Original Research Article

Antagonistic effect of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro* and molecular characterizations

Comment [h1]: Title is too lengthy; it should be short to attract reader's attention

ABSTRACT

Bananas are the earliest fruit crop cultivated by humans from ancient times in India and have great social-economic importance, merged with the country's cultural heritage. Different bio-agents were isolated from the rhizosphere of wilt affected banana plants cultivar Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 areas of Bihar. All isolated bio-agents were tested against FOC TR4 *in vitro* condition by dual culture technique. The maximum inhibition over control at 240 hrs of inoculation was recorded in *Trichoderma asperellum* 1(Tr1) (64.82%), followed by *Trichoderma asperellum* 2(Tr2) (62.70%), *Aspergillus flavus* (35.00%) and minimum in *Penicillium chrysogenum* (22.62%). The result clearly showed that *in vitro Trichoderma asperellum* 1 was highly effective while *Penicillium chrysogenum* was found least effective antagonistic against *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro.* Molecular characterizations of two morphologically different *Trichoderma spp.* ~~samples~~ were identified as two different strains of *Trichoderma asperellum* 1 and *Trichoderma asperellum* 2.

Keywords: *Fusarium oxysporum* f.sp. *cubense* TR4 (FOC TR4), *Trichoderma*, Bio-agents

Introduction

Banana (*Musa paradisiaca* L.) is a critical herbaceous perennial monocotyledonous plant, which belongs to the family Musaceae. The plant is also known as Kalpatharu, which means herb with all potential uses. It has been believed to be ~~that~~ originated from hot tropical regions of South East Asia from cultivar *Musa accuminata* and *Musa bulbisiana*. The banana is the oldest cultivated fruit crop known to humankind ~~and it's botanically known as a *Musa paradisiaca*~~. It is also known as ~~was said to be~~ the Apple of Paradise. It is also mentioned in the Great Indian epics, Ramayana (2020 BC) and Kautilya's Arthashastra (300-400 BC).

Banana pulp per 100 gm has a calorific value ranging from 67 to 137 calories. The ripe banana pulp contains about 70 % water with 23.43 % carbohydrate, 1.03 % protein, 0.48 % fat, 39.6 % K, 0.6 % Ca, 0.1 % Na, 0.03 % Fe and seven types of vitamins, including vitamins-A, B6, C, Thiamin, Riboflavin, Niacin, and Folacin (Reference). Banana provides a more balanced diet compare to other fruits and has a more therapeutic value with low salt, fat content, and cholesterol. The origin seems to be either from Malayan, Peninsula, or Asia. Subsequently, this crop was extended to many countries including Ceylon, Costa Rica, Ecuador, Mexico, Honduras, India, Jamaica, Columbia, Panama and several other Eastern countries. The banana crop was mainly cultivated in the humid tropics; it had a history in the subtropic and arid regions in the Middle East. In the 12th century, the banana was the existence in Moorish Spain and Northern Africa [1]. Banana is the world's most valuable fruit crop. In 2011, the total gross production value of US$44 Billion, and global banana production was nearly 145 Million tons [2].

Comment [h2]: Can you come up with a reference to support the phrase

About the Panama wilt

Panama wilt of banana incited by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Synd. and Hans. was first~~1~~st identified in 1874 at South East Queensland, Australia [3]. *Fusarium* wilt of banana incited by *Fusarium oxysporum* f. sp. *cubense* was ~~1~~st first time isolated and disease caused by this pathogen was proved [4-5]. The fungus produces a reddish pigment in old culture due to the formation of chlamydospores. Thise characteristic ~~is~~ separate~~d~~ it from other similar species, such as *Fusarium moniliforme* and *Fusarium solani* that also formed large quantities of microspores [5].

Formatted: Not Superscript/ Subscript

Until now, four different races of *Fusarium oxysporum* f. sp. *cubense* has been recorded [6].

Race -1: It affects varieties mainly Pome (AAB) and Silk (AAB) groups of ~~banana~~bananas in the world.

Race –2: It attacks to Bluggoe types of bananas and other closely related cooking bananas.

Race –3: Caused diseases in *Heliconia* sp. Not confined to India. It occurs in Honduras, Australia and Costa Rica.

Race -4: All known varieties of banana are susceptible against this race.

Race 4 has divided in two i. e. tropical and subtropical. It produced disease in dwarf Cavendish groups of ~~banana~~bananas and those who are susceptible to race I and race II. Subtropical race IV produced disease in dwarf Cavendish groups of bananas in different

countries like Canary Islands, Australia, Taiwan and South Africa [7]. Australia and Southeast Asia the TR 4 influences Cavendish groups of bananas in the tropical areas [8].

Material and Method

During ~~the~~ survey of Panama wilt in Bihar, the collection of a different soil sample from the rhizosphere of Panama wilt affected areas in cultivar Grand naine from different banana growing districts in Bihar. Nearly 5-10 g soil was~~is~~ taken from the rhizosphere and put a tag in a soil sample with the farmer's name, location, and date of collection. The sample was taken in polythene packets and kept~~it~~ airtight. Soil sample brought~~brings~~ to the Department of Plant Pathology, Dr. Rajendra Prasad Central Agricultural University (RPCAU), Pusa, Bihar. One gm of a soil sample was taken and ~~its~~ serial dilution ~~technique~~ of 10^{-4} and 10^{-5} was prepared and poured in Rose Bengal agar medium under aseptic condition [9]. All the plates were incubated at 27±2°C. After 72 hrs. of incubation ~~observed~~ different fungal colonies ~~and their isolate.~~ Preparations of slides and observe in a compound microscope, prepare a pure culture of separate colonies.

For ~~the testing~~ antagonistic effect of *Trichoderma asperellum* 1, *Trichoderma asperellum* 2, *Aspergillus flavus*, and *Penicillium chrysogenum*, the PDA was poured in Petri-plates ~~were distributed~~ in equal half [10]. In the first half, inoculated with seven days old pure culture of each bio agent of 5mm while, in another half, seven days old pure culture of the pathogen poured~~was put~~ on PDA ~~media~~ directly touching with fungal mycelia. ~~Control plate~~ T~~t~~he pathogenic test fungus was placed without any bio-control agent as control plate. Four replications for each treatment were prepared~~done~~. All Petri plates of each replication were incubated for 28±2°C until the growth of *Fusarium oxysporum* f. sp. *cubense* TR4 isolates in the control treatment reached up to the margin of Petri-plates. The percent inhibitions of linear mycelial growth of pathogenic fungi and ~~the~~ bio-control agent ~~were~~was calculated using the formula [11].

~~Treatments~~Treatment's mean ~~were~~was compared with F-test and L.S.D. at the level of 0.05%.

$$I = \left(\frac{C - T}{C} \right) \times 100$$

Where,

I = Percent growth inhibition

C = Control Petri plate colony diameter

T = Treated Petri plate colony diameter

Comment [h3]: Authors ae advise to rewrite the statement to make the sense clear

Comment [h4]: Rhizosphere of which plant; do author's ean banana rhizosphere? If so be ention

Formatted: Not Superscript/ Subscript

Formatted: Not Superscript/ Subscript

Comment [h5]:

Comment [h6]: Aterials and ethods are always written in past tense and in passive voice

The percent inhibition data were statistically analyzed by using a completely randomized design (C.R.D)

Result and Discussion

During the survey of Panama wilt affected areas in Bihar, collected soil sample from the rhizosphere of banana. It took 1 gm soil sample to isolate microflora and followed serial dilution at concentrations 10^{-4} and 10^{-5}. After serial dilution, it spread into Rose-Bengal agar medium up to 3-4 days colony of different microflora was observed. Each colony was a different morphological structure and colour. Isolated every colony in PDA media and prepared a slide for the identification of microflora. Four diverse microflora found during research work, i.e., *Trichoderma asperellum* 1, *Trichoderma asperellum* 2, *Aspergillus flavus*, and *Penicillium chrysogenum*. The pure culture was prepared in Petri-plates and test tubes in the PDA medium. Both *Trichoderma spp*. were observed differences in their morphological structure and colony character under a microscope. *Aspergillus flavus* colony colour green, conidia and conidiophores are visible under a microscope. *Penicillium chrysogenum* colony colour initially white and later converted into shades of green. To confirm, the identification of microflora follows available literature.

Molecular characterization of different *Trichoderma* isolates i.e., Tr1 and Tr2

Sample Name

Tr1

Sequence

TACCTTCTGTAGGGTGACCCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAA
ACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGC
GTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGT
AGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCA
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA
CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCC
TCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGGTGCC
GGCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCA
CAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTG
AAATGTTGACCTCGGATCAGGTTGGAATTCCAATCGCTGATTTTAAGCATATCAA
TAAGCGGAGAGAAAAC

Comment [h7]: Molecular characterization means authors have ***m***ined the genomic structure and different parameters i-e genetic relationship with other fungal groups (phylogenetic tree) no og intron exons protein etc should be mentioned

Identification *Trichoderma asperellum*

Percentage identity 98%

Comment [h8]: How the authors calculated %age identity

Sample Name

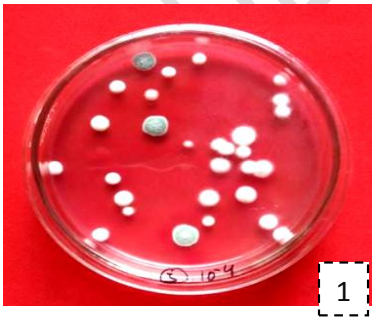
Tr2

Sequence

CTTCCGTAGGGTGAACCTGCGGAGGGATCATTACCGAAAGTTTACAACTCCCAA
ACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGC
GTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCACACTCTTTCTGT
AGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCA
AAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA
CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCC
TCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGGTGCC
GGCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGGAGTAGTTTGCA
CTACTCGCACCGGGAGCGCGGCGCGTCTACGTCCGTAGAACACCCAACTTTCTGA
AATGTTGACCTACGGATGCAGGTTGGAATTCCAA

Identification *Trichoderma asperellum*

Percentage identity 98%



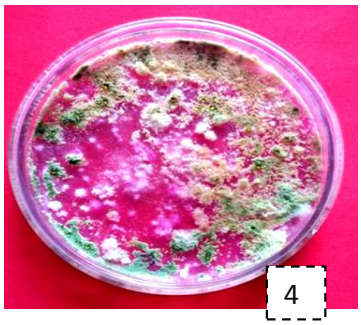
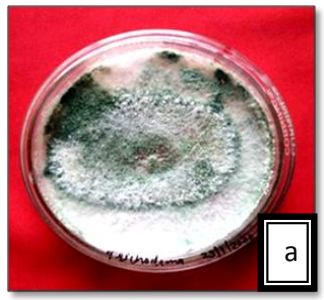


Plate- 1.1. Isolation of microflora associated with the rhizosphere of banana



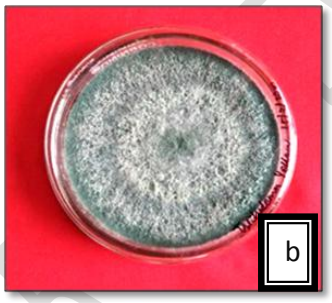


Plate-1.2. Purification and identification of rhizospheric microflora

a) *Trichoderma asperellum* 1(Tr1) ***b*) *Trichoderma asperellum* 2(Tr2)**

c) *Aspergillus flavus* d**) *Penicillium chrysogenum***

The antagonistic activity of different rhizosphere isolates microflora against the *Fusarium oxysporum* f. sp. *cubense* TR4 was determined by the dual culture technique described [12]. Seven days old pure culture having 5mm disc of different soil isolates bioagent and isolates of *Fusarium oxysporum* f. sp. *cubense* TR4 were placed equidistance in sterilized Petri plate containing PDA medium. Suitable control was also maintained without any bioagent. The growth of the pathogen was measured at 120 hrs and 240 hrs (10 days) of inoculation. Percent inhibition over control at 240 hrs of the pathogen was calculated. Data

4

are presented in Table-1.1. The maximum inhibition over control at 240 hrs of inoculation was recorded in *Trichoderma asperellum 1* (64.82%), followed by *Trichoderma asperellum 2* (62.70%), *Aspergillus flavus* (35.00%) and minimum in *Penicillium chrysogenum* (22.62%). The result clearly showed that *in vitro Trichoderma asperellum* 1 was highly effective while *Penicillium chrysogenum* was found least effective antagonistic against *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro*.

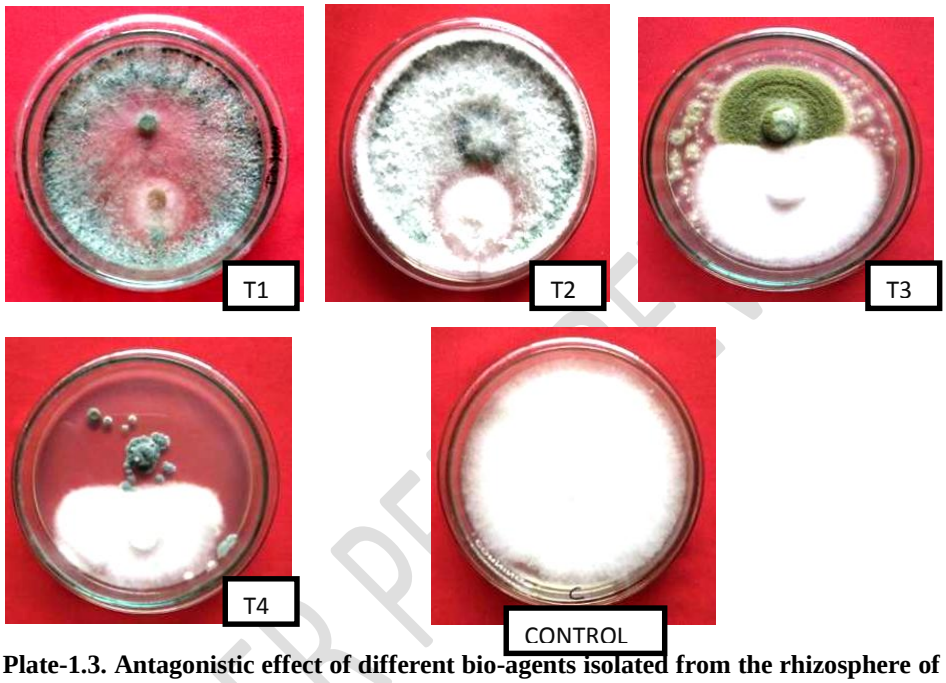


Plate-1.3. Antagonistic effect of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro* at 240 hrs

Table-1.1. Antagonistic effect of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro*

Bio-agents		**Radial growth(mm)***		
		120hrs	**240 hrs**	**Inhibition over control (%) at 240 hrs**
T_1	*Trichoderma asperellum* 1(Tr1)	13.3	31.6	64.82

T_2	*Trichoderma asperellum* 2(Tr2)	14.1	33.5	62.70
T_3	*Aspergillus flavus*	31.3	57.4	35.00
T_4	*Penicillium chrysogenum*	33.3	69.4	22.62
T_5	Control	37.4	89.7	
CD at 5%		1.24	1.21	1.27
S.Em (±)		0.41	0.39	0.41
C.V. (%)		3.15	1.41	1.76

*Mean of four replications

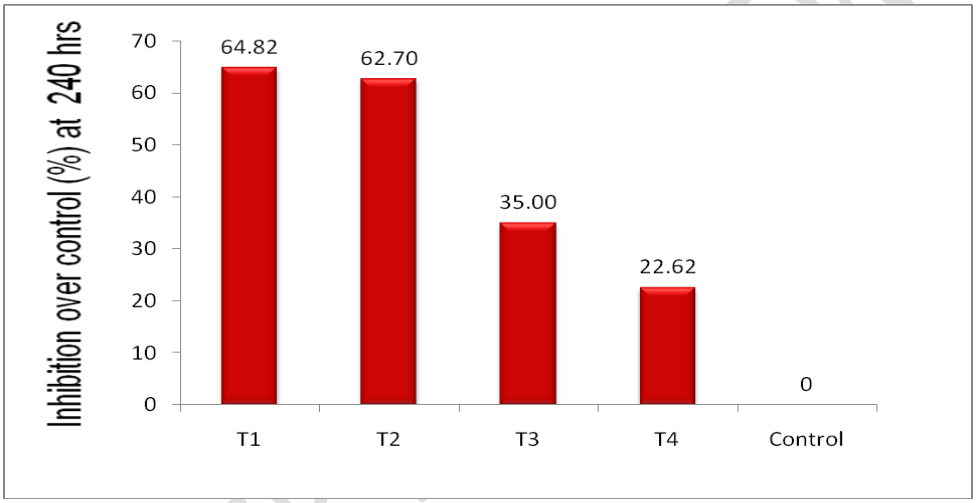


Fig-1.1. Antagonistic effect of different bio-agents isolated from the rhizosphere of wilt affected banana plants cv Grand naine on *Fusarium oxysporum* f. sp. *cubense* TR4 *in vitro*

An experiment *in vitro* and in the glasshouse in which the result found that the different *T. harzianum* isolates TH, TH 13 and UH inhibited the growth of fungus FOC TR4 of the isolate under lab condition. However, *Fusarium oxysporum* f. sp. *cubense* inoculated banana seedlings above same treatment apply in the glasshouse. Then, it was not found effective against FOC TR4 [13].New rhizospheric strain of *Trichoderma* sp. NRCB3. This was combined with the endophyte *Trichoderma asperellum* Prr2 and successfully tested against Panama wilt of banana in the field [14]. *Trichoderma harzianum* prevents up to 75.5% hindrance growth of the fungal pathogen when incubation for 72 hrs at 28±2°C *in vitro*. While,in pot conditions, *Trichoderma harzianum* will prevent disease severity in banana[15]. Experiment *in vitro* and in the poly house to determine the effects of *Trichoderma asperellum*

(B01) against the Panama wilt. Maximum 84.85% inhibition of radial growth was found in *Trichoderma asperellum* (B01) in vitro while in poly house condition spore suspension of *Trichoderma asperellum* (B01) was apply then significantly reduced Panama wilt incidence percentage up to 94.4% in comparison to control [16]. Evaluated ten strains of *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *cubense* (FOC) *in vitro* and all strains of *Trichoderma harzianum* produced volatile metabolites that inhibit the growth and development of the *Fusarium oxysporum* f. sp. *cubense* (FOC). Among the ten strains of *Trichoderma harzianum* F116 strain, the most compelling antagonist *in vitro* has a potential bioagent for biological control [17]. Studies to be found the effect of 3 isolates were *Aspergillus* against *Fusarium oxysporum* f. sp. *cubense* (FOC). *Aspergillus spp*. strain PD2, PD4, and PD5 prevent fungal mycelia growth of FOC up to 37.31, 26.52, and 12.04%, respectively [18].

References

1. De Langhe E. Banana Diversity in the Middle East (Jordan, Egypt, Oman).INIBAP. 2002; 52 pp.
2. FAOSTAT. http://faostat.fao.org/.2013.
3. Bancroft J. Report of the board appointed to enquire into the cause of disease affecting livestock and plants. In: Votes and Proceedings Queensland.1876; pp: 1011-1038.
4. Brandes EW. Banana wilt. *Phytopathology*.1919;9:339-389
5. Booth C. The Genus *Fusarium*. Commonwealth Mycol. Inst. Kew, UK.1971; pp:237.
6. Moore NY, Bentley S, Pegg KG, Jones DR. *Fusarium* wilt of banana. *Musa disease fact sheet*. INBAP, Montpellier, France.1995;5:4.
7. Ploetz RC, Herbert JP, Sebasigari K, Hernander JH, Pegg KG, Ventura JA, Mayato LS. Importance of *Fusarium* wilt in different banana growing regions. In: *Fusarium* wilt of Banana, R.C. Ploetz (ed.), APS, St. Paul, USA. 1990;pp: 9-26.
8. Bentley S, Pegg KG, Moore NY, Davis RD, Buddenhagen IW. Genetic variation among vegetative compatibility groups of *Fusarium oxysporum* f. sp. *cubense* analysed by DNA fingerprinting. *Phytopathology*. 1998;88: 1283-1288.

9. Johnson LF, Curl EA. Methods for research on the ecology of soil-borne plant pathogen. 426 So. Sixth St., Minneapolis, MN 55415: Burgess Publishing Company.1972.

10. Karunanithi K, Usman KM. Screening of *Trichoderma spp.* against *Fusarium oxysporum* f. sp. *sesame* causing wilt in sesamum. *Crop research*. 1999;18(1): 127-130.

11. Vincent JM. Determination of percent inhibition *in vitro*. *Nature.* 1927;159:850.

12. Dennis C, Webster J. Antagonistic properties of species-groups of *Trichoderma* I. Production of non-volatile antibiotics. Trans. Br. Mycol. Soc. 1971;57, 25-39.

13. Wibowo A, Santosa AT, Subandiyah S, Hermanto C, Taylor MFP. Control of *Fusarium* wilt of banana by using *Trichoderma harzianum* and resistant banana cultivars. Proceedings of the 4th international symposium on tropical and subtropical fruits. Acta Hort. 2013;975I:SHS.

14. Thangavelu R, Gopi M. Combined application of native *Trichoderma* isolates possessing multiple functions for the control of *Fusarium* wilt disease in banana cv. Grand Naine. *Biocontrol science and technology*.2015; *25*(10), 1147-1164.

15. Khan, Babri, Akash Z, Shahzad A, Javed N, Rajput NA, Jabbar A, Din UW, Atif MR. Antagonistic Potential of *T.harzianum* against *Fusarium oxysporum* f. sp. *cubense* associated with Panama wilt of Banana. *Pakistan Journal of Phytopath.* 2017;29(01):111-116.

16. Izzati MZ, Maryam SS, Azwady NA. Suppression of *Fusarium* wilt of banana with an application of *Trichoderma asperellum* inoculants. IOP Conference Series: Earth and Environmental Science. 2019; 260.012119.

17. Napitupulu TP, Ilyas M, Kanti A, Sudiana IM. In vitro evaluation of *Trichoderma harzianum* strains for the control of *Fusarium oxysporum* f. sp. *cubense.* Plant Pathology & Quarantine.2019;9(1),152–159.

18. Hidayat I, Dewi LC, Sukmawati D. Antagonistic activity of three *Aspergillus* isolates against *Fusarium* wilt of banana. Journal of Microbial Systematics and Biotechnology .2019;1(1), 1-10.