

Original Research Article

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Qualitative and Quantitative Analysis of Organic Acid Production Influenced by Phosphate Sources under Submerged Culture of *Aspergillus niger* a Phosphate Solubilising Fungi

Hruda Ranjan Sahoo and Nibha Gupta *

Division of Plant Pathology and Microbiology, Regional Plant Resource Centre,
Bhubaneswar-751015, Odisha, India

*Corresponding author

ABSTRACT

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Aspergillus niger inoculated into medium containing Tricalcium phosphate (TCP) and Rock phosphate (RP) resulted in acid production which is indicated from the measure of pH and titrable acidity. Maximum of 105 mM acid production is recorded in TCP supplemented culture of 12 days whereas it is 124 mM in nutrient medium aided with RP and 14 days old culture. At the same time, decline of pH has also been observed in media containing TCP and RP. However, fungal biomass did not show any impact of both the kind of P sources. Chromatographic analysis of culture filtrate has showed the presence of citric and oxalic acid in TCP and RP respectively. Cultural conditions of different range of pH and incubation temperature do not play an important role in organic acid secretion by the fungi but organic acid secretion is mostly influenced by the nutritional sources such as carbon and nitrogenous compounds present in the medium.

Introduction

Organic acids are the source of biotical generated H⁺ ions, which can mineralize bound phosphate present in soil and make it available for the uptake by plants (Bhattacharya and Jain, 2000). The organic acid concentration in soil solution is typically low, varying from 1 to 50 mM (Strobel, 2001). Hence, microbial production of low molecular weight organic acids enhances mobilisation of phosphate compounds (Bolan *et al.*, 1994; Goldstein, 1995) which is also accompanied by acidification of the medium. Organic acids such as acetate, citrate, gluconate, lactate, malate, oxalate, succinate

etc. can form complexes with the metals such as aluminium, calcium, and iron releasing phosphate into soil from respective metal phosphates and can also increase P availability by blocking P adsorption sites on soil particles (Jones, 1998; Rodriguez and Fraga, 1999; Gyaneswar *et al.*, 2002). It is observed that tri- and dicarboxylic acids are more effective in solubilising P as compared to monobasic and aromatic acids—aliphatic acids (Mahidi *et al.*, 2011). The solubilization of different sources of P does not depend only on the total amount of acids produced but also on type of acid produced (Cunningham and

Kuiack, 1992, Kang *et al.*, 2002). However, organic acid production is also influenced by the micro-organism and P source where it grows (Scervino *et al.*, 2010). The type of organic acid produced and their amounts may also differ with different organisms. A number of factors have been considered as they also affect the organic acid production with pH lowering by microorganisms, such as the C and N sources (Di Simine *et al.*, 1998; Reyes *et al.*, 1999).

Aspergillus niger is a fungus which is known to solubilise inorganic phosphate such as calcium and aluminium phosphates (Illmer *et al.*, 1995; Barroso and Nahas; 2005) through production of organic acids such as citric, gluconic, succinic and oxalic acid along with drop in pH of the medium (Nahas *et al.*, 1990; Illmer and Schinner, 1995). Studies by Venketeswarlu *et al.*, 1984 reported lactic acid as only acid produced by *A. niger* while Vazquez *et al.*, 2000 reported succinic acid as the only acid produced by *A. niger*. Reports also reveal that carbon as well as nitrogen sources and pH of the medium greatly influence organic acid production (Gupta *et al.*, 1976).

In the present study, we have conducted experiments to observe the organic acid production at periodic intervals when the organism *A. niger* is inoculated with different phosphate sources such as Tricalcium phosphate and rock phosphate as well as on modification of culture conditions such as temperature, pH and nutritional parameters such as carbon and nitrogen sources in presence of TCP and rock phosphate.

Materials and Methods

Modified Czapekdox medium containing Tricalcium phosphate (pH 6.8) and rock phosphate (pH 7.2) were inoculated with equal amount of inoculum of *Aspergillus*

niger and incubated at 28°C for a period of 2 weeks. At a regular interval of 48 hours from period of incubation, flasks proliferated by the organism were analysed for their pH and titrable acidity. The pH change of the culture medium was recorded with the help of pH meter. Titrable acidity (TA) was estimated using or titrating 1ml of culture supernatant against 10 mM NaOH in presence of phenolphthalein indicator at regular interval (Whitelaw *et al.*, 1999). The biomass obtained was air dried and weighed.

The culture supernatant obtained was concentrated and spotted on the chromatographic paper along with the standards of organic acids. The chromatogram was run in pre saturated chamber containing solvent mixture of n-butanol, acetic acid and water in the ratio 12:3:5. The chromatogram was air dried and sprayed with bromocresol green. After air dried the Rf values of the yellow spots of organic acids developed on blue background was measured and compared with Rf values of standard organic acid for identification.

The same Czapekdox Medium was prepared with further modification of carbon and nitrogen sources (nutritional parameters) and pH and temperature (Cultural parameters) for estimation of titrable acidity and recording of pH after completion of incubation period. For studies with nutritional modification, the carbon sources such as Fructose, Glucose, Inositol, Lactose, Maltose, Mannose, Raffinose, Sorbose and sucrose were taken in the medium and pH maintained at 7 and temperature 28°C. Likewise, nitrogen sources such as Ammonium chloride, Ammonium Sulphate, L-Glutamine, L-Phenylalanine, L-Threonine, L-valine, Potassium nitrate, Urea and Sodium nitrate were taken in the medium and pH maintained at 7 and temperature 28°C. Similarly for studies with cultural modifications, the pH of the medium was

modified in the range of 5-9 and incubated at constant temperature of 28°C. The incubation temperature was modified in the range of 20-40°C with pH of the medium at 7. The amount of organic acid produced and change in pH of the medium in all the modifications was recorded.

Results and Discussion

The titrable acidity ranged from 60-76 mM organic acids during the incubation period of 2 weeks except for 12th day where it is maximum (105 mM) in case of solubilisation of TCP. Results obtained from studies by Nenwani *et al.*, 2010 also showed that highest titrable acidity is recorded after 12 days of incubation by the organism which is similar to our findings. However, there is increase in the titrable acidity during the entire period of incubation when *Aspergillus niger* is inoculated into the medium containing rock phosphate and maximum of 124 mM of total acids is produced at the end of 14th day of incubation as shown in table 1.

A significant correlation between final pH value and titrable acidity has been observed (Nahas, 1996). When the fungal strain was tested to solubilise different types of phosphate in chemosynthetic medium, medium inoculated with *A. niger* showed greater reduction in pH (Reena *et al.*, 2013). There was decrease in the pH of the medium ranging from 2-3 both in presence of TCP and rock phosphate except for 12th day in case of TCP and 8th day in case of rock phosphate where the pH is recorded as 3.74 and 3.89 respectively. The pattern of decrease in pH is similar in case of both TCP and rock phosphate supplemented medium by *A. niger*. The biomass obtained due to the proliferation of the organism during the incubation period is recorded. At the end of 14th day, the biomass obtained from both the phosphate sources is equal (0.174g) which depicts that

the organism is able to grow unanimously irrespective of the phosphate sources present in the medium either TCP or rock phosphate. A strong positive correlation of 0.924 is observed between pH and total acids secreted by *A. niger* during TCP solubilisation which is 0.49 in case of rock phosphate solubilisation. This proves that organic acid production is the key mechanism for P solubilisation which is accompanied by lowering of the pH of the medium. This fact is confirmed that production of organic acids leads to acidification of microbial cells and their surroundings resulting in the release of orthophosphate ions from the mineral phosphate (Hwangbo *et al.*, 2003; Ben *et al.*, 2009).

It has been observed that fungal culture produced carboxylic acid confirmed as titrable acidity. The culture supernatant obtained was spotted on whatman chromatographic paper No.1 along with the standards of organic acids. The Rf values obtained for the culture supernatant obtained from *Aspergillus niger* inoculated with TCP and rock phosphate were 0.9 and 0.6 respectively when compared to the standard organic acids it coincided with oxalic acid (0.91) and citric acid (0.63) respectively (Figure 1). Similarly, Illmer and Schinner, 1995 and Alam *et al.*, 2002 also reported citric and oxalic acid to be produced by *Aspergillus niger* in large amounts along with small quantities of gluconic acid.

Modification of cultural and nutritional conditions also influences the organic acid production by the organism in presence of TCP and Rock phosphate. Nutritional modifications involve replacement of carbon and nitrogen sources in the medium. In presence of TCP, all the carbon sources were effectively utilised by the organism in order to produce organic acid in the medium except lactose, sorbose and inositol since much

reduction in the pH of the culture filtrate is not observed in presence of these three carbon sources (Figure 2a). Similar trend is also noticed in case of organic acid secretion which is reflected from the measure of titrable acidity and maximum titrable acidity during TCP solubilisation is recorded in presence of glucose (127mM) followed by sucrose (116 mM). However, in presence of rock phosphate; all the carbon sources were taken

up by *Aspergillus niger* as energy source which is indicated from the amount of organic acid present in the medium except mannose. However, amount of titrable acidity is recorded higher in glucose (45mM) followed by maltose and sucrose (34 and 32 mM). Not much change in pH is observed in presence of mannose but other carbon sources showed pH in the range of 4.4-4.8 except fructose and raffinose as depicted from figure 2b.

Table.1 Periodical analysis of Biomass, organic acid and change in pH of culture filtrate of *A. niger* grown under different phosphate sources

Incubation period	Tricalcium Phosphate			Rock Phosphate		
	organic acid production in culture filtrate (mM)	Final pH of culture filtrate	Fungal biomass (in g)	organic acid production in culture filtrate (mM)	Final pH of culture filtrate	Fungal biomass (in g)
Control	-	6.8	-	-	7.28	-
Day 2	70	2.02	0.135	42	2.48	0.276
Day 4	60	2.12	0.189	87	2.78	0.212
Day 6	73	2.35	0.162	94	3.01	0.192
Day 8	76	2.85	0.192	98	3.89	0.18
Day 10	74	2.6	0.162	105	3.08	0.172
Day 12	105	3.74	0.184	107	2.84	0.18
Day 14	66	2.49	0.174	124	2.79	0.174

Table.2 Effect of pH of the culture medium on organic acid production (titrable acidity) in presence of TCP and RP

Initial pH of the medium	Tricalcium Phosphate			Rock Phosphate		
	organic acid production in culture filtrate (mM)	Final pH of culture filtrate	Fungal biomass (in g)	organic acid production in culture filtrate (mM)	Final pH of culture filtrate	Fungal biomass (in g)
5	53±1	3.68±0.021	0.187±0.003	36±4	4.25±0.23	0.097±0.012
6	42±3.2	4.14±0.16	0.186±0.016	37±2.3	4.51±0.23	0.086±0.011
7	38±2.1	4.12±0.26	0.191±0.010	25±3.1	4.20±0.09	0.081±0.005
8	37±0	3.97±0.03	0.183±0.017	19±1.53	3.99±0.16	0.089±0.012
9	40±2	4.98±0.074	0.16±0.019	19±1.73	4.51±0.19	0.093±0.017

Table.3 Effect of Temperature of the culture medium on organic acid production (titrable acidity) in presence of TCP and RP

Temperature (°C)	Tricalcium Phosphate			Rock Phosphate		
	organic acid production in culture filtrate (mM)	Final pH of culture filtrate	Fungal biomass (in g)	organic acid production in culture filtrate (mM)	Final pH of culture filtrate	Fungal biomass (in g)
20	41±6.6	3.64±0.12	0.169±0.018	36±1.73	4.41±0.05	0.089±0.005
25	47±3.8	3.44±0.015	0.201±0.019	33±4.04	4.66±0.12	0.091±0.003
30	43±3.5	3.76±0.123	0.174±0.017	33±1.15	4.61±0.07	0.100±0.012
35	46±2.5	3.96±0.11	0.177±0.014	24±2.08	4.31±0.31	0.102±0.009
40	45±2	3.97±0.114	0.163±0.006	22±1.15	4.56±0.035	0.086±0.009

Fig.1 Qualitative analysis of organic acids produced by *Aspergillus niger* under different phosphate sources (Rf values)

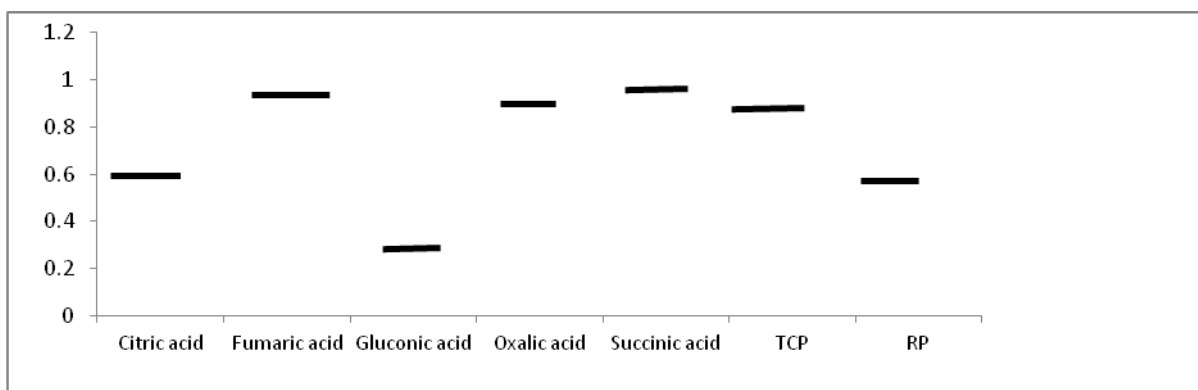


Fig.2a Organic acid secretion in form of titrable acidity in presence of TCP and RP supplemented with different carbon sources

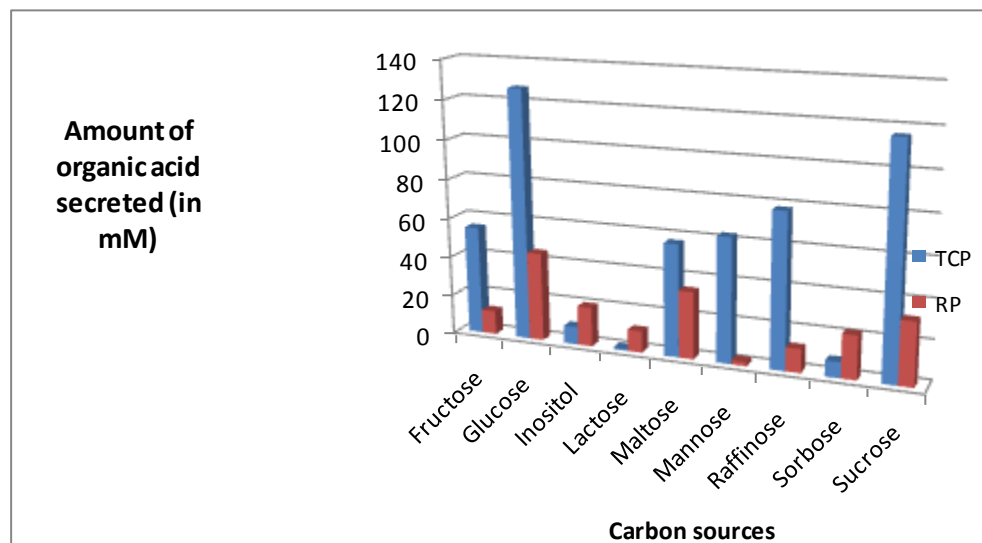


Fig.2b pH change during organic acid production supplemented with different carbon sources in presence of TCP and RP

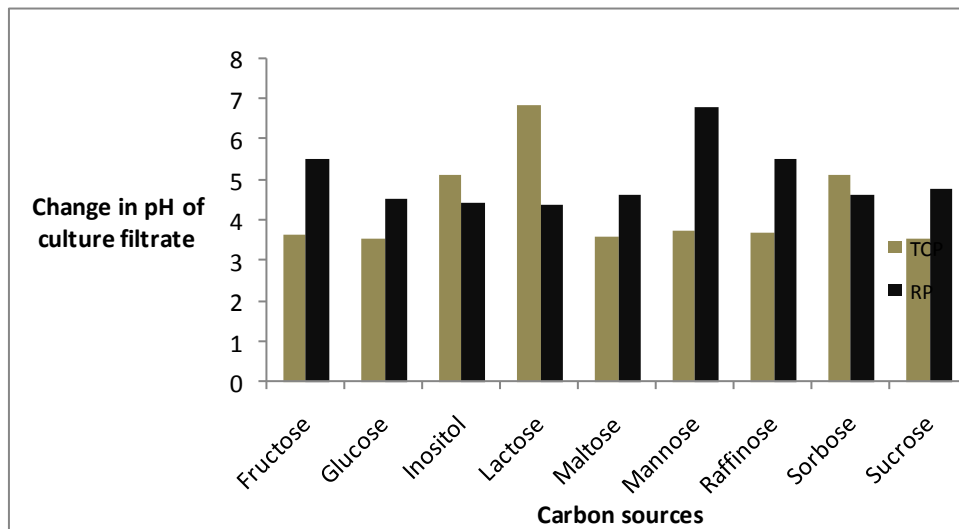


Fig.3a Organic acid secretion in form of titrable acidity in presence of TCP and RP supplemented with different nitrogen sources

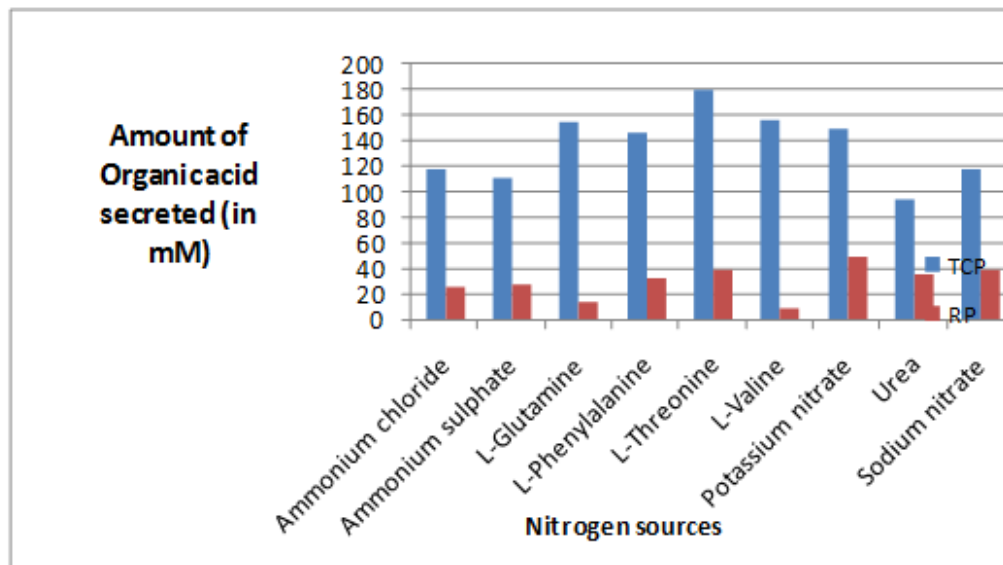
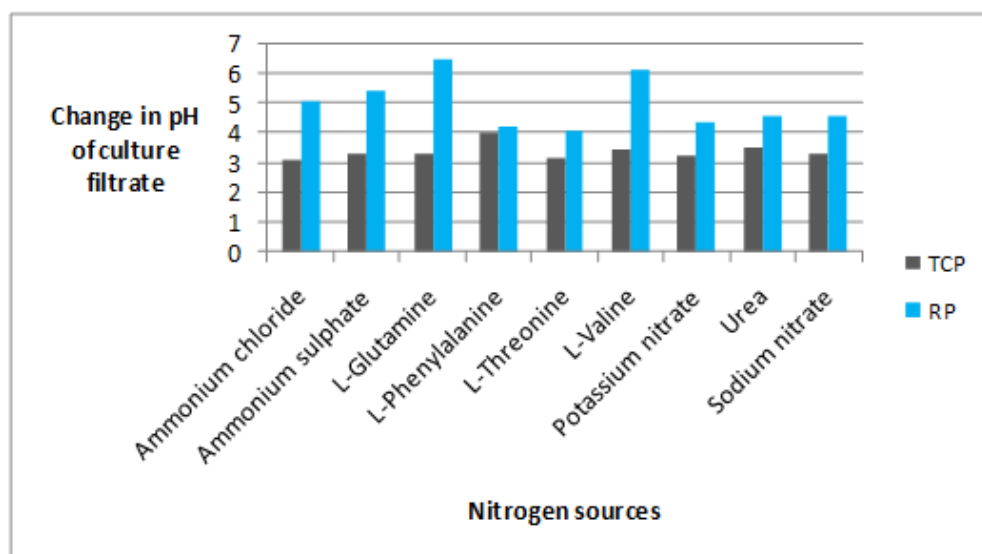


Fig.3b pH change during organic acid production supplemented with different nitrogen sources in presence of TCP and RP



For nitrogen sources, all the nitrogenous compounds and amino acids were suitably consumed by the organism for its metabolic activity since the amount of T.A. was higher in presence of TCP for all the nitrogen containing compounds with glucose as carbon source in the range of 94-179 mM suggesting that modification enhanced the organic acid producing ability of *A. niger* as shown in figure 3a. Maximum titrable acidity was recorded in medium modified with amino acid L-valine (179 mM).

Similar observation was noted for pH change in the culture filtrate with Final pH ranging from 3.12-4.03 in presence of TCP and with rock phosphate supplementation in the medium in presence of different nitrogen sources it was noticed that there is greater variation in the pH of the culture filtrate with wider pH ranging from 4.13-6.53 (Figure 3b). The titrable acidity estimated is the amount of organic acid secreted into the medium was found to be lower than that of TCP supplemented medium and highest T.A. was indicated in presence of potassium nitrate (49 mM) followed by sodium nitrate (39 mM).

Upon modification of the initial pH of the medium, the change in the final pH of the medium in presence of TCP as P source varied from 3.68 (pH 5) to 4.98 (pH 9) and amount of titrable acidity also ranged from 37-53 mM under such conditions. The pH change at different incubation temperatures also ranged from 3.44 (25 °C) to 3.97 (40°C) which suggested that organic acid is secreted into medium by lowering of pH and at ambient temperature of incubation. However, the amount of acid release at different temperature range is almost same (41-47 mM) as much difference in their values is not observed. In presence of rock phosphate, the pH change is not much significant because the final pH for all modifications is in the range of 4-4.5. Similar findings were also recorded in case of various incubation temperatures for rock phosphate also as the final pH ranged from 4.3-4.7. The amount of titrable acidity measured at different pH and temperature conditions showed that it is more in case of initial pH 6 and temperature 20°C which is 37±2.3mM and 36±1.73 mM respectively (Tables 2 and 3).

Overall, it can be concluded that cultural conditions of different range of pH and incubation temperature do not play an important role in organic acid secretion by the fungi. Hence, the organic acid secretion is mostly influenced by the nutritional sources present in the medium which regulates the metabolic pathway of the organism.

Although organic acid production influences the solubilisation process as determined from pH and titrable acidity in the present in vitro study but in vivo effect on the plant growth can only justify the potential of the strain used in this experiment.

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