). However, not all *Pseudomonas* bacteria are beneficial in casing layer, for instance, few species had been isolated with the pathogenic ability to cause mushroom disease (Godfrey *et al.*, 2001, Munsch *et al.*, 2002). This includes the brown blotch disease that is caused by two pathogenic bacterium *P. tolaasii and P. reactans* (Gill, 1995).

Rapid identification of *Pseudomonas* bacteria in casing soil is a big challenge for the mushroom producers, for their impact on mushroom yield and quality (Mohammad and Sabaa, 2015; Kosanovic, *et al.*, 2019). The API 20E which detects 20 biochemical reactions, is a traditional method for the identification of Enterobacteriaceae, it is accepted as a rapid system for the identification of enteric and non-fermentative bacteria in many clinical bacteriological laboratories, and often is used to replace conventional biochemical tests method (O'Hara *et al.*, 1992; O'Hara, 2005). However, few studies have been reported evaluating this rapid identification system to investigate bacterial isolate from the soil. This study aimed to evaluate the performance of API 20E for the identification of fluorescent *Pseudomonas* from mushroom casing soil, taking the identification by the molecular method as the gold standard.

Materials and Methods

Sampling protocols

A sample of spent casing soil was collected from the mushrooms project (University of Tikrit/ College of Agriculture), and (10 gm) of spent casing soil was suspended in 90 ml sterilized normal saline (0.85% NaCl) and shaken vigorously at 150 rpm at room temperature for one h. The resulting slurry was serially diluted, appropriate dilutions (10^{-4}) were made in normal saline, and 0.1 ml of this suspension was spread plated on King's B medium (KB) (King *et al.*, 1954). The plates were incubated at 28 °C for 48 h. Single colonies producing green/yellow fluorescent pigments streaked on KB plates. Single colonies producing green/yellow fluorescent pigments streaked on KB plates. Gram-staining was performed by following the Burke method (Beveridge *et al.*, 2007). The oxidase reaction was tested using oxidase reagent 1% (w/v) tetra-methyl-p-phenylenediamine dihydrochloride. Catalase activity was recorded by observation of bubble formation after adding 3% (v/v) hydrogen peroxide H_2O_2.

Analytical profile index (API 20E) system for bacterial identification

Fifty (n=50) *Pseudomonas* isolates cultured from the casing soil samples were randomly collected. For each isolate, an API 20E strips (bioMérieux, Marcy-I'Etoile, France) were inoculated and incubated according to the manufacturer's instructions. The likelihood of Fluorescent *Pseudomonas* was calculated, using the manufacturer's coding system based on reactions to reagents in the 20 compartments.

Polymerase Chain Reaction (PCR) identifications confirmation

A single colony of identified *Pseudomonas* species by API 20E was taken from the cultured plate and inoculated into 10 ml of KB broth. The isolated *Pseudomonas* incubated at 28 °C for 24 hours. Bacterial cells harvested from three ml of the bacterial suspension by centrifugation at 4,000 g for 10 minutes. The DNA was extracted using DNA-Sorb-B extraction kit (Sacace, Biotechnologies., Italy) The extracted DNA stored at -20 °C.

All the PCR mixes were performed in (1.5 ml) Eppendorf test tube containing ≈ 50-100 ng of bacterial genomic DNA solution, 5 µL of 10x PCR buffer, 200 µM of each deoxynucleotide triphosphate (dNTP), 2 mM of $MgCl_2$, 0.5 µM of primers and 1 U of Taq polymerase (Amersham-Pharmacia). DNA 16S region amplification was perform using the species-specific primer set (Table 1). The following thermal profile was used, 5 min at 94 °C; 35 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; 35 cycles

consisting of 92 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min; final extension of 72 °C for 2 min. The amplifications performed in DNA thermal cycle (Eppendorf, Germany).

Table 1. Primers used in the study

Primer*	Sequence	References
16SPSEfluF	*5′–TGCATTCAAAACTGACTG–3′*	Scarpellini *et al.* (2004)
16SPSEfluR	*5′–AATCACACCGTGGTAACCG–3′*	Scarpellini *et al.* (2004)
16SPSEaeF	*5′–TGCATCCAAAACTACTGA–3′*	This study
16PSEaeR	*5′–AATCACTCCGTGGTAACCG–3′*	This study
16SPSEtolF	*5′–TGCATTCAAAACTGACTG–3′*	This study
16SPSEtolR	*5′–CCCGGGAACGTATTCACCG–3′*	This study
16SPSEputF	*5′–TGCATCCAAAACTGGCAAG–3′*	This study
16SPSEputR	*5′–AATCACACCGTGGTAACCG–3′*	This study

*Database of 16S rRNA sequence in National Center for Biotechnology Information (NCBI) gave necessary information to design these species-specific primers, using the Basic Local Alignment Search Tool (BLAST).

PCR amplification products were analysed on agarose gel (1.0%) using a horizontal electrophoresis unit. The gel was immersed in 1X TAE buffer containing 0.2 μg ml^{-1} ethidium bromide (Scarpellini *et al.*, 2004) and photographed by Bio-Rad gel doc system.

Results

All the considered bacterial isolates were gram-negative, produced yellow-greenish florescent pigmentation on KB medium and gave a positive reaction to oxidase and catalase tests.

According to the results from API 20E system, all Bacterial isolates from King's B medium belonged to the genus *Pseudomonas* (Table 2); however, only 74% were to species level, and the accuracy of identified bacteria to species level was 60% when compared to the molecular method.

Table 2. Identification and accuracy results for *P. putida, P. fluorescens, P. tolaasii, P. aeruginosa* by API 20E (*n* is the number of bacteria isolates)

	P. putida *n=8*	*P. fluorescens* *n=16*	*P. tolaasii* *n=8*	*P. aeruginosa* *n=5*	Total isolates *n*= 50
Total identification to species level	8	16	8	5	37 (74%)
Correct identification at the species level	7 (87.5%)	13 (81.3%)	5 (63%)	5 (100%)	30 (60%)
Inaccurate identified to species level	1(12.5%)	3 (18.7%)	3 (37%)	0 (0%)	20 (40%)
Identification to genus level	8 (100%)	16 (100%)	8 (100%)	5 (100%)	50 (100%)

To confirm the identification of *Pseudomonas* isolates at the molecular level, the 16S rRNA gene sequence was amplified to identify *Pseudomonas* spp. isolated from the casing soil.

A pair of primer were used for the amplification of 16S rRNA genes in *P. fluorescens*; forward primer (16SPSEfluF) and the reverse primer (16SPSEfluR). The PCR result showed that there is a fragment of 848 bp of 16S rRNA, which is a specific amplification for *P. fluorescens* species. Scarpellini *et al.* (2004b) obtained similar results by using the same pair of primers to identify *P. fluorescens*. To identify *P. tolaasii,* a species-specific amplification of the 757 bp of 16S rRNA fragment obtained using two primers forward primer 16SPSEtolF and reverse primer 16SPSEtolR. Eight isolates identified as *P. tolaasii* using API 20E system but only five of them confirmed to be accurate using PCR amplification assay. For confirming the identification of tested *P. aeruginosa* and *P. putida* to the species level, based on the alignment of the 16S rRNA gene sequence available in GenBank, two primer pairs were used to confirm species identification. PCR products of the 874 bp and 848 bp were obtained in confirmation of *P. aeruginosa* and *P. putida* species identification, respectively.

Discussion

The API 20E diagnostic is a traditional method for the identification of *Enterobacteriaceae* (Koneman *et al.*, 1997). In this study, we have examined the suitability of API 20E system in the identification of Pseudomonas bacteria from mushroom casing soil. The results show 40% of *Pseudomonas* isolates were not identified to species level. Previous studies about the use of API 20E have reported both good and inaccurate sample classifications (O'Hara *et al.*, 1992; Popovic *et al.*, 2004). Topic Popovic et al., (2004) found that the accuracy API 20E to identify *Pseudomonas* spp bacteria was 37%. Biochemical testing is frequently required to positively identify these organisms and require the use of multiple assays, with a composite result predicting the most likely identification (Kiska and Gilligan, 2003).

API 20E system identified 37 bacterial samples to species level from these 30 isolates (60%) confirmed to be correct using gene amplification. Two isolates that according to API 20E belonged to *P. fluorescens*, we found that they belonged to *P. putida* using the molecular method. Furthermore, we found that 26% of species were not sufficiently identified to species level. The overall accuracy of the API 20E system to classify florescent *Pseudomonas* to species level was 60%. Furthermore, in this study, the accuracy of API 20E system were 100%, 87.5%, 81.3% and 63% in identifying *P. aeruginosa, P. putida, P. fluorescens* and *P. tolaasii* respectively (Table 2).

In a previous study, Drancourt *et al.* (2000) suggested that Some bacteria are difficult to identify phenotypically using the schemes of identification with commonly used outside reference laboratories. For this reason, the 16S rRNA gene sequence offers a useful method for the identification of bacteria because these genes are conservative. The 16S rRNA gene sequence had long been used as a taxonomic method in determining the phylogenies of bacterial species. Selective amplification of *Pseudomonas* 16S rRNA gene sequence and PCR chain reaction was used to reveal and differentiate *Pseudomonas* species from clinical and environmental samples (Porteous *et al.*, 2002). Clarridge (2004) described that 16S ribosomal RNA sequencing could be claimed as the reference method for bacteria species identification in research laboratories. Genotypic identification is emerging as an alternative or complement method to conventional phenotypic identification (Kolbert and Persing, 1999; Franzetti and Scarpellini, 2007). The public databases (GenBank, Nucleotide Sequence Database at the European Molecular Biology Laboratory, DNA Data Bank of Japan, RDP II) contain a significant amount of bacterial 16S rRNA sequences that allows rapid analysis and provide phylogenetically meaningful information (Bosshard *et al.*, 2006). The classification of the *Pseudomonas* genus employing physiological and biochemical characteristics is useful, but it is not enough to distinguish the *Pseudomonas* species: genomic studies are needed to confirm the exact taxonomic position of *Pseudomonas* spp.

Conclusions

Physiological and biochemical characteristics (API 20E system) could be regarded as a fast identification approach, but it is not enough to distinguish the *Pseudomonas* species; genomic studies are needed to confirm the exact taxonomic position of *Pseudomonas* spp.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Beveridge TJ, Lawrence JR, Murray RGE (2007). Sampling and staining for light microscopy. Methods for General and Molecular Microbiology 3:19-33. *https://doi.org/10.1128/9781555817497.ch2*

Bosshard PP, Zbinden R, Abels S, Böddinghaus B, Altwegg M, Böttger EC (2006). 16S rRNA gene sequencing versus the API 20 NE System and the VITEK 2 ID-GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory. Journal of Clinical Microbiology 44:1359-1366. *https://doi.org/10.1128/JCM.44.4.1359-1366.2006*

Clarridge JE (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clinical Microbiology Reviews 17:840-862.

Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D (2000). 16S Ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. Journal of Clinical Microbiology 38:3623-3630.

Franzetti L, Scarpellini M (2007). Characterisation of *Pseudomonas* spp. isolated from foods. Annals of Microbiology 57:39-47. *https://doi.org/10.1007/BF03175048*

Gill WM (1995). Bacterial diseases of *Agaricus* mushrooms. Reports of the Tottori Mycological Institute (Japan).

Godfrey SAC, Harrow SA, Marshall JW, Klena JD (2001). Characterization by 16S rRNA sequence analysis of pseudomonads causing blotch disease of cultivated *Agaricus bisporus*. Applied and Environmental Microbiology 67:4316-4323. *https://doi.org/10.1128/AEM.67.9.4316-4323.2001*

King EO, Ward MK, Raney DE (1954). Two simple media for the demonstration of pyocyanin and fluorescin. The Journal of Laboratory and Clinical Medicine 44:301-307.

Kiska DL, Gilligan PH (2003). Pseudomonas. In: Manual of Clinical Microbiology. ASM Press, Washington DC, pp 719-728.

Kolbert CP, Persing DH (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Current Opinion in Microbiology 2:299-305.

Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC (1997). The Enterobacteriaceae. Color atlas and textbook of diagnostic microbiology. B Lippincott Co, Philadelphia, pp 71-230.

Kosanovic D, Sheehan G, Grogan H, Kavanagh K (2019). Characterisation of the interaction of *Pseudomonas putida* and *Pseudomonas tolaasii* with *Trichoderma aggressivum*. European Journal of Plant Pathology 1-11.

Long PE, Jacobs L (1974). Aseptic fruiting of the cultivated mushroom, *Agaricus bisporus*. Transactions of the British Mycological Society 63:99-107. *https://doi.org/10.1016/S0007-1536(74)80140-3*

Mohammad A, Sabaa A (2015). *In vitro* and *in vivo* impact of some *Pseudomonas* spp. on growth and yield of cultivated mushroom (*Agaricus bisporus*). The Egyptian Society of Experimental Biology 11(2):163-167.

Munsch P, Alatossava T, Marttinen N, Meyer J-M, Christen R, Gardan L (2002). *Pseudomonas costantinii* sp. *nov.*, another causal agent of brown blotch disease, isolated from cultivated mushroom sporophores in Finland. International Journal of Systematic and Evolutionary Microbiology 52:1973-1983. *https://doi.org/10.1099/ijs.0.02090-0*

Noble R, Fermor TR, Lincoln S, Dobrovin-Pennington A, Evered C, Mead A, Li R (2003). Primordia initiation of mushroom (*Agaricus bisporus*) strains on axenic casing materials. Mycologia 95:620-629.

O'Hara CM (2005). Manual and automated instrumentation for identification of Enterobacteriaceae and other aerobic gram-negative bacilli. Clinical Microbiology Reviews 18:147-162. *https://doi.org/10.1128/CMR.18.1.147-162.2005*

O'Hara CM, Rhoden DL, Miller JM (1992). Re-evaluation of the API 20E identification system versus conventional biochemicals for identification of members of the family Enterobacteriaceae: a new look at an old product. Journal of Clinical Microbiology 30:123-125.

Porteous LA, Widmer F, Seidler RJ (2002). Multiple enzyme restriction fragment length polymorphism analysis for high resolution distinction of Pseudomonas (*sensu stricto*) 16S rRNA genes. Journal of Microbiological Methods 51:337-348. *https://doi.org/10.1016/S0167-7012(02)00108-2*

Scarpellini M, Franzetti L, Galli A (2004). Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. FEMS Microbiology Letters 236:257-260.

Topic Popovic N, Benussi Skukan A, Strunjak-Perovic I, Coz-Rakovac R, Hacmanjek M, Hunjak B (2004). Comparison of the API 20E and BBL crystal E/NF identification systems for differentiating bacterial isolates from apparently healthy reared sea bass (*Dicentrarchus labrax*). Veterinary Research Communications 28:93-101. *https://doi.org/10.1023/B:VERC.0000012113.95479.2f*