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A plate assay to screen manganese-tolerant *Aspergillus niger* strains

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Two different physiological effects of Mn[II] interactions with *Aspergillus niger* are known. Excess Mn[II] levels are toxic and restrict fungal radial growth on plates and second, an Mn[II] limited fungal growth is the key to successful citric acid fermentation. While studying Mn[II] toxicity we noted that the Mn[II]-supplemented minimal medium (MM) agar plates were translucent. Also, clearing zones around the fungal colonies owing to organic acid release were observed. Simultaneous scoring of these two parameters was exploited to screen for Mn[II] tolerant/ insensitive *A. niger* strains with improved acid production. Clear halos were best viewed on MM plates supplemented with ≥5.0 mM of Mn[II]. Accordingly, an acid unit (AU; the ratio of diameter of the clear zone to that of colony diameter) was defined. Colonies that produced larger zones of clearing (and higher AUs) were picked up and sub-cultured. A Mn[II] tolerant/ insensitive spontaneous mutant of *A. niger* was isolated by this screen and characterized. It produced significantly higher amounts of citric acid and the yield was not adversely affected by the added Mn[II]. While separate screens for metal tolerance and citric acid production were reported earlier, the method presented here is simple and directly combines the two features for strain selection/screening.

Keywords: Acidogenesis, Mn[II] sensitivity, Strain selection

Metal ions play an important part in microbial growth and physiology. While their deficiency impairs microbial physiology, an excess is often toxic because of the wrong metal ion insertion into metalloproteins and metalloenzymes, leading to impaired protein/ enzyme function[1]. The microbial action (chiefly by bacteria and fungi) in transforming inorganic substrates of biogeological and environmental importance has long been appreciated[2]. Molds promote metal corrosion by producing organic acids. Several fungi, including Aspergilli, are capable of mobilizing metal ions like Co[II], Zn[II], Mn[II] and Sr[II] as their corresponding oxalates and/or citrates[3-5]. Divalent metal ions in turn play significant roles in fungal organic acid accumulation[6]. It is argued that *Aspergillus niger* secretes citrate to increase iron bioavailability[7] and bio-recovery of cerium by this fungus occurs through oxalate formation[8]. The fungus is also significant in manganese mobilization and transformation[4]. Influence of Mn[II] bioavailability on mycelial morphology and organic acid production in fungi (more particularly *Aspergillus* sp.) have attracted much attention[9-11].

Two different physiological effects of Mn[II] interactions with Aspergilli are known. Excess concentrations of Mn[II] are toxic and restrict fungal radial growth on plates and organic acids are released to solubilize and complex with the metal ion[4,12]. Second, a Mn[II]-deficient fungal growth is an essential feature of citric/ itaconic acid production by submerged fermentations[6,13-18]. Methods to screen for solubilizing ability and metal tolerance[12] and citric acid production[19,20] are reported. Screening for Mn[II] resistant and/or insensitive fungal strains is therefore of value. The present work presents a simple agar plate screen to isolate *A. niger* strains that are tolerant/ resistant to high Mn[II] levels and also to pick citrate producers that are potentially insensitive to this metal ion during fermentation.

Materials and Methods

Aspergillus niger NCIM 565, a citric acid producing wild type isolate, was obtained from the culture collection at NCIM, Pune, India. *Aspergillus terreus* NCIM 656, used in a few experiments was also procured from the same source. These cultures (and their mutant isolates) were maintained on potato

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dextrose agar. Fully sporulated plates were flooded with sterile Tween-20 (0.01% in saline) solution, surface mixed and the suspension was filtered through sterile glass wool and the spore count was recorded.

The fungal strains were cultured either on the minimal medium (MM; which contained 10.0 g/L glucose, 3.0 g/L KH_2PO_4, 6.0 g/L Na_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 2.25 g/L NH_4NO_3, 10 mg/L $ZnSO_4.7H_2O$, 3.0 mg/L $MnSO_4.7H_2O$, 1.5 mg/L $Na_2MoO_4.H_2O$, 20.0 mg/L $FeCl_3.6H_2O$ and 1.0 mg/L $CuSO_4.H_2O$. The medium pH was adjusted with 0.1 N HCl to 5.5–6.0) or on the acidogenic medium (AM; which contained 140.0 g/L sucrose, 1.0 g/L KH_2PO_4, 0.1 g/L $Fe(NH_4)_2(SO_4)_2$, 2.25 g/L NH_4NO_3 and 0.25 g/L $MgSO_4.7H_2O$; medium pH was adjusted with 0.1 N HCl to 2.3)[21]. Since citric acid fermentation is highly sensitive to presence of trace metals (especially Mn[II] ions) all the glassware was treated first with 20% nitric acid and then thoroughly washed with double distilled water.

Aspergillus niger growth and acid production experiments were conducted in liquid culture at 30°C either as surface cultures or under shake flask conditions (using baffled flasks to achieve better aeration during submerged fermentations and at 220 rpm). Typically, 10^8 spores (harvested from suitable solid media in Petri plates) were inoculated into 100 mL of respective liquid media in one liter Erlenmeyer flasks. The samples of spent media were obtained for citrate and oxalate analysis at suitable time points as desired.

Plates were prepared by including 2% agar in liquid MM (it normally contains ~0.01 mM of Mn[II]). When required, the MM was appropriately amended with various concentrations of Mn[II]. Stock solution of $MnCl_2$. $4H_2O$ (1.0 M) was prepared in sterile double distilled water and suitable aliquots of this stock were added to achieve the final concentration (0.05, 1.0, 5.0, 10, 20, 30 and 50 mM) of Mn[II] in the MM[22]. However, for concentrations above 20 mM, the stock solution pH (being very acidic!) was pre-adjusted to 6.2 before adding to the MM. Typically 25 µL of spore suspension (2.5×10^2 spores/mL) was spread over MM agar plates. The inoculated plates were incubated at 37°C to measure fungal growth (as colony diameter) and diameter of clearing zone, after 45 to 65 h of incubation. The acid unit (AU) was defined as the ratio of diameter of the clearing zone to that of colony diameter.

Both citrate and oxalate present in the spent media was estimated by HPLC. The Hewlett Packard HPLC system [model HP 1100 series, fitted with a Zorbax SB C-18 column (4.6 mm × 25 cm), a Rheodyne 7725i injector (50 µL sample loop) and Diode Array Detector (at 210 nm) was used. The acids were quantified using HP CHEM STATION software. Spent media samples (1.0 mL; drawn on different days) were centrifuged (at 10,000×g for 10 min), the supernatant was made up to 5% perchloric acid and the resultant precipitate was cleared by centrifugation (5 min). These samples were neutralized with 1.0 M KOH and centrifuged and then filtered through 0.2 µ Millipore filter; typically 20 µL of the sample was used for injection. The standard curves (from peak areas) for citrate (run at pH 2.2) and oxalate (run at pH 6.0) were obtained using methanol in 20 mM $(NH_4)H_2PO_4$ (3:97, v:v) as mobile phase (flow rate, 1.0 mL/min). The standard solutions of the acids (AR grade) were prepared in HPLC grade water as well as in MM to clearly identify them in the background of media components.

All liquid culture tests were conducted as four separate experiments with each set in duplicates. The date represents the average of four separate runs. For the Acid Unit (AU) calculations, diameters of discrete colonies (n=13) and clear zones (n=8) on plates were used and the values presented are averages of replicates with standard deviation.

Results and Discussion

As an essential nutrient, manganese is involved in many cellular functions while only a handful of strictly Mn[II]-dependent enzymes are known[22]. Being toxic at high concentrations, microbes utilize various strategies to immobilize manganese and limit its bioavailability. Production and secretion of organic acids is one such response to solubilize and/or scavenge metal ions[4,6]. While evaluating Mn[II] toxicity, in *A. niger* strains with altered arginase expression, we observed that the MM agar plates were turbid (possibly due to metal ion precipitation by phosphate, at higher Mn[II] levels)[22]. Besides a compact, restricted radial growth due to toxicity, appearance of clear zones around the fungal colonies (owing to organic acid release) was noted (Fig. 1). Such simultaneous scoring of two parameters offers an opportunity and could be exploited to screen for Mn[II]-resistant/ insensitive *A. niger* strains with improved acid production. In another plate screen for

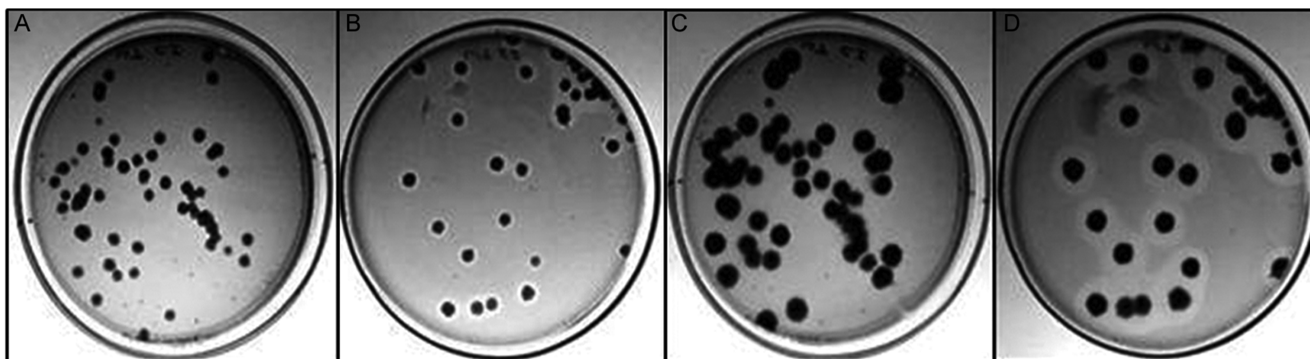

Fig. 1 — *Aspergillus niger* colony growth and appearance of clear zones around the colonies. Spores of *A. niger* (NCIM 565) were plated on (A and C) Minimal Medium (MM) or (B and D) MM supplemented with 5 mM Mn[II]. Growth on the plates was recorded after (A and B) 45 h and (C and D) 65 h of incubation.

solubilizing ability and metal tolerance with metal ions, a zone of crystal formation (rather than clear zones) was observed with *A. niger*[12].

Restricted growth of *A. niger* upon increasing Mn[II] levels (tested up to 50 mM) in the MM agar medium (with 0.05% Triton X-100) is shown in Figure 2. The colony diameter was not significantly affected up to 5 mM Mn[II] but measurable growth inhibition occurred at higher concentrations. The colonies were also characterized for the formation of zones of clearing. Well defined clear zones were seen and could be scored when the MM was supplemented with ≥5.0 mM of Mn[II]. Fungal colonies that produced larger zones of clearing could be picked up and sub-cultured. Clear halos were visible on plates supplemented with 5 mM Mn[II] (or higher) and an AU (ratio of diameter of the clearing zone to that of colony diameter meter) of about 2.00 was observed after 45 h of growth (Fig. 2).

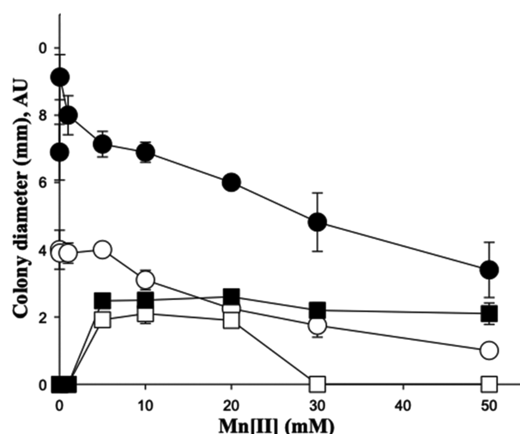

Fig. 2 — Effect of supplemented Mn[II] on radial growth and the formation of clear zones around the *A. niger* colonies on MM. The colony diameters (○,●) and AU values (□,■) recorded after 45 h (open symbols) and 65 h (closed symbols) of growth are shown.

The standard screen (MM supplemented with 5 mM Mn[II]) was used to isolate many mutants (spontaneous as well as UV mutants) of *A. niger* NCIM 565 (parent strain). One spontaneous mutant (strain spm2; with AU 2.73±0.32 versus 2.0±0.22 for the parent) was chosen for further study. The solubilization activity of fungi is typically due to the release of organic acids in the surrounding medium[4,12]. *A. niger* is a well-known producer of citric acid and is also capable of secreting oxalic and gluconic acids under appropriate growth conditions[6,16-18,23]. A filter paper culture technique was developed and later adopted to screen for acid producing *A. niger* strains[19,20]. Here, the citric acid was detected by spraying a specific color reagent (4% p-dimethyl aminobenzaldehyde in acetic anhydride); the acid producing capacity of different colonies was presented in acid units (AU - dividing the diameter of acid zone by the corresponding colony diameter).

The screening protocol presented here is much simpler but is not specific to citric acid. Any other organic acid secreted may also be scored. It was therefore of interest to characterize the organic acids produced by *A. niger* strains (both the spm2 isolate and the parent NCIM 565) on different growth media. With liquid surface culture, no significant acid (either oxalate or citrate) was formed by the parent strain on MM whereas citrate (≥12 g/L, after 14 days) accumulated on AM (not shown). Both the strains were also grown as submerged culture on AM or on AM supplemented with 182 nM (10 ppb; that adversely affects citric acid yield) and 5 mM Mn[II] (used in this plate screen). The citrate yield of the parent (NCIM 565) strain was negatively affected by

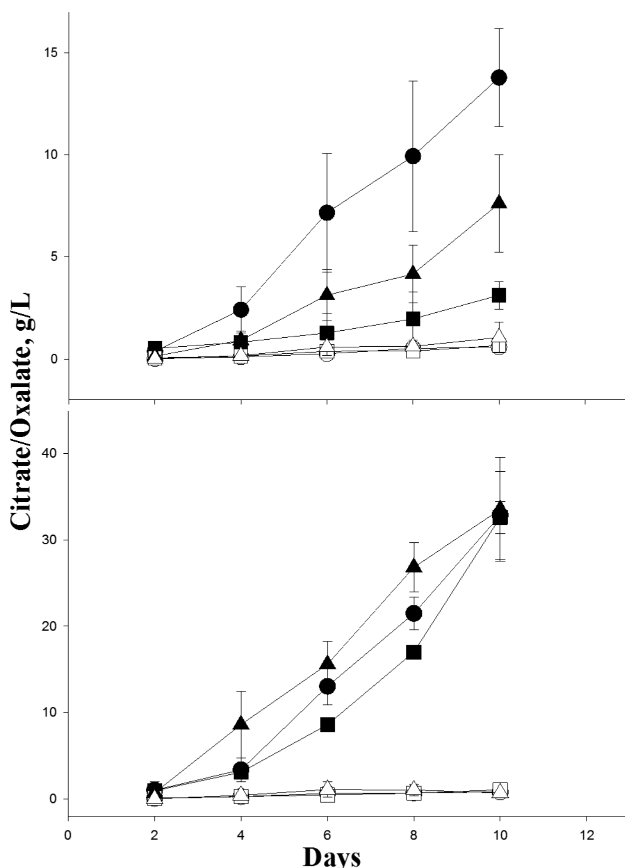

Fig. 3 — Effect of Mn[II] supplementation on organic acid production by *A. niger*. *A. niger* (NCIM 565) (top panel) and the isolated spm2 strain (bottom panel) were grown on AM or AM supplemented with Mn[II]. Acids produced on AM (●, citrate; ○, oxalate), AM supplemented with 182 nM (~10 ppb) Mn[II] (▲, citrate; Δ, oxalate) or AM supplemented with 5 mM Mn[II] (■, citrate; □, oxalate), were monitored.

Mn[II] addition (at both concentrations) whereas very little oxalate was formed in both the cases (Fig. 3A). The observed Mn[II] sensitivity of this strain is consistent with earlier reports on acidogenesis[6,13-18]. Results of a similar study with isolated spm2 strain are shown in Fig. 3B. This strain produced significantly higher amounts of citric acid and the yield was not adversely affected by the added Mn[II]. However, the acid yields are nowhere comparable to those from industrial producer strains. This screening method appears to work well in isolating Mn[II] tolerant/ insensitive strains of acid producing *A. niger*; it provides a more direct method for selecting/ evaluating such strains.

Various effects of Mn[II] on *A. niger* biochemistry and physiology are documented. A bioassay was developed based on *A. niger* growth sensitivity to Mn[II] ions[24]. The fungus possesses a specific transport system for this metal ion[25]. Lack of Mn[II] ions from the medium is of major importance for efficient citric acid fermentation and concentrations >5.0 µg/L (i.e., 5 ppb) significantly reduce the acid yield[15,26]. An effort to map the *A. niger* genes associated with this Mn[II] effect was made earlier[9]. While a number of enzymes from Aspergilli employ Mn[II] for their activity[22], the primary target(s) of Mn[II] deficiency in the *A. niger* acidogenic metabolism remain elusive even today[27,28]. Also, citric acid producing *A. niger* strains with varying degree of Mn[II] sensitivity are known[29,30]. In the context of Mn[II] biology of *A. niger*, the present screen is useful in obtaining better producer strains. Lastly, the method may be more generally adopted (based on our preliminary results on *A. terreus*) for other fungal acid producers[6,31].

Conclusion

Separate screening techniques for filamentous fungi, for metal tolerance and citric acid production are reported in the literature. The plate assay method presented here combines the features of Mn[II] tolerance and organic acid production. The colony size relates to metal ion resistance while the diameter of the clearing zone scores for organic acid formation; their ratio defines the acid producing capacity of the strain in AU. Utility of this plate assay was demonstrated to select the citrate producing *Aspergillus niger* strains, as an example. The screen could have broad applicability in strain selection/ screening of other fungal organic acid producers.

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Conflict of interest

Authors declare no competing interests.

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