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Bioprospecting Metal Tolerant Rhizospheric and Xerophytic Phylloplanic Fungi for Organic Acid Production Using Agro-Waste

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ABSTRACT

The ability of fungi to grow in contaminated and less favourable conditions makes them a significant vehicle for the synthesis of economically important chemicals like organic acids. This potential of fungi is a promising factor in the field of industrial biotechnology as the use of microbe lessens the economic burden. This study systematically investigated the bio-prospect of 26 fungal strains comprising metal-tolerant rhizospheric and xerophytic phylloplanic fungi for extracellular production of organic acid. Based on the qualitative screening (by bromocresol green plate assay) and quantitative screening (by extracellular pH profile assay), *Aspergillus niger* NJP09 was identified as the most efficient strain, exhibiting significant extracellular acidification and organic acid production. Under solid state fermentation (SSF) with rice straw as the sole carbon source, *A. niger* NJP09 produced 1.7 ± 0.15 g L^{-1} of total organic acids. High performance liquid chromatography (HPLC) analysis revealed the presence of significant concentrations of oxalic acid (2.719 mg mL^{-1}) and citric acid (0.377 mg mL^{-1}). The findings emphasize the dual advantage of metal tolerance and agro-waste utilization ability of *A. niger* NJP09 which offers economic and eco-friendly synthesis of organic acids at industrial scale. The present approach contributes to advancing circular bioeconomy strategies utilizing agro-waste for production of value-added products.

Keywords: Metal-tolerant rhizospheric fungi, Xerophytic phylloplanic fungi, Minimal salt media, Solid state fermentation, pH, agro-waste

INTRODUCTION

A specific category of bulk chemicals known as organic acids are biomolecules containing a carboxyl group, and have widespread applications in the food, textile, detergent, pharmaceutical, cosmetic, and polymer industries as food additives, chelators, and acidulants to nanomedicines (Yang *et al*., 2017). The most abundant organic acid generated annually (1.6 million tons) is citric acid, which has an average annual growth rate (AAGR) of 3.5% to 4.0% (Angumeenal & Venkappayya, 2013, Vandenberghe *et al*., 2017). Citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid), an intermediate of the Kreb's cycle is a weak organic acid. Bulk amount of citric acid has applications in the food, beverage and confectionary industries and being utilized as flavour enhancer, buffer, chelator, emulsifier, aniti-oxidant, and acidulant (Latif *et al*., 2025). Alongside, oxalic acid (ethanedioic acid) is a naturally occurring di-carboxylic acid, and is indirectly involved in various processes like bioremediation of toxins, wood degradation, rock weathering, soil nutrient availability, metal mobilization, and biodeterioration (Grąz, 2024). Oxalic acid plays a significant role in soil weathering due to its acidic and complexing properties. It chelates metal cations and enhances the release of other nutrients from mineral surfaces (Lapeyrie *et al*., 1987). Organic acids are conventionally produced using chemical processes and can also be accurately synthesised through biological processes. The growing need for more economically and ecologically acceptable bio-based processes for the production of biochemicals has led to an expansion in the market for microbe-assisted organic acid manufacturing (Ghai *et al*., 2024). With an estimated 1.5 million species worldwide, fungi are a varied collection of microorganisms that synthesizes an extensive spectrum of biomolecules viz., organic acids, secondary metabolites and enzymes in order to acclimatize to different surroundings, hence, developing a number of survival strategies (Afifa *et al*., 2022). Fungal synthesized organic acids broadly belong to four major categories, i.e., monocarboxylic acids (lactic acid), dicarboxylic acids (oxalic, malic, fumaric, itaconic, succinic and trans-epoxy succinic acid), tricarboxylic acids (citric acid) and lastly sugar acids (gluconic acid)

(Karaffa *et al*., 2021). Fungi are highly efficient to survive in harsh environmental conditions. The primary cause of their exceptional flexibility and functional adaptability to various habitats are their competent extracellular enzymatic actions that are not impacted by harsh environmental conditions (Awasthi *et al*., 2022). Their unique metabolic capabilities and stress tolerance makes them invaluable for applications in bioremediation, and other environmentally sustainable processes as well as for production of value-added products such as organic acids (Liaud *et al*., 2014). The inherent capability of fungi to naturally produce organic acids is thought to have many key roles in their survival (Ojo & de Smidt, 2023). These roles are either due to the decrease in cytoplasmic pH consecutive to their secretion or due to direct interaction of organic acids with the environment (Huang *et al*., 2024). It has been well reported that genus *Aspergillus* has the potential to use a variety of carbon sources and spontaneously accumulate large amounts of organic acids (Yang *et al*., 2017). There are two distinct categories of A*spergillus* strains that are employed for organic acid production; first are the established and well-studied strains (termed as industrial mainstays or workhorses) and second are the strains that have the potential to produce organic acids, but are not used at an industrial scale. Strains of *A. niger* viz. ATCC 1015, ATCC 11414, ATCC 9142, ATCC 12846 etc. have been reported to be used at commercial scale for the large-scale production of organic acids. The filamentous fungus *A. niger*, in particular, has been regarded as the cornerstone for the synthesis of organic acids like citric acid at an industrial scale (Yang *et al*., 2017). Moreover, fungal synthesized organic acids are generally recognized as safe (GRAS), have a high tolerance to stress conditions, and can be synthesized utilizing a variety of inexpensive waste substrates (Angumeenal & Venkappayya, 2013; Safdar *et al*., 2024). Metal tolerance in fungi embodies a notable evolutionary adaptation that enables survival in environments with high concentrations of toxic heavy metals, conditions that are typically lethal to most of the microorganisms (Khan & Gupta, 2017). Metal-tolerant fungi have evolved an array of sophisticated biological and physiological strategies to cope with and even thrive in such extreme settings. These adaptive mechanisms play vital roles in habitats that are heavily affected by industrial pollutants, mining operations, and in naturally occurring metalliferous soils (Dusengemungu *et al*., 2021). The resilience and versatility of metal-tolerant fungi highlight their ecological importance and underscore their potential for applications in bioremediation and sustainable industrial processes (Zhran *et al*., 2022). The mechanistic basis of metal tolerance in fungi involves multiple interconnected mechanisms that includes blocking target protein's binding site, inducing conformational changes in protein or by creating a stable and inactive enzyme-inhibitor complex (Robinson *et al*., 2021). Moreover, some filamentous fungi such as *Aspergillus* and *Penicillium* species incorporate metal ions into their hyphal cell walls, effectively immobilizing them and preventing metal toxicity (George *et al*., 2012; Zhang *et al*., 2023). Fungi growing in metal contaminated sites have been reported for enhanced organic acid production. Sazanova *et al*. (2015) reported high oxalic acid production by fungi in presence of zinc which helped in detoxification of Zn by forming Zn-containing crystals in fungal cultures. Organic acid production is a fundamental strategy used by fungi for nutrient mobilization and environmental adaptation. Another eminent property of fungi is their exceptional production and secretion of extracellular enzymes, constituting to their ecological success and biotechnological potential (Tian *et al*., 2019). These exoenzymes break down complex macromolecules into smaller, assimilable units, and enable fungi to exploit a broad range of nutrient sources. Enzymes like cutinases, proteases, lipases, esterases, carboxylesterases, lignocellulolytic enzymes, and some pro-oxidant ions synthesized and released by fungi are reported to degrade plastics as well (Nasrabadi *et al*., 2023; Srikanth *et al*., 2022). Several fungal genera, such as *Fusarium* and *Penicillium*, have been shown to increase their organic acid secretions in response to the presence of minerals, which fascilitate the solubilization and uptake of otherwise inaccessible nutrients (Gulliani *et al*., 2023). The utilization of inexpensive and renewable waste substrates from a range of industries, including the oil, distillery, animal fat industries, and as well as agriculture wastes (rice straw, molasses, plant oils, oil wastes, starchy substances, and lactic whey) for organic acid fermentation, has been extensively documented and examined in several research (Banat *et al*., 2014). These organic wastes contain large portions of cellulose rich components (Awasthi *et al*., 2022).

Various *Aspergillus* species viz. *A. oryzae, A. niger, A. tereus*, and *A. sojae* have been reported to efficiently breakdown the lignocellulosic compounds due to their array of extracellular enzymes and secondary metabolites like organic acids. The low molecular organic acids are the key feature of fungal machinery responsible for degradation of lignocellulose complexes. Organic acids act as diffusible agents that allow the action of fungal enzymes for degradation (Grąz, 2024). Optimization of various process parameters in the microbial fermentation can aid in enhanced organic acid production (Du *et al*., 2015).

MATERIALS AND METHODS

Fungal strains

A total of 26 fungal strains previously isolated by our group from zinc metal-rich areas of Zawar mine, Udaipur, Rajasthan (Jain *et al*., 2013), iron rich region of Udaipur, Rajasthan (Bhargava *et al*., 2013), Khetri copper mine, Khetri Nagar, Rajasthan and the phylloplanic surfaces of xerophytic plants (*Salvadora persica* and *Calotropis procera*) growing naturally in western Rajasthan, respectively, were screened in order to assess their potential for organic acid production. These fungal strains were revived from glycerol stocks (20%) and maintained on potato dextrose agar (PDA) media (pH 5.6) at 28°C.

Qualitative screening of fungal strains for organic acid production

All 26 fungal strains were qualitatively screened for the extracellular production of organic acids using bromocresol green plate assay. This assay is based on the pH-induced colour change in growth media indicated by bromocresol green dye which shifts from blue-green to yellow as the pH decreases due to the accumulation of organic acids (Dezam *et al*., 2017). For this, round pieces (0.4 mm) of fresh fungal culture were aseptically removed from a pure culture plate using sterile inoculation loop and inoculated at the centre of petri plate containing sterilized minimal Salt media (30.0 g L^{-1} glucose, 0.01 g L^{-1} $FeSO_4.7H_2O$, 0.5 g L^{-1} KCl, 1.0 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 2.0 g L^{-1} $NaNO_3$, 15.0 g L^{-1} agar, surfactant triton X-100 (1.0 mL L^{-1}), and bromocresol green (0.1 g L^{-1}) as pH indicator. The initial pH of the medium was adjusted to 7. After inoculation, the petri plates were incubated at 28°C for 7 days and the diameter of yellow halo zone formed around the fungal inoculum was measured every 24 h. An uninoculated petri plate was setup alongside as negative control. Petri plate inoculated with *A. niger* MTCC12975 culture was used as positive control as it has been reported for high organic acid production (Latif *et al*., 2025). The ability of the fungal strains to produce organic acid was indicated by the development of yellow halo zones (Dezam *et al*., 2017). Acid unitage (AU) values were calculated for the strains showing development of yellow halo zones on 4th day of incubation, as after this time-point dispersal of spores was observed over the entire media surface in petri plates (Shaikh & Qureshi, 2013).

$$\text{Acid unitage(AU)} = \frac{\text{Diameter of yellow zone}}{\text{Diameter of fungal colony}}$$

The fungal strains forming significant yellow halo zones with higher AU values were selected for further quantitative screening.

Quantitative screening of fungal strains for organic acid production

On the basis of qualitative screening results, three fungal strains were further selected for quantitative screening through pH profiling. For this, round pieces (0.4 mm) of fresh cultures were inoculated in 100 mL minimal salt media (MSM) (KH_2PO_4 7.0 g L^{-1}, K_2HPO_4 2.0 g L^{-1}, $C_6H_5Na_3O_7$ 0.5 g L^{-1}, $MgSO_4$ 0.10 g L^{-1}, $(NH_4)_2SO_4$ 1.0 g L^{-1}, and glucose 50.0 g L^{-1}) in 250 mL Erlenmeyer flasks. The initial pH of media was adjusted to 7. The flasks were incubated in shaking incubator (130 rpm) at 28°C. The pH of the inoculated culture media was measured using an electronic pH meter (Eutech, pH 700) at regular intervals of 24 h for 7 days. A negative control flask without inoculation containing sterilized MSM at pH 7 was also setup for 7 days. Flask inoculated with similar amount of *A. niger* MTCC12975 inoculum was used as positive control as it has been reported for high organic acid production.

Solid state fermentation

Based on the results of quantitative screening experiment, the best tested fungal isolate was further evaluated for its potential to synthesize organic acids under solid state fermentation (SSF) utilizing rice straw waste as the sole carbon source. Briefly, fungal spores (1×10^7 spores) were inoculated in 100 mL Erlenmeyer flasks containing rice straw and MSM in a ratio of 1:2 w/v and

incubated at 28°C under static conditions for 7 days for extracellular organic acid synthesis. A negative control flask with uninoculated sterilised rice straw and MSM (1:2 w/v) was setup alongside. The synthesized organic acids were extracted after 24 h consecutively for 7 days by adding distilled water (1:15 w/v) in the flasks followed by shaking at 150 rpm for 30 min. Then, the mixture was filtered through a cheesecloth and collected in a sterilized beaker. The pH of filtered extract was measured using an electronic pH meter (Eutech, pH 700).

Quantification of total organic acid

The quantification of total organic acid was performed for extracellular extract of cultures growing in MSM + 5% glucose media as well as in SSF utilizing rice straw and MSM (1:2 w/v) by calculating the volumetric titratable acidity using standard titration method with 0.01 M NaOH solution and 1% phenolphthalein as an indicator (Dezam *et al*., 2017). Briefly, the sample containing 3 mL extract and 7 mL distilled water was titrated with 0.01M NaOH solution. The quantity of total organic acid synthesized was measured using citric acid as the equivalence factor in grams. The titratable acidity was calculated using the following formula:

$$\text{Titratable acidity } (g\,L^{-1}) = \frac{V_{NaOH} \times M_{NaOH} \times \text{Mol. wt. of citric acid}}{V_{sample} \times n}$$

Where, V_{NaOH}, is the volume of NaOH used; M_{NaOH} is the molarity of NaOH; V_{sample} is the sample volume used for titration; and n is the acid equivalence factor (n = 3 for citric acid).

Characterization of organic acids

The concentration and type of synthesized organic acids were determined using High-Performance Liquid Chromatography (HPLC) analysis on Agilent 1260 Infinity II system (Agilent Technologies, USA). The extracellular filtrate was sterilized by passing through syringe filter (0.45 µm) and separation was performed on a Poroshell 120 EC- C18 column (4.6 mm × 150 mm, 4 µm particle size) under isocratic conditions using 50 mM KH_2PO_4 solution as the mobile phase at a flow rate of 0.6 mL min^{-1}. The column temperature was maintained at 40°C and the detection was performed using a UV-vis diode detector set at 210 nm. Citric acid monohydrate (Merck) and oxalic acid (Thermo Fisher Scientific) were used as standard for the HPLC characterization.

Graphical representation of the overall methodology is depicted in **Figure 1**.

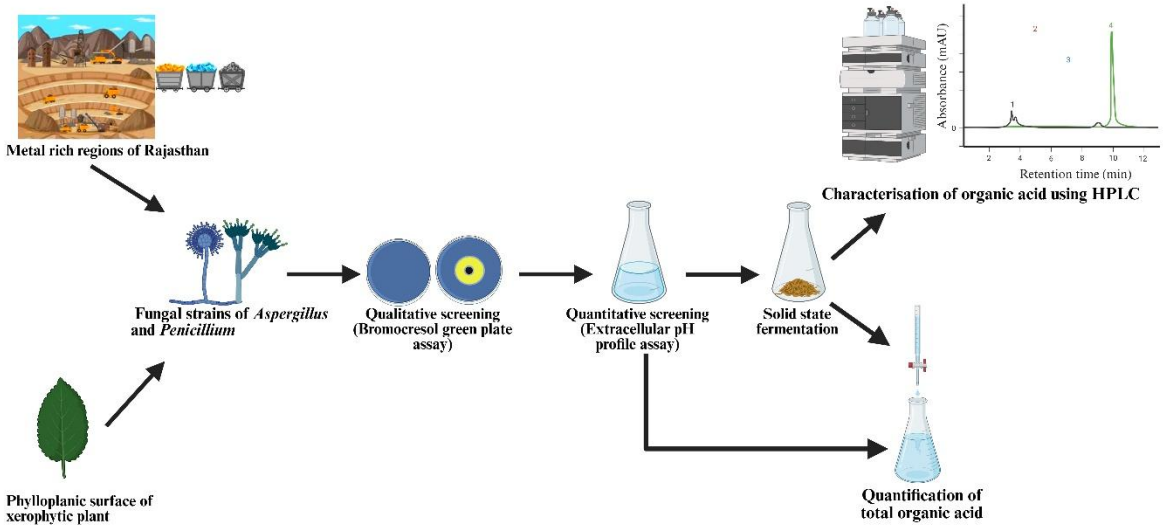

Figure 1: Graphical representation of overall methodology used in the present study.

Statistical analysis

All the experiments were carried out in triplicates and the values were reported as mean ± standard deviation (SD) represented by bars. Microsoft Excel software was used to calculate SD and GraphPad Prism V 8.0 was used for the construction of graphs.

RESULTS

Qualitative screening of selected fungal strains for organic acid production

Table 1 represents the list of 26 fungal strains (18 metal-tolerant rhizospheric fungi and 8 xerophytic phylloplanic fungi) evaluated for their potential to synthesize extracellular organic acids. The fungal strains belong to 2 different genera (*Aspergillus* and *Penicillium*), and were previously isolated by our research group from zinc and iron metal mines of Rajasthan, and phylloplane surface of xerophytic plants (*Salvadora persica* and *Calotropis procera*) growing naturally in western Rajasthan.

Table 1: Qualitative screening of metal-tolerant rhizospheric and xerophytic phylloplanic fungal strains for extracellular organic acid synthesis using bromocresol green plate assay.

Fungal strains	NCBI accession number	Colour change
Rhizospheric fungi from iron rich region, Udaipur, Rajasthan		
A. japonicus AJP01	JF770435	+++
A. flavus AJP02	JF770436	- - -
A. tamarii AJP10	KM873047	- - -
Rhizospheric fungi from Khetri copper mines, Khetri Nagar, Rajasthan		
A. tamarii DJP01	KU707929	- - -
A. ochraceus DJP05	KU707931	- - -
A. flavus DJP07	KU707928	- - -
Rhizospheric fungi from Zawar mines, Udaipur, Rajasthan		
A. oryzae NJP01	HQ710532	- - -
Aspergillus sp. NJP02	HM222932	- - -
P. commune NJP03	HQ710533	+
P. commune NJP07	HQ710537	+
A. oryzae NJP06	HQ710536	- - -
A. flavus NJP08	HM222933	- - -
A. niger NJP09	HQ710538	+++
A. oryzae NJP10	HQ710539	- - -
A. aeneus NJP12	HM222934	- - -
A. ochraceus NJP13	HQ710541	- - -
A. oryzae NJP15	HQ710543	- - -
A. oryzae NJP18	HQ710546	- - -
Phylloplanic fungi of xerophytic plants of western Rajasthan		
Aspergillus sp. VJP05	OM945716	+++
A. ochraceus VJP06	OM945723	- - -
A. ochraceus VJP10	OM952173	- - -
A. nidulans VJP11	OM945811	- - -
A. foveolatus VJP13	OM946598	- - -
Aspergillus sp. VJP15	OM946588	- - -
A. quadrilineatus VJP16	OM946591	- - -
A. welwitschiae VJP20	OM952175	- - -

(- - -) no halo zone formation; (+) halo diameter < 3 cm; (++) halo diameter ≥ 5 cm; (+++) halo diameter ≥ 8 cm

The diameter of yellow halo zone formed around tested fungal inoculums were measured and utilized for the qualitative classification of fungal strains for extracellular organic acid production (Shaikh & Qureshi, 2013). Among 26 fungal strains, 3 strains namely *A. niger* NJP09, *A. japonicus* AJP01 and *Aspergillus* sp. VJP05 showed formation of significant yellow halo zone (≥8 cm diameter) progressively by 7 days in the plate screening assay (**Figure 2**). The acid unitage (AU) values calculated for *A. niger* NJP09, *A. japonicus* AJP01 and *Aspergillus* sp. VJP05 were found to be 5.4, 3.9 and 4.1, respectively after 4 days of incubation (**Table 2)**. Based on these results, *A. niger* NJP09, *A. japonicus* AJP01 and *Aspergillus* sp. VJP05 were selected for further quantitative evaluation for extracellular organic acid production through pH profiling

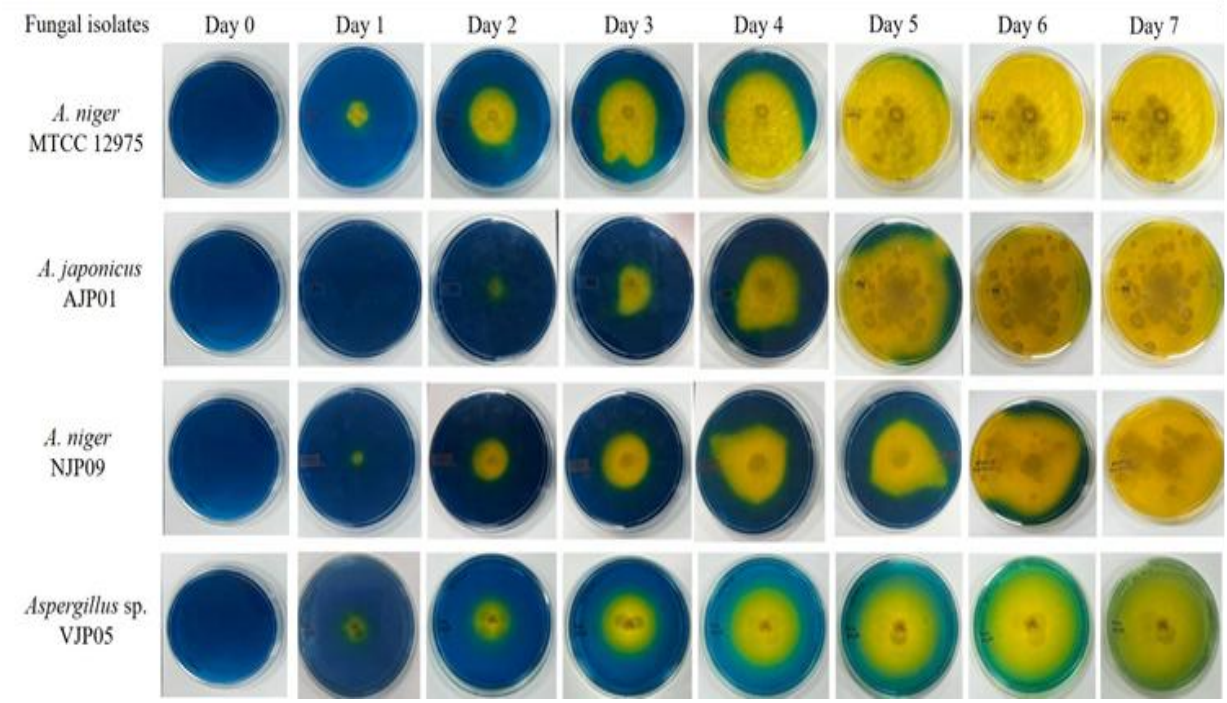

Figure 2: Bromocresol green plate assay showing extracellular organic acid production indicated by formation of yellow zone on MSM + 5% Glucose plates progressively for 7 days by *A. niger* MTCC 12975 (poitive control), *A. japonicus* AJP01, *A. niger* NJP09, and *Aspergillus* sp. VJP05.

Table 2: Acid unitage values of selected fungal strains after 4 days of qualitative screening.

Fungal isolate	Diameter (mm) of yellow zone on 4th day	Diameter (mm) of fungal colonies	Acid unitage (AU)
A. niger MTCC12975	56	10	5.6
A. niger NJP09	49	9	5.4
A. japonicus AJP01	47	12	3.9
Aspergillus sp. VJP05	46	11	4.1

Quantitative screening of selected fungal strains for organic acid production

The time-dependent extracellular pH profile was recorded in order to quantitatively estimate the amount of extracellular organic acid produced by the three selected *Aspergillus* strains in comparison to controls (**Figure 3**). Amongst the three tested strains, *A. niger* NJP09 showed maximum pH reduction (2.43 ± 0.12) in the culture media at 7th day, which was almost in the same range of the tested positive control i.e. *A. niger* MTCC 12975 (pH 2.44 ± 0.14). It was followed by *Aspergillus sp.* VJP05 and *A. japonicus* AJP01 which showed pH values of 3.33 ± 0.57 and 3.31 ± 0.55 respectively at 7th day in MSM + 5% glucose broth.

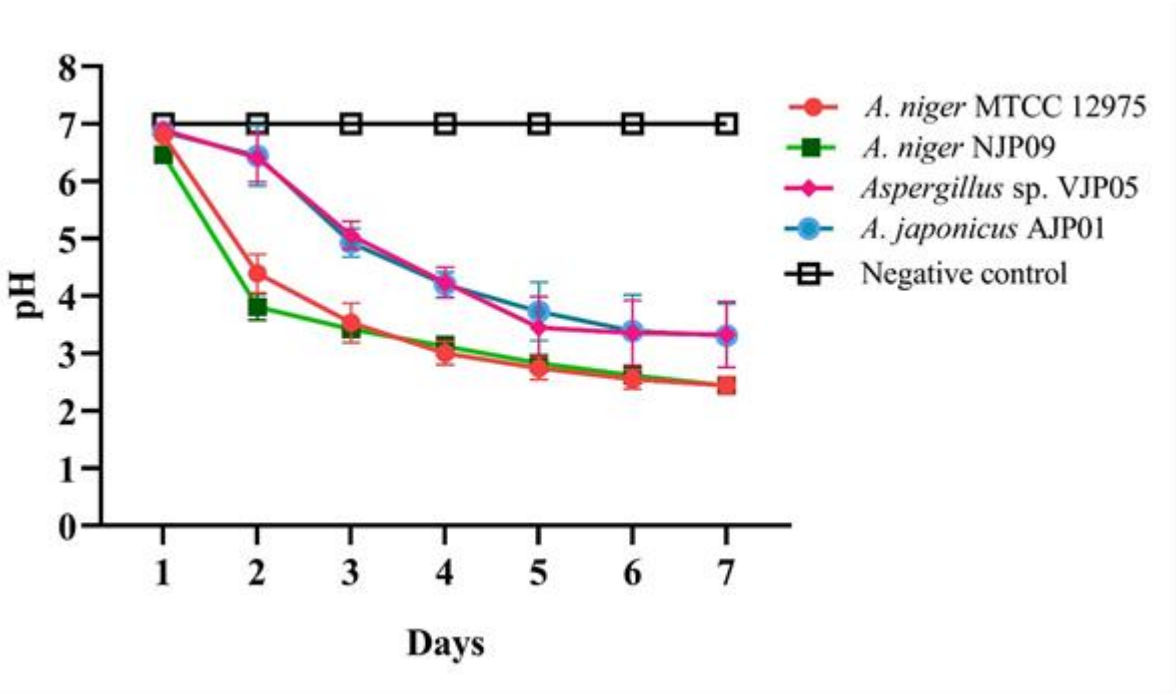

Figure 3: Extracellular pH profile of *A. niger* NJP09, *A. japonicus* AJP09, and *Aspergillus* sp. VJP05 in MSM + 5% Glucose broth during 7 days. Negative control: without fungi, positive control: *A. niger* MTCC 12975. Bar represents the standard deviation; N = 3.

Solid state fermentation for production of organic acids

On the basis of quantitative screening experiment results, *A. niger* NJP09 was further evaluated for its potential to utilize rice straw agro-waste as sole carbon source with MSM (1:2 w/v) for production of organic acids under SSF conditions. Significant reduction was observed in the extracellular pH of filtered extract after growth of *A. niger* NJP09 from day 1 to day 7 with a pH value of 3.48 ± 0.02 at 7th day in comparison to the positive control *A. niger* MTCC 12975 which showed a pH value of 3.44 ± 0.04 at 7th day (**Figure 4**). The obtained results suggested the potential of *A. niger* NJP09 to utilize rice straw waste as carbon source.

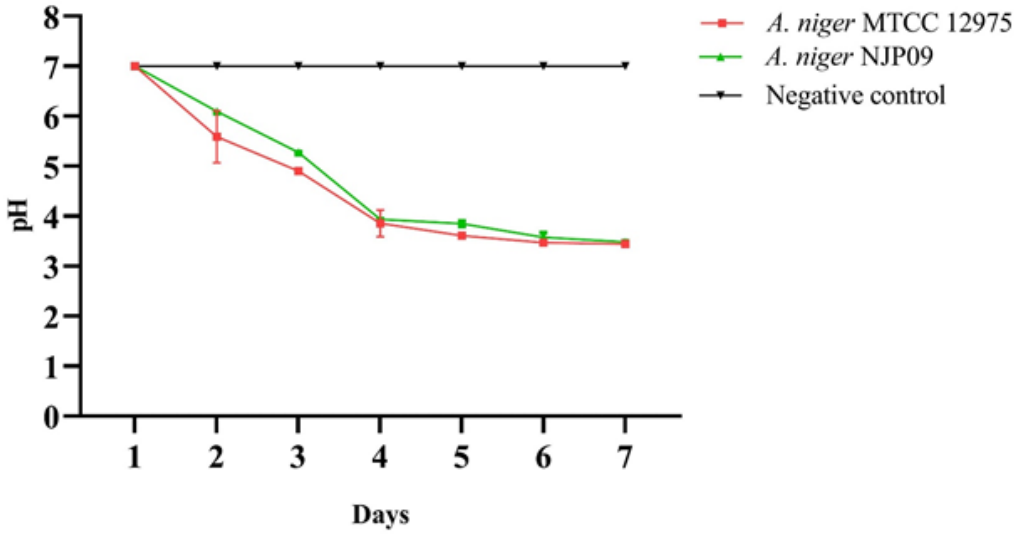

Figure 4: Extracellular pH profile of *A. niger* MTCC 12975 (positive control) and *A. niger* NJP09 under SSF using rice straw as the sole carbon source and MSM (1:2 w/v) during 7 days. Negative control: without fungi. Bar represents the standard deviation; N = 3.

Quantification of total organic acid

The total amount of organic acid produced (g L^{-1}) in MSM + 5% glucose media as well as in SSF media were calculated using titration with 0.01 M NaOH by keeping citric acid as the acid equivalence factor. The obtained results of volumetric titratable acidity profile showed that *A. niger* NJP09 produced 22 ± 0.91 g L^{-1} of total organic acid in the MSM + 5% glucose media in comparison to 20.3 ± 0.3 g L^{-1} total organic acids produced by *A. niger* MTCC 12975 (**Figure 5**). Under SSF conditions with rice straw as sole carbon source and MSM (1:2 w/v), *A. niger* NJP09 produced 1.7 ± 0.14 g L^{-1} of total organic acid, which was found to be similar to the total organic acid produced by *A. niger* MTCC 12975 (1.7 ± 0.04 g L^{-1}) after 7 days (**Figure 6**).

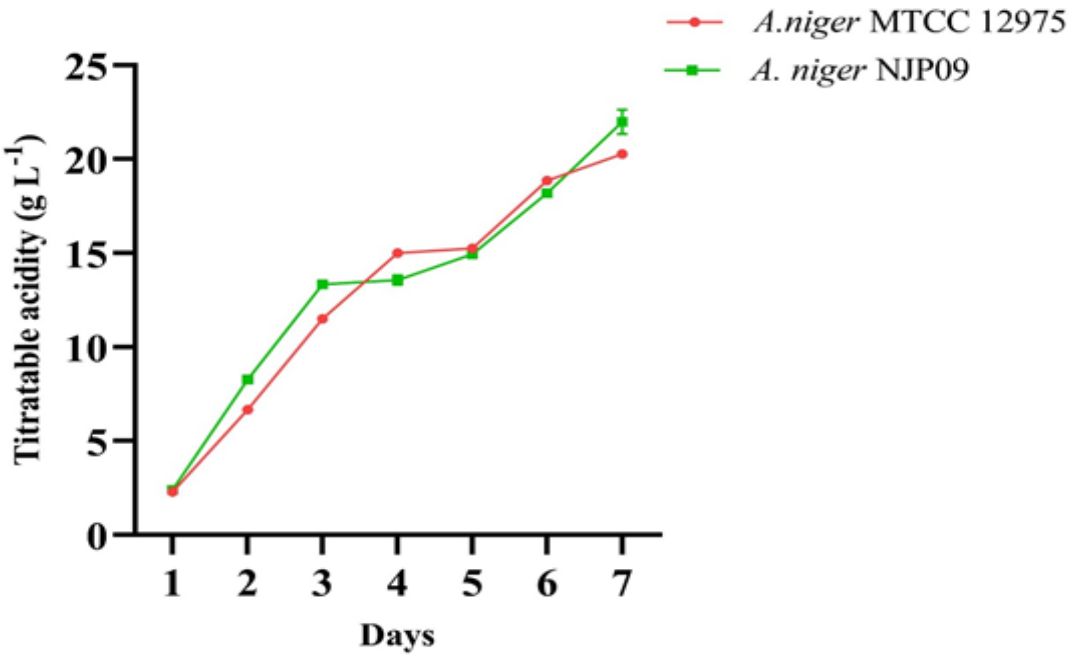

Figure 5: Volumetric titratable acidity profile of *A. niger* MTCC 12975 and *A. niger* NJP09 in MSM + 5% Glucose during 7 days. Bar represents the standard deviation; N = 3

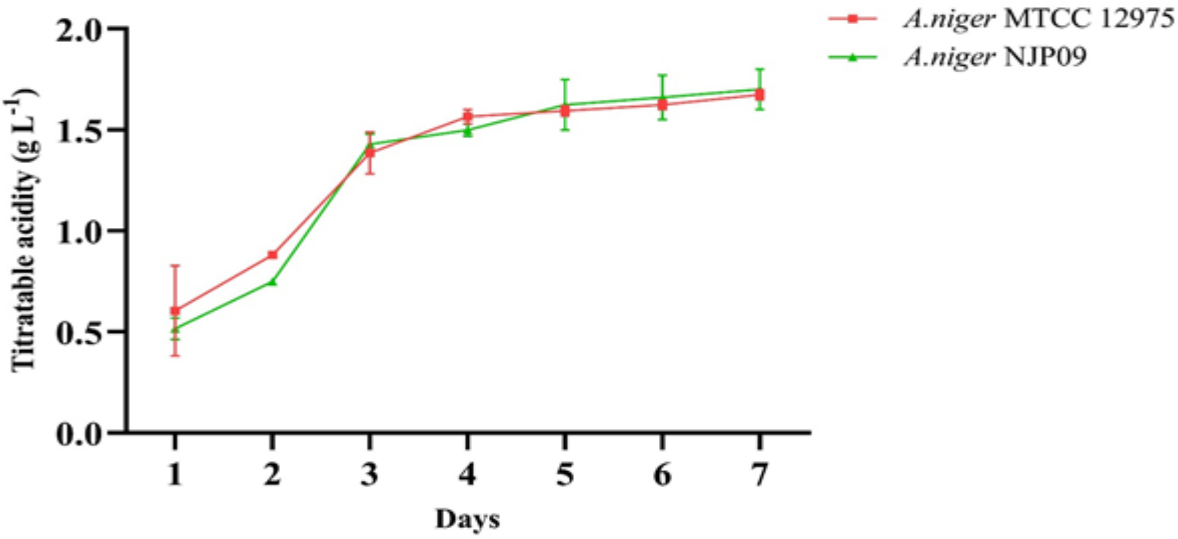

Figure 6: Volumetric titratable acidity profile of *A. niger* MTCC 12975 and *A. niger* NJP09 under SSF using rice straw as sole carbon source and MSM (1:2 w/v) during 7 days. Bar represents the standard deviation; N = 3.

Characterization of organic acid

The HPLC analysis of the organic acid being produced by *A. niger* NJP09 and *A. niger* MTCC 12975 under SSF cultivations using rice straw and MSM (1:2 w/v), revealed that the organic acids produced were mostly oxalic acid, followed by citric, tartaric, and malic acid (**Figure 7**). SSF cultivation using 2 g rice straw substrate and 4 mL MSM resulted in production of organic acids which were separated as oxalic acid (Rt = 2.182 min), tartaric acid (Rt = 2.432), malic acid (Rt = 2.866), and citric acid (Rt = 3.47) after 7 days (Arnetoli *et al*., 2008, Park *et al*., 2017). With major peaks, the concentration of oxalic acid and citric acid were found to be 2.719 mg mL^{-1} and 0.377 mg mL^{-1}, respectively. In comparison, *A. niger* MTCC 12975 showed production of 2.803 mg mL^{-1} of oxalic acid and 0.267 mg mL^{-1} of citric acid after 7 days.

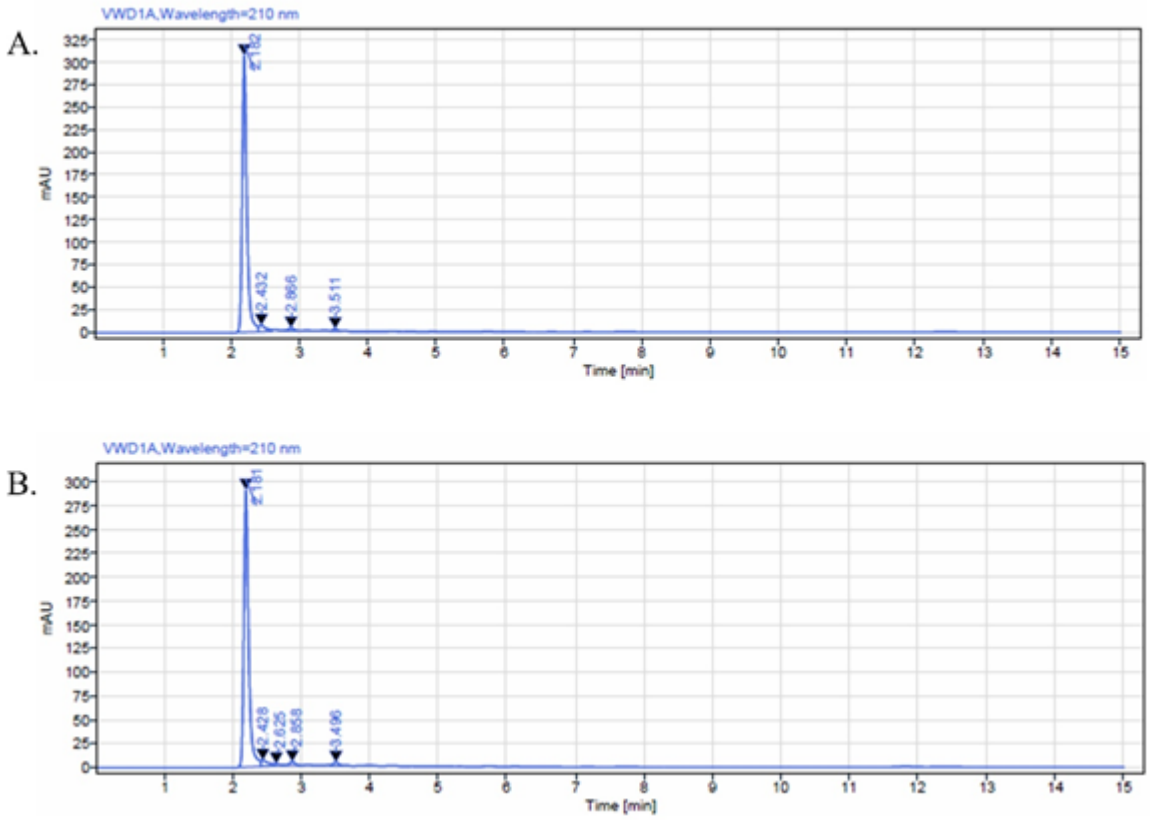

Figure 7: HPLC chromatogram of organic acids produced by (A) *A. niger* MTCC 12975 and (B) *A. niger* NJP09 under SSF.

DISCUSSIONS

The ability of fungi to grow in harsh and inhabitable conditions makes them an interesting organism for research in order to understand their various intracellular and extracellular mechanisms which leads to their robustness (Ilyas *et al*., 2013). This study demonstrates the potential of metal-tolerant rhizospheric and xerophytic phylloplane-derived fungal strains for production of valuable organic acids under laboratory conditions. The organic acids are metal chelators that help in metal detoxification, and help the fungi to tolerate metal stress conditions, therefore, metal-tolerant species of fungi are the best suited candidates for bioleaching (Kubicek *et al*., 2010). Previous studies have confirmed and established the use of different strains of *A. niger* for the production of organic acids at industrial scales (Show *et al*., 2015; Yang *et al*., 2017). Among 26 available fungal strains where species of *Penicillium* and *Aspergillus* genera were screened for extracellular production of organic acids, strains of genus *Aspergillus* showed better result than genus *Penicillium*. These results are consistent with the earlier reports suggesting outstanding capacity of *Aspergillus* species, *A. niger* in specific to produce organic acids (Li *et al*., 2016). Among all the tested fungal strains, *A. niger* NJP09, *A. japonicus* AJP01, and *Aspergillus* sp. VJP05 produced significant quantity of organic acids as indicated by the formation of yellow halo zone with higher diameter.

Bromocresol green is a pH indicator dye and changes its colour from blue (at neutral pH) to yellow (at acidic pH), indicating the formation of organic acids by fungi (Dezam *et al*., 2017). Qualitative plate assay results were supported by the semi-quantitative acid unitage (AU) assay values of yellow halo zone forming fungi, which was found to be more than 2 (Shaikh & Qureshi, 2013). AU values (>2) give a relative estimate of the ability of microbial strains to produce acid. The higher AU value indicate the ability of fungi to produce substantial amount of acid (Jadhav & Mulla, 2018). Based on the quantitative screening using pH profiling of extracellular culture extract of three selected fungal strains *A. niger* NJP09*, A. japonicus* AJP01*,* and *Aspergillus* sp. VJP05, *A. niger* NJP09 which showed the maximum decline in pH of culture media was selected for further studies. According to reports, the most effective acidification of the medium based on the external pH is achieved by synthesis of organic acids, particularly oxalate and citrate by *A. niger* (Šimonovičová *et al*., 2020). In contrast, production of extracellular gluconic acid does not lead to pH drop below 3 as it is a weak acid in comparison to oxalic and citric acids. In the present study, *A. niger* NJP09 showed significant drop in pH from 7 to 2.43 ± 0.12 in 7 days in minimal salt media + 5% glucose inferring to the possibility of oxalic acid and citric acid production in majority. The metal tolerance feature of *A. niger* NJP09 may be rendered by the extracellular oxalic acid production which is a pivotal adduct in fungal mediated biomineralization (Gadd *et al*., 2014). Hence, to further confirm the variant of produced organic acid, HPLC analysis was performed using oxalic acid and citric acid as standards.

The strong performance of *A. niger* NJP09 suggested that it can also utilize agro-waste residues effectively, which offers a low-cost and eco-friendly alternative for chemical synthesis of organic acids (Jiménez-Quero *et al*., 2017). *A. niger* MTCC 12975 available in the Microbial Type Culture Collection, CSIR-IMTech, a well reported strain for the significant production of organic acid was used as the positive control during the present study (Yang *et al*., 2017). The amount of organic acid produced by *A. niger* NJP09 were found to be comparable with that of *A. niger* MTCC 12975, making it a potential candidate for further studies into mechanistic aspect of organic acid production by metal-tolerant fungi, and to understand the role of synthesized organic acids in its survival under metal rich conditions.

An increasing trend in the quantity of total organic acid produced justifies the gradual decrease observed in pH over the course of 7 days of experimentation. The difference observed in the total organic acid produced by both *A. niger* MTCC 12975 and *A. niger* NJP09 in media containing MSM + 5% glucose and under SSF with rice straw and MSM (1:2 w/v), highlights the restricted ability of fungi to use rice straw in raw form as carbon source which is due to the lignocellulosic nature of untreated substrate (Singh & Arya, 2021). The steep increase observed in the amount of titratable acidity calculated for MSM + 5% glucose media in comparison to the SSF with rice straw and MSM (1:2 w/v) was due to the readily available carbon source i.e., glucose in the former media. This difference points towards the need for optimized utilization of rice straw as substrate for organic acid production as rice straw substrates contain sub-optimal levels of easily accessible proteins and sugars and on the contrary have higher levels of cellulosic and hemi cellulosic content (Chen *et al*., 2018). Hence, many studies reported use of pre-treated rice straw to ease the degradation of lignocellulosic substrate for utilisation by fungi (Zhao *et al*., 2022). Results obtained in the present experimental setup contribute to the theory of using lignocellulosic substrates for organic acid production (Dezam *et al*., 2017), hence, contributing towards appreciation of agricultural wastes as valuable substrates in industrial technologies and biorefineries (Li *et al*., 2021). The prevalence of *Aspergillus* species among acid-producing strains aligns with their well-known metabolic flexibility and stress tolerance. Future research should focus on optimizing SSF parameters, understanding metabolic pathways, and evaluating large-scale feasibility to fully exploit *A. niger* NJP09 for industrial applications. It has been well reported that oxalic acid, citric acid, tartaric acid, malic acid, succinic acid, fumaric acid, and malonic acid are the majorly synthesized organic acid by fungi (Das *et al*., 2019). This study investigated the organic acids produced on rice straw and MSM (1:2 w/v) by *A. niger* NJP09, majority of which was found to be oxalic acid followed by citric acid. Previous reports also showed synthesis of itaconic acid and fumaric acid by *Aspergillus* species by utilization of agro-wastes like corn cob and wheat bran as substrates under SSF conditions (Jiménez-Quero *et al*., 2017). Din *et al*. (2020) reported HPLC analysis of organic acid produced by *A. niger* showing significant peaks,

identified as oxalic acid, gluconic acid and fumaric acid, where gluconic acid was produced in maximum concentration followed by oxalic acid. It suggests that the stark difference in the type and concentration of organic acid produced by *Aspergillus* species may be greatly influenced by the type of carbon source used for SSF. Moreover, the organic acid produced may be strain specific, i.e., different strains of *A. niger* can produce different set of organic acids in varying ratios. According to Li *et al*. (2016), synthesis and extracellular secretion of organic acids by fungi depends on the pH of surrounding media. They reported that concentration of citric acid produced by fungi increases with an increasing trend in the acidity of media. On the contrary, oxalic acid and fumaric acid concentration decreases with the increase in acidity of surrounding media. Considering the potential of *A. niger* NJP09 for high production of extracellular organic acids using carbon from rice straw substrates, further studies should focus on understanding the metabolic pathways and regulatory mechanisms as well as optimization of process parameters for industrial scale production of organic acids.

CONCLUSION

In this study, 26 fungal strains isolated from rhizospheric soils of metal rich regions and the leaf phylloplanic surfaces of xerophytic plants of Rajasthan were screened to assess their potential to manufacture extracellular organic acids. Based on the qualitative and quantitative screening experiments, *A. niger* NJP09 was selected as best strain for extracellular synthesis of organic acids. Using rice straw and MSM (1:2 w/v) under SSF conditions, the chosen *A. niger* NJP09 strain produced 1.421 mg L^{-1} of citric acid and 0.166 mg L^{-1} of oxalic acid. These findings suggest the possibility of bioprospecting metal-tolerant fungal strains for extracellular production of organic acids utilizing agro-wastes. This would be in line with the United Nations sustainable development goals (SDGs) and the current demands of bioeconomy by offering a sustainable substitute for organic acid production. *A. niger* NJP09 stood out for its consistent and substantial pH reduction, highlighting its potential as a robust producer of organic acids. Factors like metal tolerance and high production of organic acids by utilizing carbon from agro-wastes substrates, makes *A. niger* NJP09, a potential strain for further studies involving bioremediation approaches. The prevalence of *Aspergillus* among the most effective acid producers reflects its well-known adaptability to stressful environments and its ability to utilize organic acid secretion as a strategy for both metal tolerance and niche exploitation. Further investigations are required to fully understand the metabolic pathways and regulatory mechanisms underlying the organic acid synthesis trait.

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