ORGANIC ACID PRODUCTION EFFICIENCY OF DIFFERENT PHOSPHATE SOLUBILIZING *TALAROMYCES PINOPHILUS* STRAINS

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Abstract. Soils are generally low in phosphorus (P) readily available for plants affecting their growth and yield. Microorganisms play an important role in improving available P content in the soil through the solubilization process. Although the mechanism of phosphate solubilization is still not well documented now, the most important aspect is recognized to be responsible for the solubilization of phosphorus is the production of different types of organic acids. Previously we studied the P solubilization potential of 17 *Talaromyces pinophilus* strains. Therefore, the present study aimed to evaluate the organic acid production efficiency of these fungal strains in broth containing insoluble tricalcium phosphate [$Ca_3(PO_4)_2$], aluminum phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). Results showed that both the type and quantity of organic acid production depended on the P source and fungal strains. The test fungal strains produced highest amount of acetic, formic and lactic acids in the medium supplemented with $Ca_3(PO_4)_2$, citric and oxalic acids were produced in the medium supplemented with $AlPO_4$ and tartaric and malic acids were produced in the medium supplemented with $FePO_4$. In conclusion, the strain NBRC106907 has the strongest ability to produce organic acids considering the whole study followed by SI-4URAgr and JCM5593. These strains may become a potential bioresource for agricultural and industrial purposes.
Keywords: *phosphorus, Phosphate solubilizing fungi, organic acids, biofertilizer, crop production*

Introduction

Phosphorus (P) is a key nutrient required for plant growth and development which plays an influential role in biochemical and physiological activities of plants (Mittal et al., 2008; Chai et al., 2011; Li et al., 2020). Most of the soils in the world having insoluble phosphate that can't be utilized by the plants unless solubilization (Singh et al., 2011). There is little available inorganic phosphorus to be absorbed and utilized by crops in the soil, whereas the majority of phosphorus is in insoluble forms that crops can't utilize (Busato et al., 2017). Acidic environment can enhance the solubility of P minerals significantly which is a possible major pathway to increase soluble P release from phosphate minerals (Li et al., 2016). Soil microbes solubilize insoluble inorganic

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phosphates and convert them available to the plants (Pradhan and Sukla, 2005; Wang et al., 2018). Although the precise mechanism of phosphate solubilization utilized by different phosphate solubilizing microorganisms (PSM) still not clear now, the production of organic acid is recognized as main mechanism responsible for P solubilization (Nahas, 1996; Alam et al., 2002; Siddique and Robinson, 2003). There are many significant roles of organic acids in agriculture. Soil organic acids have been reported to have both direct and indirect roles in crop production (Barea et al., 2005) and their presence in the soil improves the physico-chemical properties of the soil and may help facilitate uptake of deficient/unavailable/ insoluble nutrients (Morgan et al., 2005). Organic acids also have huge industrial applications as food supplement, pharmaceutical and cosmetic diluents and they are fully degradable molecules and can be used as chemical intermediates or as for the production of biodegradable polymers replacing synthetic chemicals (Sauer et al., 2008).

A large number of phosphate solubilizing microorganisms including bacteria, fungi and actinomyces have the ability to secrete organic acids (Kavanagh, 2011) and they help to dissolving insoluble P through the process of acidification, chelation and exchange reaction, which promote plant growth (Gerretsen, 1948; Singh et al., 2011; Behera et al., 2017). The ability of organic acids secretion by fungi is 10 times higher than bacteria (Kavanagh, 2011). Among the microorganisms, fungal species belonging to *Aspergillus, Penicillium, Talaromyces* and *Eupenicilium* have shown potential for solubilization of insoluble P compounds and they are considered as "key organisms" in the P cycle (Whitelaw, 2000; Achal et al., 2007; Jain et al., 2017).

The organic acid producing capabilities of phosphate solubilizing microorganisms are primarily determined by gene but can also be affected by environmental conditions. For example, carbon and nitrogen could affect the types of organic acids and phosphate solubilizing (Narsian and Patel, 2000).

In the previous study, 17 *Talaromyces pinophilus* (anamorph: *Penicillium pinophilum;* Thom, 1910*)* strains solubilized different insoluble phosphate compounds such as $Ca_3(PO_4)_2$; TCP, $AlPO_4$; Al-P and $FePO_4$; Fe-P (Majumder et al., 2019). However, the organic acid production abilities of these strains in different P substrates are unidentified. In this study, we evaluated the organic acid production ability of phosphate solubilizing *T. pinophilus* in the medium supplemented with different insoluble phosphate compounds.

Materials and Methods

Fungal strains

Seventeen *Talaromyces pinophilus* fungal strains were used in this study where 4 strains isolated from subtropical soils in Okinawa, Japan and 13 strains collected from different institutions in Japan (*Table 1*). The isolates were cultured on potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, MD, USA) slant and kept at 4^{o}C for further study.

Culture medium

Pikoveskaya's (PKV) broth medium consisted of 10.0 g glucose, 5.0 g $Ca_3 (PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.02 g NaCl, 0.02 g KCl, 0.003 g $FeSO_4 \cdot 7H_2O$, 0.003 g $MnSO_4 \cdot H_2O$, 0.5 g yeast extract and 1000 ml distilled water was used for this

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study (Rao, 1982). In this medium $Ca_3(PO_4)_2$ was used as source of insoluble phosphate compound that was replaced by insoluble $FePO_4$ and $AlPO_4$. The medium was autoclaved at 121 ^{0}C for 15 minutes. Chloramphenicol (Wako Pure Chemical Corporation, Osaka, Japan) was also used to avoid bacterial growth.

Table 1. *List of the fungal strain used in the study*

Isolates	Strain in GenBank	Source	Country of origin	Fungi	Institution
1	SI-4URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
2	SI-15URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
3	SI-17URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
4	SI-19URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
5	IFM 64651	Sputum	Japan	*Penicillium pinophilum*	Chiba University
6	IFM 57309	Sputum	Japan	*Penicillium pinophilum*	Chiba University
7	NBRC 6345	Radio set	Papua New Guinea	*Talaromyces pinophilus*	NITE BRC
8	NBRC 100533	Polyvinyl chloride plastic	France	*Talaromyces pinophilus*	NITE BRC
9	NBRC 33285	Polyvinyl chloride plastic	France	*Talaromyces pinophilus*	NITE BRC
10	NBRC 106907	Soil	Japan	*Penicillium pinophilum*	NITE BRC
11	NBRC 9575	Polyvinyl chloride plastic	France	*Penicillium allahabadense*	NITE BRC
12	JCM 9928	Soil	India	*Penicillium pinophilum*	RIKEN
13	JCM 5593	Radio set	Papua New Guinea	*Penicillium pinophilum*	RIKEN
14	JCM 22801	Wood stakes	Australia	*Penicillium pinophilum*	RIKEN
15	JCM 22802	Barley grain	Australia	*Penicillium pinophilum*	RIKEN
16	JCM 22803	Moldy sorghum grain	Australia	*Penicillium pinophilum*	RIKEN
17	JCM 23043	Radio set	Papua New Guinea	*Penicillium pinophilum*	RIKEN

NITE; National Institute of Technology and Evaluation, Biological Resource Center, NITE (NBRC), Japan. RIKEN; is a large scientific research institute in Japan

Spore suspension preparation

For conducting organic acid production experiment, fungal cultures were made from the re-slanting of pure culture slants that preserved at 4^0C. Sporulated culture slants were selected for preparation of spore suspension by using standard procedure. A total volume of 5 ml sterile water with 0.02% of tween 80 (Polyoxyethylene sorbitan monooleate, Nacalai Tesque, Inc, Kyoto, Japan) was added in culture slants and the fungal colony surface was lightly scraped by a sterile inoculation loop (Thermo Scientific^{TM}, Nunc^{TM} Disposable Loops and Needles, Thermo Scientific^{TM} 251586, Fisher Scientific, Tokyo, Japan).Then cultures were passing through a syringe with a 4×4 cm sheet of a sterile absorbant cotton (Kyualet, Kawamoto Sangyo, Osaka,Japan). Spore count was done by a hemocytometer and the suspension was adjusted to approximately10^6 spores mL^{-1}.

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Incubation

The experiments were carried out using Erlenmeyer flask containing 40 ml Pikoveskaya's (PKV) broth medium supplemented with 0.5% tricalcium phosphate (Ca_3PO_4; TCP), aluminium phosphate ($AlPO_4$; AL-P) and iron phosphate ($FePO_4$; Fe-P). After sterilization, the medium of each flask was inoculated with the 5 % (v/v) spore suspension of a particular fungal strain containing 10^6 spore mL^{-1}. Sterile distilled water inoculated flaks was treated as control (*Fig. 1*). Three replicates were maintained for each test isolate. Incubation was done at 25^0C in an incubator shaker at 120 rpm up to 7 days. The samples were autoclaved and centrifuged at 5000 rpm for 25 minutes to remove any suspended solids and mycelial parts. The culture supernatants were filtered through 0.22 µm pore size syringe filter unit (Merck KGaA, Darmstadt, Germany).

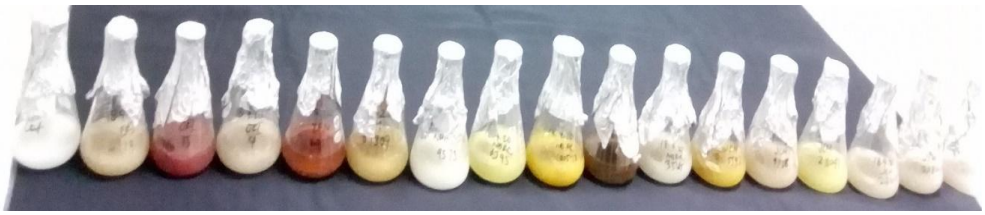

Figure 1. *Farmented Pikovskaya broth culture for organic acid determination by HPLC inoculated with phosphate solubilizing T. pinophilus strains*

Organic acids analysis

Detection and quantification of organic acids were done by High Performance Liquid Chromatography (Prominence HPLC system, Shimadzu-CBM-20A, Japan) equipped with diode array detector (SPD-M20A), refractive index detector (RID-10A), column ICE-ION-300 (300 mm X 7.8 mm), auto sampler (LC-20AD) and fraction collector (FRC-10A). The injection volume, temperature and flow rate was 50 µl, 50ºC and 0.5 ml/min, respectively. Sulfuric acid (0.01N) (Merck, Germany) was used as solvent of mobile phase. Peaks were identified against a set of standards from known organic acids (oxalic, citric, tartaric, malic, lactic, formic and acetic acids; Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

All experiments were conducted in triplicate and data were analyzed using Microsoft Excel program (version 2016). The mean values were compared by Fisher test and significant differences were detected at $p< 0.05$ level.

Results

We detected and quantified seven different organic acids, oxalic, citric, tartaric, malic, lactic, formic and acetic from medium containing TCP, Al-P and Fe-P.

Organic acid production by fungal strains in the medium supplemented with insoluble P compounds [$Ca_3(PO_4)_2$; TCP, $AlPO_4$; AL-P and $FePO_4$; Fe-P]

In the medium supplemented with TCP, all the strains produced oxalic, citric, tartaric, lactic, formic and acetic acids except malic acid in the medium. The amount ranged

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from 2.3-26.7, 3.0-404.3, 7.3-77.0, 8.0-285.0, 17.3-484.0, 9.0-142.0 µg/ml, respectively. Excepting SI-15URAgr and JCM 9928, other strains produced malic acid ranged from 17.0-266.0 µg/ml. The highest amount of oxalic (26.7 µg/ml), citric (404.3 µg/ml), tartaric (77.0 µg/ml), malic (266.0 µg/ml) and formic (484.0 µg/ml) were produced by the strain JCM 22803, NBRC 9575, NBRC 6345, NBRC 106907 and SI-17URAgr, respectively whereas the strain SI-19URAgr produced higher both lactic (285.3 µg/ml) and acetic acids(142.3 µg/ml) (*Table 2*).

Table 2. *Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with $Ca_3(PO_4)_2$, (TCP) compound by phosphate solubilizing T. pinophilus strains*

Strains	Fungi	Organic acid (µg/ml)						
		Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic
SI-4URAgr	*T. pinophilus*	22.0±2.5^{b}	7.3±0.5^{d}	42.7±9.0^{b}	115.3±2.5^{def}	126.3±6.5^{cd}	107.0±8.0^{efg}	24.0±4.0^{fi}
SI-15URAgr	*T. pinophilus*	15.3±3.0^{bc}	19.7±3.0^{d}	42.7±4.5^{b}	N. D.	21.0±4.0^{h}	167.0±11.0^{c}	37.0±4.0^{efg}
SI-17URAgr	*T. pinophilus*	9.7±3.0^{cf}	24.3±6.1^{d}	50.7±3.5^{b}	17.0±1.0^{ij}	104.3±13.5^{e}	484.0±13^{a}	46.0±5.5^{def}
SI-19URAgr	*T. pinophilus*	5.7±1.5^{efg}	6.7±1.5^{d}	25.0±4.6^{cd}	121.0±14.1^{def}	285.3±19.5^{a}	140.0±17.5^{cde}	142.3±19.2^{a}
IFM 64651	*P. pinophiilum*	9.0±2.0^{cg}	6.0±1.0^{d}	25.7±3.1^{cd}	45.7±1.5^{hi}	162.7±4.0^{c}	138.0±22.6^{cde}	45.0±8.2^{def}
IFM 57309	*P. pinophiilum*	2.3±0.6^{g}	10.0±1.0^{d}	21.0±4.6^{de}	88.7±9.5^{cd}	62.3±5.0^{fg}	73.3±5.5^{gh}	117.0±11.0^{b}
NBRC 6345	*T. pinophilus*	12.0±2.6^{cde}	6.0±1.0^{d}	77.0±9.5^{a}	250.0±19.6^{a}	25.7±5.5^{gh}	120.7±7.0^{def}	50.7±7.6^{de}
NBRC100533	*T. pinophilus*	11.0±2.0^{cf}	20.3±2.5^{d}	52.0±5.5^{b}	81.7±8.7^{g}	94.0±4.6^{ef}	116.3±10.5^{def}	54.3±7.5^{de}
NBRC 33285	*T. pinophilus*	8.0±2.6^{dg}	144.7±20.6^{c}	7.3±2.5^{e}	67.3±14.0^{gh}	9.0±2.0^{h}	70.3±16.3^{h}	21.3±2.5^{ghi}
NBRC106907	*P. pinophilum*	9.7±3.0^{cf}	254.3±34.5^{b}	36.7±1.5^{bc}	266.0±19.0^{a}	8.0±1.0^{h}	145.0±11.0^{cd}	33.0±4.0^{eh}
NBRC 9575	*P. allahabadense*	7.0±2.0^{dg}	404.3±17.2^{a}	36.7±9.5^{bc}	154.7±18.0^{bc}	13.0±3.0^{h}	17.3±4.0^{i}	82.0±7.0^{c}
JCM 9928	*P. pinophiilum*	4.3±1.5^{fg}	5.7±1.5^{d}	12.0±3.0^{de}	N. D.	14.0±2.6^{e}	101.7±11.3^{fgh}	66.3±5.0^{cd}
JCM 5593	*P. pinophiilum*	10.3±3.5^{cf}	14.5±2.1^{d}	49.0±2.0^{b}	156.7±4.5^{bc}	162.7±26.0^{c}	131.3±10.5^{def}	42.0±9.0^{efg}
JCM 22801	*P. pinophiilum*	13.0±2.0^{cd}	12.0±1.0^{d}	18.7±3.5^{de}	126.3±7.5^{cde}	109.3±10.7^{de}	216.7±12.0^{b}	28.3±2.5^{fi}
JCM 22802	*P.pinophiilum*	12.0±2.6^{cde}	11.0±2.0^{d}	10.7±3.1^{de}	94.0±3.0^{efg}	98.3±10.7^{ef}	168.3±5.5^{c}	14.0±3.0^{hi}
JCM 22803	*P. pinophiilum*	26.7±4.0^{a}	3.0±1.0^{d}	23.0±2.0^{cd}	173.0±10.0^{b}	211.3±22.5^{b}	81.3±3.5^{gh}	9.0±2.0^{i}
JCM 23043	*P. pinophiilum*	11.0±2.0^{cf}	9.7±2.5^{d}	10.7±1.5^{de}	129.0±11.5^{cd}	143.7±17.0^{cd}	125.3±8.5^{def}	36.7±9.0^{efg}

Values given are the mean of three replicates ± standard deviation of the mean. Values with common letters in each column do not differ statistically according to Duncan's Multiple Range Test (DMRT) at $p<0.05$. N.D.: Not detected. Organic acid calculated as micrograms per milliliter

In the medium supplemented with AL-P, most of the strains produced oxalic acid, citric, tartaric, malic, lactic, formic and acetic acids ranged from 1.3-46.0, 2.0-857.7, 4.7- 218.0, 31.0-132.3, 3.3-169.7, 4.3-112.7 and 10.7-122.3 µg/ml, respectively. Exception was that the strain SI-4URAgr, SI-15URAgr, SI-17URAgr and NBRC33285 could not produce malic acid, and JCM 22803 could not produce formic acids. The highest amount of citric (857.7 µg/ml), tartaric (218.7 µg/ml), malic (132.3 µg/ml), formic (112.7 µg/ml) and acetic (122.3 µg/ml) acids were produced from Al-P containing broth by the strain SI-4URAgr, NBRC 9575, JCM5593, SI-19URagr and NBRC100533, respectively whereas NBRC 6345 produced highest amount both oxalic acid (46.0 µg/ml) and lactic acid (169.7 µg/ml) (*Table 3*).

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Table 3. *Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with $AlPO_4$ (Al-P) compound by phosphate solubilizing T. pinophilus strains*

Strains	Fungi	Organic acid (µg/ml)						
		Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic
SI-4URAgr	*T. pinophilus*	22.7±4.0^{bc}	857.7±77.0^{a}	27.3±2.1^{bcd}	N.D	8.6±3.0^{ef}	38.3±10.5^{dg}	10.7±2.1^{h}
SI-15URAgr	*T. pinophilus*	2.3±0.5^{f}	3.3±1.5^{d}	30.0±4.0^{bc}	N.D.	17.0±4.0^{def}	59.0±12.0^{bcd}	21.7±3.5^{fgh}
SI-17URAgr	*T. pinophilus*	2.0±1.0^{f}	5.3±1.5^{d}	44.0±2.0^{b}	N.D	17.0±4.0^{def}	60.3±17.0^{bcd}	19.7±2.5^{fgh}
SI-19URAgr	*T. pinophilus*	6.3±1.5^{def}	2.0±1.0^{d}	25.0±6.0^{bcd}	46.0±5.0^{de}	38.0±11.0^{d}	112.7±14.2^{a}	29.0±5.1^{fgh}
IFM 64651	*P. pinophiilum*	17.0±4.0^{be}	7.0±2.0^{d}	5.0±2.0^{d}	52.3±1.5^{de}	10.0±1.0^{ef}	56.0±14.5^{be}	33.7±7.3^{efg}
IFM 57309	*P. pinophiilum*	5.3±1.5^{ef}	5.0 ±1.0^{d}	16.7 ±5.5^{cd}	86.0 ±2.0^{c}	151.0 ±27.0^{a}	19.0±8.0^{ghi}	21.3 ±3.5^{fgh}
NBRC 6345	*T. pinophilus*	46.0±13.4^{a}	6.0 ±2.0^{d}	12.7±3.2^{cd}	88.0±2.0^{b}	169.7±10.5^{a}	72.0±9.0^{bc}	86.3±10.5^{b}
NBRC100533	*T. pinophilus*	44.7±12.0^{a}	5.0 ±1.0^{d}	35.7±5.5^{bc}	62.7±17.0^{cd}	24.7±6.0^{def}	24.0±5.5^{fi}	122.33±19.1^{a}
NBRC 33285	*T. pinophilus*	7.3±2.1^{def}	7.3±1.5^{d}	5.3±1.5^{d}	N.D	12.7±2.5^{def}	4.3±1.5^{hi}	22.7 ±4.5^{fgh}
NBRC106907	*P. pinophilum*	1.3±0.5^{f}	513.7±73.5^{b}	24.7±6.5^{bcd}	68.3±17.0^{bcd}	33.7±7.0^{de}	7.0±1.0^{hi}	60.3±5.0cd
NBRC 9575	*P. allahabadense*	9.7±2.5^{cf}	58.7±12.0^{cd}	218.7±26.5^{a}	31.0±5.5^{e}	20.0±2.0^{def}	29.3±6.0^{eh}	26.7±6.0^{fgh}
JCM 9928	*P. pinophiilum*	1.3±0.5^{f}	140.3±19.2^{c}	22.7±2.5^{bcd}	N. D.	34.0±9.5^{de}	37.0±6.0^{dg}	52.7±5.5^{cde}
JCM 5593	*P. pinophiilum*	23.0±4.0^{bc}	6.3±1.5^{d}	6.3±1.5^{d}	132.3±6.5^{a}	120.3±16.5^{b}	52.7±11.5^{bf}	69.3±11.4^{bc}
JCM 22801	*P. pinophiilum*	24.0±3.0^{bc}	9.7±1.5^{d}	13.0±1.0^{cd}	51.0±3.0^{de}	158.0±7.0^{a}	75.7±12.0^{b}	42.3±6.5^{def}
JCM 22802	*P. pinophiilum*	26.0±5.0^{b}	8.3±2.5^{d}	15.7±2.5^{cd}	56.0±9.1^{cd}	3.3±1.5^{f}	110.3±6.5^{a}	32.3±8.3^{eh}
JCM 22803	*P. pinophiilum*	20.0±4.0^{bcd}	6.7±1.5^{d}	30.0±5.0^{bc}	61.3±14.6^{cd}	9.0±2.0^{ef}	N.D	17.7±3.0^{gh}
JCM 23043	*P. pinophiilum*	24.0±4.0^{bc}	7.0±2.0^{d}	4.7±1.5^{d}	79.7±4.5^{bc}	88.7±4.5^{c}	44.7±11.0^{cg}	24.3±5.0^{fgh}

Values given are the mean of three replicates ± standard deviation of the mean. Values with common letters in each column do not differ statistically according to Duncan's Multiple Range Test (DMRT) at $p<0.05$. N.D.: Not detected. Organic acid calculated as micrograms per milliliter

In the medium supplemented with Fe-P, all strains produced oxalic, citric, tartaric and lactic acids ranged from 1.3-54.7, 2.0-238.7, 4.0-288.0 and 8.0-140.0 µg/ml, respectively. The malic acid was produced ranged 9.0-727.0 µg/ml by the strain SI-4URAgr, SI-17URAgr, SI-19URAgr, IFM64651, JCM5593, JCM22802, JCM22803 and JCM23043. Most of the strains produced formic acid ranged from 6.3-119.7 µg/ml excepting IFM64651, IFM57309, NBRC33285, JCM5593, JCM22802, JCM22803 and JCM23043. All the strains except NBRC 6345 and JCM 22803 produced acetic acid ranged from 16.3-104.3 µg/ml. The highest amount of formic (119.7 µg/ml), lactic (140.0 µg/ml) and malic acids (727.0 µg/ml), whereas, acetic acid (104.0 µg/ml) and citric acid (238.7 µg/ml) were produced by the strain NBRC106907 and the strain SI-19URAgr produced highest in both oxalic (54.7 µg/ml) and tartaric acids (288.0 µg/ml) respectively (*Table 4*).

Effect of phosphate compounds in medium on the quantities of different organic acids

Insoluble phosphate compounds strongly affect the quantities of different organic acids produced by *T. pinophilus* strains. The highest amount of acetic acid, formic acid and lactic acid were produced in the medium supplemented with TCP followed by Al-P and Fe-P. On the other hand oxalic acid and citric acid were produced in the medium containing Al-P followed by TCP and Fe-P. Tartaric acid and malic acid were produced in the medium containing Fe-P followed by TCP and AL-P (*Table 5*).

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Table 4. *Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with $FePO_4$ (Fe-P) compound by phosphate solubilizing T. Pinophilus strains*

Strains	Fungi	Organic acid (µg/ml)						
		Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic
SI-4URAgr	*T. pinophilus*	3.0±1.0^{d}	21.0±4.0^{b}	4.0±1.0^{g}	371.0±55.0^{de}	107.0±7.2^{cd}	8.0±2.0^{d}	82.0±6.0^{b}
SI-15URAgr	*T. pinophilus*	4.3±1.5^{d}	8.7±2.1^{b}	31.0±6.0^{def}	N.D	24.0±6.0^{e}	15.7±3.0^{d}	23.0±4.0^{cd}
SI-17URAgr	*T. pinophilus*	35.0±2.0^{b}	2.0±1.0^{b}	245.7±6.0^{b}	9.0±5.0^{f}	54.0±6.5^{d}	119.0±21.5^{a}	71.3±24.5^{b}
SI-19URAgr	*T. pinophilus*	54.7±1.5^{a}	7.0±2.0^{b}	288.0±5.0^{a}	71.0±4.0^{f}	43.0±6.0^{a}	103.7±16.2^{ab}	64.3±8.5^{b}
IFM 64651	*P. pinophiilum*	14.0±3.6^{c}	25.3±2.5^{b}	8.0±2.0^{g}	294.7±30.8^{e}	140.0±8.5^{bc}	N.D	60.0±13.0^{b}
IFM 57309	*P. pinophiilum*	1.3±0.5^{d}	7.3±2.5^{b}	28.0±1.0^{ef}	N.D	23.3±5.6^{e}	N.D	26.3 ±9.5^{c}
NBRC 6345	*T. pinophilus*	6.0±1.0^{d}	8.0±3.0^{b}	40.0±3.0^{d}	N.D	19.0±4.0^{e}	19.33±3.5^{d}	N.D
NBRC100533	*T. pinophilus*	3.0±1.0^{d}	216.0±26.7^{a}	41.0±2.0^{d}	N.D	19.0±2.0^{e}	83±5.0^{b}	23.0±2.0^{cd}
NBRC 33285	*T. pinophilus*	3.3±1.5^{d}	8.0±2.0^{d}	7.0±2.0^{g}	N.D	8.0±1.0^{e}	N.D	22.0±2.0^{cd}
NBRC106907	*P. pinophilum*	3.0±2.0^{d}	238.7±39.3^{a}	29.7±0.5^{def}	N.D	15.33±4.5^{e}	6.3±1.5^{d}	104.0±15.5^{a}
NBRC 9575	*P. allahabadense*	4.7±2.0^{d}	6.3±1.5^{b}	66.7±6.5^{c}	N.D	35.0±16.0e	45.3±2.5^{c}	23.0±2.0^{cd}
JCM 9928	*P. pinophiilum*	2.3±0.5^{d}	21.3±4.5^{b}	20.0±1.0^{f}	N.D.	14.7±3.2^{e}	45.3±3.0^{c}	31.3±5.5^{c}
JCM 5593	*P. pinophiilum*	2.0±1.0^{d}	19.0±2.0^{b}	37.7±6.0^{de}	727.0±67.1^{a}	110.0±19.0^{d}	N.D	23.0±2.0^{cd}
JCM 22801	*P. pinophiilum*	2.0±1.0^{d}	7.0±1.0^{b}	33.0±2.0^{de}	N.D	37.0±4.0^{e}	119.7±6.0^{a}	21.3±2.5^{cd}
JCM 22802	*P. pinophiilum*	2.0±1.0^{d}	20.0±2.6^{b}	33.3±3.5^{de}	446.0±39.5^{cd}	19.0±7.0^{e}	N.D	16.3±4.0^{cd}
JCM 22803	*P. pinophiilum*	2.7±2.1^{d}	9.3±1.5^{b}	31.7±3.2^{de}	534.0±54.8^{bc}	94.3±15.5^{b}	N.D	N.D
JCM 23043	*P. pinophiilum*	2.3±1.1^{d}	20.7±5.7^{b}	29.7±1.5^{def}	571.7±48.2^{b}	87.0±21.0^{d}	N.D	19.3±1.5^{cd}

Values given are the mean of three replicates ± standard deviation of the mean. Values with common letters in each column do not differ statistically according to Duncan's Multiple Range Test (DMRT) at $p<0.05$. N.D.: Not detected. Organic acid calculated as micrograms per milliliter

Table 5. *Phosphate sources effect on the quantities of different organic acids produced by phosphate solubilizing T. Pinophilus strains*

Insoluble P sources	Organic acid (µg/ml)						
	Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic
TCP	179.3	959.5	541.3	1886.3	1651.0*	2403.7*	849.0*
Al-P	283.0*	1649.3*	537.3	814.0	915.7	802.3	693.3
Fe-P	145.7	645.7	974.3*	3024.3*	850.7	565.3	610.3
Mean±SD	202.7±71.6	1145.9±501.9	684.3±251.2	1917.8±1105.2*	1139.3±444.5	1256.0±1000.0	717.5±121.2

An asterisk (*) indicated outstanding values of produced organic acids, It was higher than sum of the mean and standard deviation of organic acids produced by 17 *T. pinophilus* strains, Values given are the mean ± standard deviation of organic acids produced by 17 *T. pinophilus* strains

Comparison of produced organic acids from different P substrate

The strongest organic acid production ability of *T. pinophilus* strains was found in medium containing TCP followed by Fe-P and Al-P. The produced organic acid ranged between 204.0-752.7 µg/ml, 59.7-965.3 µg/ml and 48.3-918.7 µg/ml in TCP, Al-P and Fe-P medium, respectively. Among the isolates, the highest amount of organic acid was produced by strain SI-4URAgr (965.3 µg/ml) followed by JCM5593 (918.7 µg/ml) and NBRC106907 (752.7 µg/ml). The strain SI-4URAgr, SI-17URAgr, SI-19URAgr, NBRC106907, NBRC9575, JCM5593, JCM22803 and JCM 23043 were considered as

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outstanding because their produced organic acid was higher than sum of the mean and standard deviation of organic acid produced by 17 *T. pinophilus* strains (*Table 6*). Investigated result showed that the stain NBRC106907 had outstanding performance in both TCP and AL-P broth medium. HPLC chromatograms of highest organic acid producing fungal strains shown in (*Fig. 2*).

Table 6. *Comparison between different P substrate (TCP, Al-P and Fe-P) in Pikoveskaya's broth based the quantity of produced organic acids by T. pinophilus strains*

Strain	Fungi	Organic acid (µg/ml) released in medium		
		TCP	AL-P	Fe-P
SI-4URAgr	*Talaromyces pinophilus*	435.0	965.3*	596.0
SI-15URAgr	*Talaromyces pinophilus*	302.7	133.3	106.7
SI-17URAgr	*Talaromyces pinophilus*	736.0*	148.3	536.3
SI-19URAgr	*Talaromyces pinophilus*	726.0*	259.3	631.7
IFM 64651	*Penicillium pinophiilum*	432.0	181.3	542.7
IFM 57309	*Penicillium pinophiilum*	374.7	304.3	86.3
NBRC 6345	*Talaromyces pinophilus*	542.0	480.7	92.3
NBRC100533	*Talaromyces pinophilus*	429.7	318.3	385.0
NBRC 33285	*Talaromyces pinophilus*	328.0	59.7	48.3
NBRC106907	*Penicillium pinophilum*	752.7*	709.0*	397.0
NBRC 9575	*Penicillium allahabadense*	715.0*	394.0	181.0.
JCM 9928	*Penicillium pinophiilum*	204.0	288.0	135.0
JCM 5593	*Penicillium pinophiilum*	566.5	410.0	918.7*
JCM 22801	*Penicillium pinophiilum*	524.3	373.7	220.0
JCM 22802	*Penicillium pinophiilum*	408.3	252.0	536.7
JCM 22803	*Penicillium pinophiilum*	527.3	144.7	672.0*
JCM 23043	*Penicillium pinophiilum*	466.0	273.0	730.7*
	Mean±SD	498.2±162.0*	335.0±223.6	401.0±268.7

TCP: tricalcium phosphate; Al-P: aluminium phosphate and Fe-P: iron phosphate, An asterisk (*) indicated outstanding values of produced organic acids. It was higher than sum of the mean and standard deviation of organic acids produced by 17 fungal strains. It also indicated the best substrate for organic acid production, Values given are the mean ± standard deviation of organic acids produced by 17 *T. pinophilus* strains

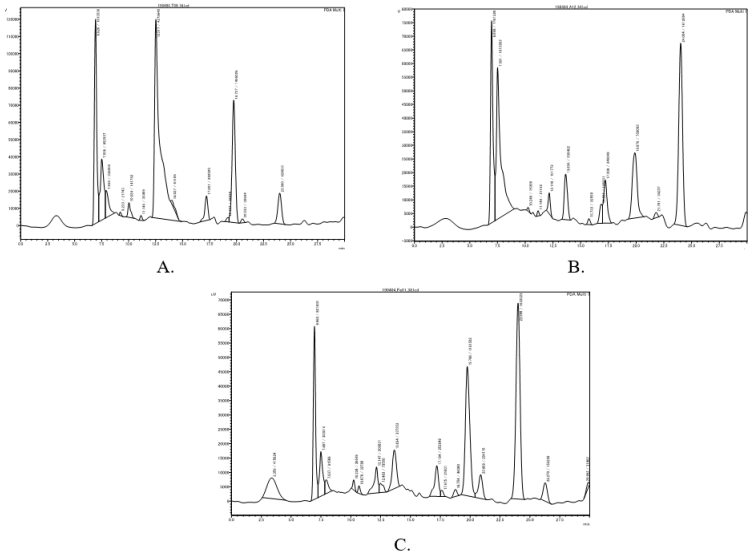

Figure 2. *Chromatograms of organic acids analyzed by HPLC. The highest organic acids were produced by P solubilizing T. pinophilus strains; A. SI-4URAgr, B. JCM5593, C. NBRC106907*

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Discussion

In the present study, results showed that the type and the amount of organic acids produced by *T. pinophillus* strains were varied on both fungal strains and phosphate substrate. TCP supplemented medium enhanced acetic acid, lactic acid and formic acid production but Fe-P medium enhanced the production of malic and tartaric acid. The Al-P medium enhanced the production of oxalic and citric acid. Based on the total quantity of organic acids, TCP medium is the best followed by Fe-P and Al-P. It might be the result of interaction between fungal strains and the P sources (Scervino et al., 2013; Zang et al., 2018).

In our study, eight strains of *T. pinophilus* (SI-4URAgr, SI-17URAgr, SI-19URAgr, NBRC106907, NBRC9575, JCM5593, JCM22803 and JCM23043) could produce higher amount of organic acids in the medium supplemented with TCP, Al-P and Fe-P. Among the isolates, the strain SI-17URAgr and NBRC 9575 could produce higher amount of organic acids in the medium supplemented with TCP, whereas NBRC106907 produced higher organic acids in both TCP and AL-P medium. The strain SI-4URAgr also could produce higher organic acids in the medium supplemented with AL-P. The strain JCM5593, JCM22803 and JCM23043 were capable to produce higher amount of organic acids in the medium supplemented with Fe-P. It suggested that the type and the quantity of organic acids produced by each fungal species varied according to the P source (Jose et al., 2010). Vyas and Gulati (2009) have reported that organic acid production by microorganism is independent of their genetic relatedness and each strain has its own ability of producing organic acid during the P solubilization. Jose et al. (2010) suggested that the production of organic acids depends both on the microorganism and source of P in which the microorganisms grows. Cunningham and Kuiack (1992) reported that total amount and type of acids produced in the medium affect the solubilization of the different sources of P. Phosphorus is fixed as insoluble iron and aluminium phosphates in acidic soil or as calcium phosphates in alkaline soils (Zhang et al., 2001). Soil microbes use organic acids for various purposes such as making available relatively insoluble elements in the soil for themselves and plants (Morgan et al., 2005; Balogh-Brunstad et al., 2008). These organic acids decrease the pH of the medium that is responsible for solubilization of the insoluble phosphates (Chen et al., 2006). Some of the phosphorus-solubilizing microbial strains produce phosphorus-hydrolyzing enzymes known as phosphatases and phytases, in addition to organic acids (Rodriguez et al., 1999; Pawar et al., 2009). These enzymes convert insoluble phosphates to the soluble phosphorus. Bacterial cells and fungal mycelia require phosphorus for their own growth; thus, they produce phosphorus-hydrolyzing enzymes. Large quantities of phos-phates are available in soil, and when the microbial culture is added in the form of biofertilizer, they solubilize the phos-phates. The soluble phosphorus is then easily available for plant.

T. pinophilus has received increasing attention in mycological research for its ability to act as a fungal antagonist and plant-growth promoter (Nicoletti et al., 2004; Pandey et al., 2008; Wani et al., 2016). They are capable of dissolving insoluble phosphate compounds in soil. Out of P solubilization activitiy, *T. phinophilus* are considered as the significant source of enzymes, pigments and secondary metabolites that are essential for sustainable crop production and industrial utilization (Zhai et al., 2015; Koul et al., 2016; Yao et al., 2017; Caro et al., 2017). They also play an important role to biomass degradation and act as bio control agent against some plant pathogen (Fujii et al., 2014; Ismail and Kamal, 2018).

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Conclusions

The results implied that both type and the quantity of organic acid production depended on the P source and the type of fungal strains. The *T. pinophilus* strains produced highest amount of acetic, formic and lactic acids in the medium supplemented with $Ca_3(PO_4)_2$, citric and oxalic acids in the medium supplemented with $AlPO_4$, and tartaric and malic acids in the medium supplemented with $FePO_4$. Overall, the strain NBRC106907 could be considered as higher organic acid producing fungi in the medium supplemented with TCP, whereas SI-4URAgr and JCM5593 produced higher amount of organic acids in the medium supplanted with Al-P and Fe-P, respectively. These strains could be potential bio-resources in agricultural production. Further research should be conducted to evaluate the performance of these outstanding strains in vivo condition.

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