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Phosphate limitation alleviates the inhibitory effect of manganese on itaconic acid production by *Aspergillus terreus*

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ABSTRACT

Lignocellulosic biomass has the potential to serve as a low-cost source of sugars for production of itaconic acid (IA, a building block platform chemical) by fermentation with *Aspergillus terreus*. However, the IA production from biomass hydrolysate was severely inhibited. Mn^{2+} was found to inhibit the IA production strongly. The effect of Mn^{2+} on each medium component (KH_2PO_4, NH_4NO_3, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $FeCl_3{\cdot}6H_2O$, $ZnSO_4{\cdot}7H_2O$, and $CuSO_4{\cdot}7H_2O$) was evaluated for sugar utilization and IA production by *A. terreus* NRRL 1972. Both K^+ and PO_4^{-3} were necessary for IA production. Low PO_4^{-3} in the medium greatly alleviated the inhibitory effect of Mn^{2+} on IA production. However, high PO_4^{-3} (K^+) was detrimental for IA production in the presence of Mn^{2+}. The inhibitory effect of Mn^{2+} on IA production was partly eliminated by increasing the $CuSO_4{\cdot}7H_2O$ level in the medium. This is the first report on the effect of phosphate limitation to alleviate the inhibition of IA production by Mn^{2+} and on the relationship of Mn^{2+} on the medium components for utilization of sugar and production of IA.

1. Introduction

Itaconic acid (methylene succinic acid or methylene butanedioic acid, IA) is a five carbon (C5) unsaturated dicarboxylic acid with one carboxyl group conjugated to the methylene group. It is produced industrially from glucose by submerged fermentation using the filamentous fungus *Aspergillus terreus* (Nubel and Ratajak, 1962; Batti and Schweiger, 1963; Willke and Vorlop, 2001). In recent years, IA has gained importance as a fully sustainable building block chemical (platform chemical) for its broad applications in the manufacture of various synthetic resins, coatings and polymers (Saha, 2017; Robert and Friebel, 2016; Kumar et al., 2017). It has applications in super-absorbents, phosphate free detergents and cleaners, and bioactive compounds particularly in the pharmaceutical industry and agriculture. IA is regarded as α-substituted acrylic or methyl methacrylic acid (also known as Plexiglass) and its potential to replace fossil-based acrylic acid and use of its derivatives for production of plastics is high. The conversion of IA to methyl methacrylate provides a giant market potential of up to 3.2 million tons per year (Choi et al., 2015). IA can be used for synthesis of 3-methyltetrahydrofuran, a potential biofuel. It plays an important role during inflammation and acts as an endogenously produced antimicrobial compound against pathogens (Cordes et al., 2015). The market of IA is about 80,000 tons per year and the selling price is \$2.00 kg^{-1} (\$ 0.91 lb^{-1}) (Okabe et al., 2009). The world market is expected to exceed \$ 216 million by 2020 because of an increasing demand for bio-based chemicals (Cruz et al., 2018). The US represents the single largest market for IA but imports all its needs from overseas. However, the production cost must be lowered in order to expand the market of IA (Klement and Büchs, 2013). It is one of the 12 building block molecules identified as potential new platform chemicals that can be produced from lignocellulosic sugars (Werphy and Peterson, 2004).

Recently, only a handful of research articles are available on the IA production by *A. terreus* strains using lignocellulosic hydrolysates. Tippkötter et al. (2014) reported that *A. terreus* NRRL 1960 could not grow on untreated organosolv beech wood hydrolysate. They used several detoxification methods to remove the fermentation inhibitors. First, the fiber fraction was washed with 0.5 M NaOH at 50 °C overnight to remove lignin derived phenolics prior to hydrolysis. The hydrolysate was then treated with zeolite for complete removal of monomeric phenolic compounds. The hydrolysate was finally treated with an equal mixture of anion- and cation exchangers to remove metal ions. The fungal strain grew in the form of small pellets and produced 7.2 g IA from the detoxified hydrolysate containing 20.5 g glucose and 4.0 g

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xylose (total 24.5 g) per L at a productivity of 0.1 g L^{-1} h^{-1} (41.7% of theoretical IA yield). Jimenez-Quero et al. (2016) performed liquid state fermentation of acid and enzymatic hydrolysates of wheat bran and corn cob with a 10-times dilution and glucose added by two *A. terreus* strains (DSM 826 and 62071). No IA was formed by either of the two *A. terreus* strains tested. Pedrosa et al. (2017) reported that *A. terreus* ATCC 10020 produced 1.9 g IA (yield of 49 mg g^{-1} sugar utilized) from H_3PO_4 pretreated and detoxified rice husk hydrolysate (41.0 g total sugars) utilizing 38.9 g sugar per L under optimized conditions. The detoxification of the hydrolysate was carried out by active carbon and CaO treatments. Krull et al. (2018) pretreated wheat chaff with NaOH at room temperature, washed and enzymatically saccharified with a commercial enzyme preparation. The hydrolysate as such gave only 0.6 g IA per L by fermentation with *A. terreus* DSM 23081. They obtained 27.7 g IA per L with a yield of 0.41 g g^{-1} total sugars from the enzymatically saccharified hydrolysate after purifying it through heat denaturation of protein, evaporation and subsequently treating it with a cation exchanger. The concentrations of all cations were reduced by > 90% and in particular the Mn^{2+} concentration decreased from 1.72 mg L^{-1} to 0.5 μg L^{-1}. It is evident that more research needs to be performed in order to cost-effectively utilize lignocellulosic hydrolysates as substrate for IA production.

The biosynthesis of IA from glucose involves metabolizing it to pyruvate mainly via Embden-Myerhof Parnas (EMP) pathway (glycolysis). Pyruvate is converted to *cis*-aconitic acid and then decarboxylated to IA by the action of *cis*-aconitic acid decarboxylase (EC 4.1.1.6) in the cytoplasm (Bonnarme et al., 1995; Steiger et al., 2013). Recently, 100 *A. terreus* strains were evaluated for growth and IA production from glucose, xylose and arabinose (Saha et al., 2017). Twenty strains showed good production of IA from all three sugars. Six strains (NRRL 1960, 1961, 1962, 1972, 66125 and DSM 23081) were then chosen for investigation on growth and IA production from lignocellulosic hydrolysates. However, these strains could not grow on dilute acid pretreated and enzymatically saccharified wheat straw hydrolysates (DAWSH) containing ~ 80 g mixed sugars per L (Saha and Kennedy, 2018). In order to find out the reasons for such failure, the effects of typical inhibitory compounds and metal ions present in DAWSH on growth and IA production from model mixed sugars substrate mimicking DAWSH by one selected *A. terreus* strain (NRRL 1972) were investigated. Karaffa et al. (2015) showed that Mn^{2+} reduced IA production by about 60% at 2500 ppb, while Saha et al. (2018) had a much higher inhibitory effect at Mn^{2+} concentration of 50 μg L^{-1} level inhibiting the IA production by 97%. DAWSH contained 11,600 ± 200 μg Mn^{2+} per L, which is 232 times higher than 50 μg L^{-1} level. The effect of Mn^{2+} on the production of IA by the fungal strain and its relationship with each medium component for sugar utilization and IA production at five concentration levels (0, 1, 10, 20 and 50 μg L^{-1}) were investigated. In this paper, the results are reported. In addition, relationship between K^+ and PO_4^{-3} for production of IA with (50 μg L^{-1}) or without Mn^{2+} is reported. Time courses of sugar utilization and IA production along with monitoring PO_4^{-3} and dissolved oxygen levels during fermentation at low and high PO_4^{-3} levels with and without Mn^{2+} were studied.

2. Materials and methods

2.1. Materials

Czapek-Dox-agar, glucose, glycerol, Tween 80, all medium components, KOH, KCl, NaH_2PO_4, H_2SO_4, IA, propionic acid, succinic acid, α-ketoglutaric acid, *cis*-acotinic acid, and *trans*-acotinic acid were purchased from Sigma Chemical Co., St. Louis, MO, USA. Aminex HPX 87H column (300 × 7.8 mm), Cation H micro-guard cartridge (30 × 4.6 mm) and Dowex 50-X8 cation exchange resin (100/200 mesh) were obtained from Bio-Rad Laboratories, Inc., Hercules, CA, USA. Ninety-six-well (400 μL) Nunc Edge microtiter plate (MTP) was purchased from ThermoFisher Scientific, Waltham, MA, USA. Flasks with dissolved oxygen (DO) sensor spots (PreSens Precision Sensing GmbH, Regensburg, Germany) were obtained from Applicon Biotechnology, Inc. (Dover, NJ, USA).

2.2. Fungal strain and inoculum preparation

A. terreus NRRL 1972 obtained from ARS Culture Collection (Peoria, IL, USA) was used. The strain was stored in the form of spores at – 80 °C as 70% (v/v) glycerol stock. The stock culture was maintained on Czapek-Dox (Merck) agar plates. The conidiospores were collected from the 7-day culture on Czapek-Dox-agar plates incubated at 30 °C by shaving and extracting spores using sterile water with 0.04% (v/v) Tween 80. The spore suspension was adjusted by dilution with sterile deionized water such that the final inoculum concentration was 10^6 spores mL^{-1} of the medium.

2.3. Cultivation of A. terreus strain

The medium for both MTP and flask fermentations contained 0.8 g KH_2PO_4, 3 g NH_4NO_3, 1 g $MgSO_4{\cdot}7H_2O$, 5 g $CaCl_2{\cdot}2H_2O$, 1.67 mg $FeCl_3{\cdot}6H_2O$, 8 mg $ZnSO_4{\cdot}7H_2O$, and 15 mg $CuSO_4{\cdot}7H_2O$ per L, unless otherwise stated (Hevekerl et al., 2014). Glucose (80 g L^{-1}) was used as carbon source. For all experiments, glucose was dissolved in deionized water and passed through a column (44.0 × 4.5 cm) of Dowex 50-X8 cation exchange resin (100/200 mesh) to remove Mn^{2+}, even though it may be present in trace quantity (Karaffa et al., 2015). Glucose and other medium components were added from sterile stock solutions. The pH of the medium without $CaCl_2$ was adjusted to 3.1 with 0.5 M H_2SO_4 before inoculating the spore preparation for each strain. MTP cultivation was performed with 100 μL medium in a 96-well (400 μL) Nunc Edge MTP with sterile deionized water added to the surrounding moat, lidded, and wrapped in parafilm. To improve consistency of inoculums, the volume of spores introduced was always 20 μL of the 100 μL MTP fermentation. MTP cultures were incubated in a thermoshaker incubator (MB 100-4A, Allsheng Inst. Co., Hangzhou City, China) at 33 °C and 950 rpm for 7 days (Saha and Kennedy, 2018). After the fermentation, the plate was withdrawn from the plate shaker, centrifuged using a centrifuge (Model 5430R, Eppendorf North America, Hauppauge, NY, USA) with a rotor for MTPs at 2,000 rpm for 10 min and the supernatant solutions were analyzed for sugar utilization and IA production.

Flasks (125 mL) with dissolved oxygen (DO) sensor spots containing 25 mL of the medium were mounted in a rotary shaker incubator equipped with a shake flask reader (SFR). The fermentation was carried out at initial pH 3.1, 33 °C and 200 rpm for 7 days. DO was continuously monitored and recorded every 6 min using the provided software (PreSens SFR Software V1.2.0). The pH was not controlled during either MTP or shake flask fermentation. All experiments were performed in triplicate.

2.4. Analytical methods

Glucose and organic acids (IA, propionic acid, succinic acid, α-ketoglutaric acid, malic acid, *cis*-acotinic acid, and *trans*-acotinic acid), were quantified by using high-performance liquid chromatography (HPLC). A Shimadzu Prominence Series (Shimadzu America, Inc., Columbia, MD) HPLC system was used. The Aminex HPX 87H column, 300 × 7.8 mm with Cation H microguard cartridge was used for analysis of glucose and organic acids. The column was maintained at 65 °C and glucose and organic acids were eluted with 5 mM H_2SO_4 prepared using Milli-Q filtered water (Millipore Corp., Bedford, MA, USA) at a flow rate of 0.5 mL min^{-1}. Peaks were detected by refractive index or UV absorption at 210 nm, and were identified and quantified by comparison with authentic standards. Propionic acid (1%, w/v) was used as internal standard in order to estimate the liquid lost during the shaken

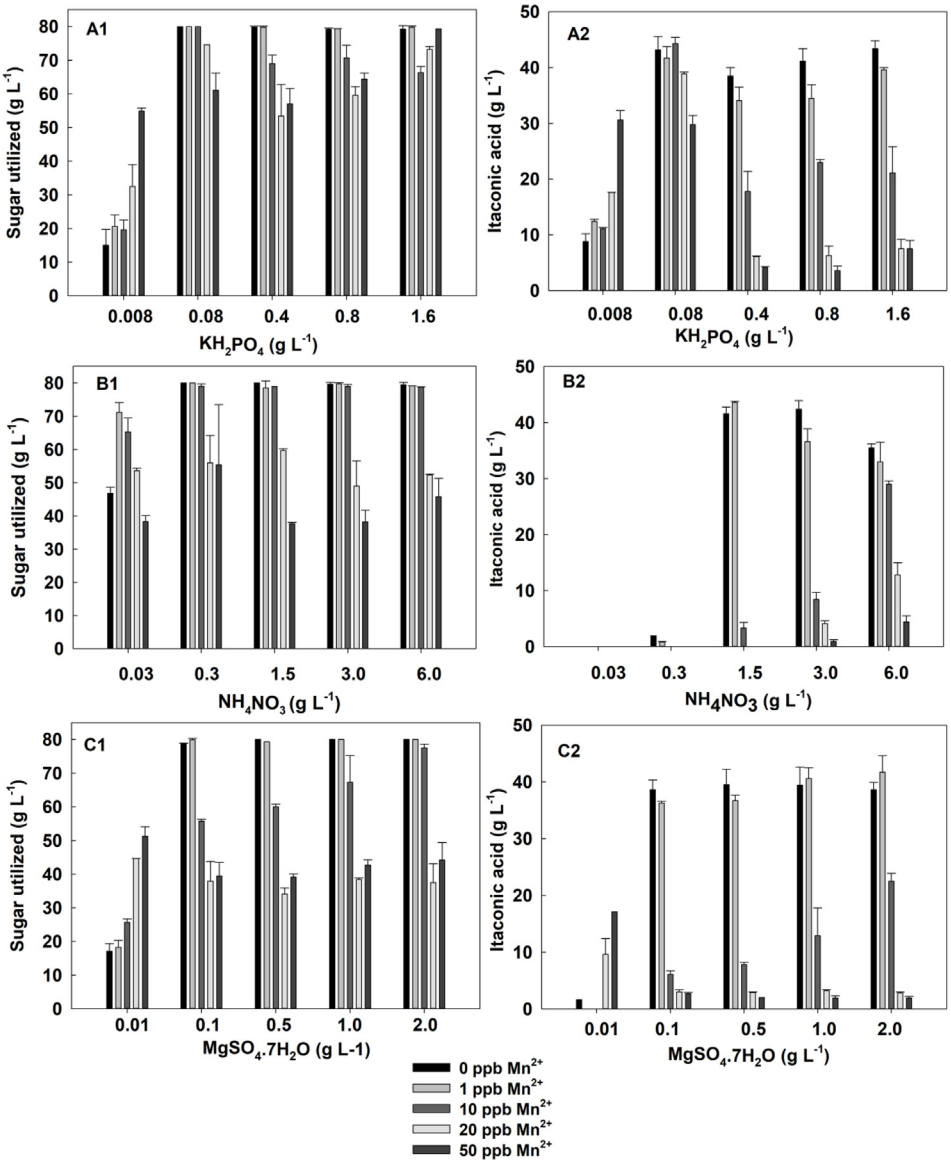

Fig. 1. Effects KH_2PO_4 (A1, A2), NH_4NO_3 (B1, B2) and $MgSO_4{\cdot}7H_2O$ (C1, C2) concentrations on sugar (80 g L^{-1}) utilization and IA production by *A. terreus* NRRL 1972 at pH 3.1, 33 °C and 200 rpm grown for 7 days with variable amounts of Mn^{2+}. The data presented are means ± standard deviations for triplicate experiments. The standard medium contained 0.8 g KH_2PO_4, 3 g NH_4NO_3 and 1 g $MgSO_4{\cdot}7H_2O$ per L. ppb is equivalent to μg L^{-1}.

aerobic fermentation at 33 °C. PO_4^{-3} content was determined using the PO_4^{-3} Colorimetric Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). It reacted with a chromogenic complex, which resulted in a colorimetric (650 nm) product proportional to the amount of PO_4^{-3} present. Cell mass was quantified using dry weight of twice rinsed cell pellet which was dried at 60 °C until constant weight was obtained.

3. Results and discussion

3.1. Relationship of each medium component with Mn^{2+} for sugar utilization and production of itaconic acid

Unless otherwise mentioned, the fermentation experiments were performed in MTP. The effect of KH_2PO_4 (0.008–1.6 g L^{-1}) in the presence of Mn^{2+} at 5 different concentrations (0, 1, 10, 20, 50 μg L^{-1}) on sugar utilization and IA production by *A. terreus* NRRL 1972 at pH 3.1, 33 °C grown for 7 days was investigated. The data are presented in Fig. 1 (A1, A2). KH_2PO_4 was found to be essential for sugar utilization and IA production. The sugar utilization was better at higher KH_2PO_4 concentrations tested up to 1.6 g L^{-1} but the IA production was better at low KH_2PO_4 concentration (0.08 g L^{-1}) in the presence of Mn^{2+}. At the lowest KH_2PO_4 concentration (0.008 g L^{-1}) used, the sugar utilization was slow but it was enhanced in the presence of high Mn^{2+}. The IA production was greatly enhanced at low KH_2PO_4 concentration (0.08 g L^{-1}) in the presence of Mn^{2+} relative to the standard KH_2PO_4 concentration (0.8 g L^{-1}). The data clearly indicate that KH_2PO_4 limitation is essential for alleviating the inhibitory effect of Mn^{2+} on IA production by the fungal strain. A recent study using Mn^{2+} free sugars as substrate shows that PO_4 limitation is not required for production of IA by *A. terreus* NRRL 1972 using mixed sugars of glucose, xylose and arabinose (4:3:1) (Saha et al., 2018). Kuenz et al. (2012) reported that more IA was produced using higher PO_4 concentration by *A. terreus* DSM 23081 using glucose as substrate. Hevekerl et al. (2014) also reported that the highest IA concentration could be reached by increasing KH_2PO_4 concentration from 0.1 to 0.8 g L^{-1} by the same *A. terreus* strain using glucose as substrate.

The effect of NH_4NO_3 (0.03–6.0 g L^{-1}) in the presence of Mn^{2+} (0, 1, 10, 20, 50 μg L^{-1}) on sugar utilization and IA production was studied

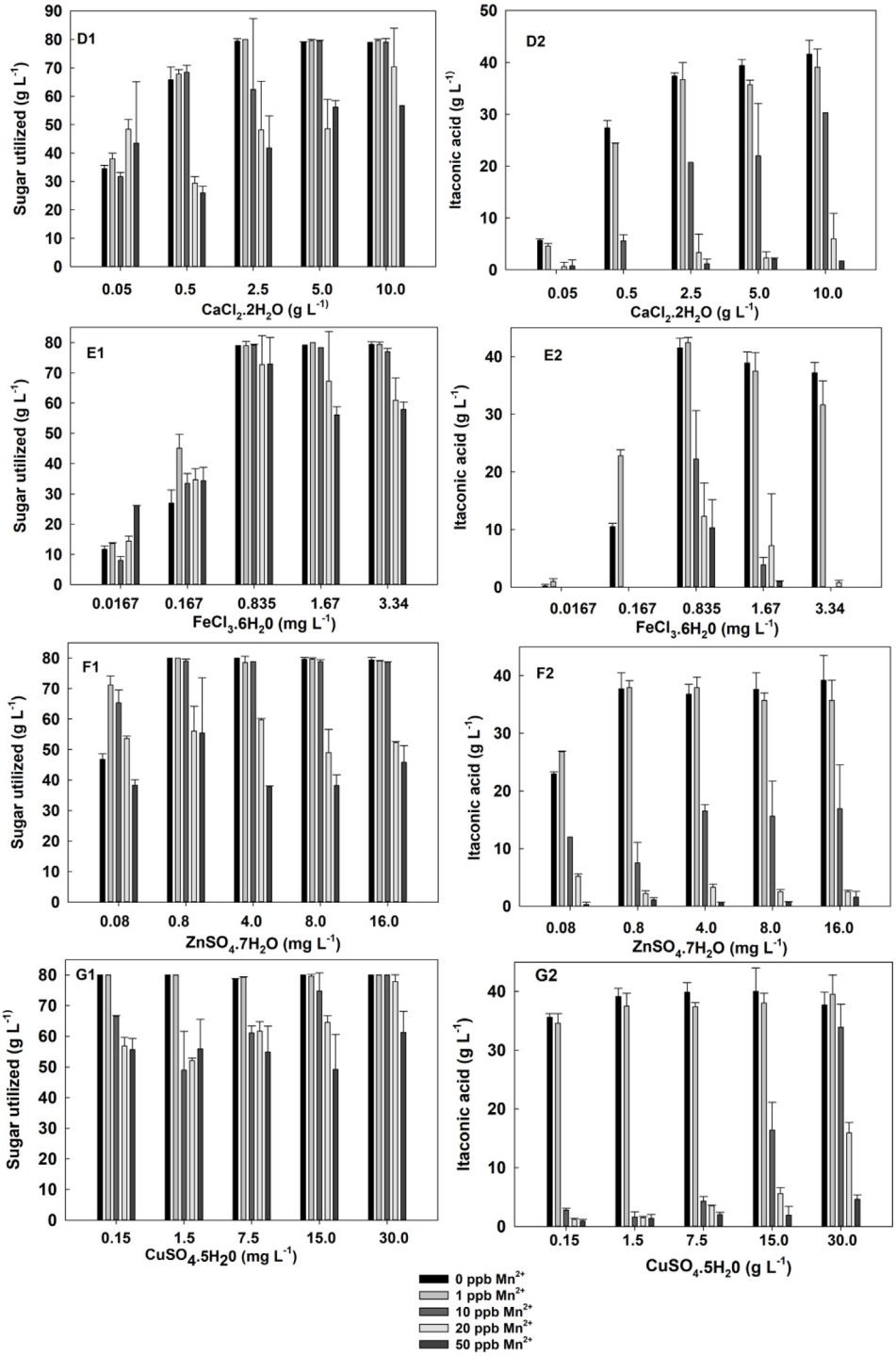

Fig. 2. Effects $CaCl_2{\cdot}2H_2O$ (D1, D2), $FeCl_3{\cdot}6H_2O$ (E1, E2), $ZnSO_4{\cdot}7H_2O$ (F1, F2) and $CuSO_4{\cdot}7H_2O$ (G1, G2) concentrations on sugar (80 g L^{-1}) utilization and IA production by *A. terreus* NRRL 1972 at pH 3.1, 33 °C and 200 rpm grown for 7 days with variable amounts of Mn^{2+}. The data presented are means ± standard deviations for triplicate experiments. The standard medium contained 5 g $CaCl_2{\cdot}2H_2O$, 1.67 mg $FeCl_3{\cdot}6H_2O$, 8 mg $ZnSO_4{\cdot}7H_2O$ and 15 mg $CuSO_4{\cdot}7H_2O$ per L. ppb is equivalent to μg L^{-1}.

(Fig. 1 B1, B2). At the very low NH_4NO_3 (0.03 g L^{-1}), no IA was produced even though 46.8 ± 1.8, 71.2 ± 2.9, 65.3 ± 4.2, 53.6 ± 0.8 and 38.3 ± 1.8 g glucose was consumed per L at Mn^{2+} concentration of 0, 1, 10, 20 and 50 μg L^{-1}, respectively. NH_4NO_3 at 0.3–6.0 g L^{-1} did not affect the sugar utilization up to 0–10 μg Mn^{2+} per L concentrations. NH_4NO_3 at 6.0 g L^{-1} was found to be better for IA production in the presence of 10, 20 and 50 μg Mn^{2+} per L even through IA production was still inhibited.

$MgSO_4{\cdot}7H_2O$ (0.01–2.0 g L^{-1}) did not help much to alleviate the inhibitory effects of Mn^{2+} on glucose utilization and IA production (Fig. 1 C1, C2). At 10 μg Mn^{2+} L^{-1}, 2.0 g $MgSO_4{\cdot}7H_2O$ L^{-1} gave the highest sugar utilization and IA production.

Varying the concentration (0.05–10.0 g L^{-1}) of $CaCl_2{\cdot}2H_2O$ also did not help to alleviate the effects of Mn^{2+} on glucose utilization and IA production (Fig. 2 A1, A2). However, $CaCl_2{\cdot}2H_2O$ at 10 g L^{-1} was marginally better for both sugar utilization and IA production at high Mn^{2+}.

$FeCl_3{\cdot}6H_2O$ at 0.835 mg L^{-1} was found to be better for both sugar

utilization and IA production in the presence of Mn^{2+} (Fig. 2 B1, B2). Higher $FeCl_3{\cdot}6H_2O$ (1.67 and 3.34 mg L^{-1}) along with raised Mn^{2+} worked to impede both sugar utilization and IA production by the fungal strain.

$ZnSO_4{\cdot}7H_2O$ at the concentrations tested (0.8–16.0 mg L^{-1}) had almost no effect on sugar utilization and the inhibition of IA production in the presence of Mn^{2+} (Fig. 2 C1, C2).

Mn^{2+} was tolerated better at higher $CuSO_4{\cdot}5H_2O$ per L with respect to sugar utilization and IA production (Fig. 2 D1, D2). High $CuSO_4{\cdot}5H_2O$ concentration (30 mg L^{-1}) was able to alleviate the inhibitory effect of Mn^{2+} at 10 μg L^{-1} level. Kuenz et al. (2012) reported an increased effect of IA yield with the increase of Ca^{2+} or Cu^{2+} ions. Cu^{2+} ions were shown to antagonize the effect of Mn^{2+} probably by inhibiting the transport of Mn^{2+} (Karaffa et al., 2015; Schweiger, 1961; Hockertz et al., 1987; Netik et al., 1997).

3.2. Effect of K^+ and PO_4^{-3} on sugar utilization and IA production in the presence of Mn^{2+}

The effects of K^+ and PO_4^{-3} on sugar utilization and IA production in the presence of Mn^{2+} (0, 50 μg L^{-1}) were evaluated using either KCl or NaH_2PO_4 and both compounds at different concentrations in the medium instead of KH_2PO_4 in order to determine which of the two ions needs limitation or both K^+ and PO_4^{-3} limitations are needed to alleviate the inhibitory effect of Mn^{2+} on IA production. The results are presented in Table 1. The fungal strain could not utilize sugar well and produce IA in the absence or presence of Mn^{2+} without either K^+ or PO_4^{-3} (# 1 and # 2). PO_4^{-3} had more pronounced effect than K^+ with respect to sugar utilization and IA production by *A. terreus* NRRL 1972. The sugar utilization and IA production were restored by adding 0.042 g KCl and 0.063 g NaH_2PO_4 in the medium equivalent to 0.08 g KH_2PO_4 per L (# 3). With 0.168 g KCl and 0.063 g NaH_2PO_4 in the medium, the effect of Mn^{2+} on sugar utilization and IA production were greatly minimized (# 4). Using 0.336 g KCl and 0.063 g NaH_2PO_4 in the medium, effect of Mn^{2+} on sugar utilization and IA production were also greatly minimized (# 5). However, with 0.042 g KCl and increasing the NaH_2PO_4 concentration to 0.254 g/L, sugar utilization and IA production were decreased by 35.8 and 82.7%, respectively at 50 μg L^{-1} Mn^{2+} concentration (# 6). With 0.042 g KCl and increasing the NaH_2PO_4 concentration to 0.507 g L^{-1}, the sugar utilization and IA production were decreased by 38.2 and 95.6%, respectively at 50 μg L^{-1} Mn^{2+} concentration (# 7). These data clearly indicate that high K^+ concentration did not have any adverse effect on sugar utilization and IA production, but low PO_4^{-3} concentration was better for both sugar utilization and IA production in the presence of Mn^{2+}. Thus, PO_4^{-3} limitation is essential for alleviating the inhibitory effect of Mn^{2+} on IA production by *A. terreus* NRRL 1972.

3.3. Measurement of PO_4^{-3} and dissolved oxygen in low and high PO_4^{-3} medium in the presence of Mn^{2+}

Two levels of KH_2PO_4 (0.1 and 2.4 g L^{-1}) with or without Mn^{2+} (50 μg L^{-1}) were used to monitor the changes in pH, PO_4^{-3} concentration and DO along with sugar utilization and IA production by *A. terreus* NRRL 1972 in shake flasks. The data are presented in Fig. 3 (A-E). At low PO_4^{-3} concentration (0.07 g L^{-1}, 0.74 mM), almost all (90%) of the PO_4^{-3} was consumed in 2 days in both cases (Fig. 3C). The sugar utilization patterns were the same in both cases with no sugar left unutilized in 7 days (Fig. 3A). The IA production decreased by 15.4% in 7 days in presence of 50 μg Mn^{2+} per L (Fig. 3B). The final pH dropped to 2.04 ± 0.06 and 1.88 ± 0.02 in the absence and presence of Mn^{2+}, respectively (Fig. 3E). At high PO_4^{-3} concentration (1.67 g L^{-1}, 17.64 mM), PO_4^{-3} level decreased slowly to 29% without Mn^{2+} and 55% with Mn^{2+} (1.9 fold more) in 7 days (Fig. 3C). The sugar utilization rate was faster, and the sugar was fully utilized in 4 days with 50 μg Mn^{2+} per L (Fig. 3A). The IA production decreased from 34.7 ± 0.8 g L^{-1} without Mn^{2+} to 0.3 ± 0.1 g L^{-1} with 50 μg Mn^{2+} per L that is 99.1% less (Fig. 3B). The final pH dropped to 2.16 ± 0.08 for no Mn^{2+} but the final pH was increased to 3.33 ± 0.1 with Mn^{2+} (Fig. 3E). One possible reason for higher final pH of the fermentation broth in the case of 2.4 g KH_2PO_4 with 50 μg Mn^{2+} per L is that after all sugars were consumed, the fungus started consuming itaconic acid. Depending on the amount of itaconic acid present in the fermentation broth, the itaconic acid consumption by the fungus may lead to pH change to higher level. Recently, Chen et al. (2016) identified an itaconic acid degrading pathway in itaconic acid producing *A. terreus*. It is evident that low PO_4^{-3} concentration was desirable for IA production in the presence of Mn^{2+}. The DO decreased gradually to 30% at 3 days, remained almost the same (27%) at 4 days and then increased to 63% in 7 days in the cases of low KH_2PO_4 without Mn^{2+} (Fig. 3D). For low KH_2PO_4 with 50 μg Mn^{2+} per L the DO decreased gradually to 40% after 3 days, remained almost the same (37%) at 4 days and then increased to 50% in 7 days. With high KH_2PO_4, the DO decreased to 24% in 2 days, 9% in 3 days and remained the same at 4 days without Mn^{2+}. It then increased to 56% in 6 days and decreased again to 43% in 7 days. The sugar was fully utilized in 6 days and IA production level was 37.4 ± 3.5 g L^{-1} in 6 days (3A, B). However, the DO decreased to about 10% in 2 days, remained more or less the same up to 4 days and then continued to increase to 50% in 7 days with 50 μg Mn^{2+} per L. The sugar was fully utilized in 4 days but IA production was severely inhibited. The DO content seemed to show no clear distinctive pattern in all four experiments. An adequate oxygen supply is essential for the cultivation of *A. terreus* because anaerobic conditions will irreversibly damage the filamentous fungus (Willke and Vorlop, 2001). Riscaldati et al. (2000) reported that rapid phosphorus consumption during the

Table 1
Relationship among Mn^{2+}, KCl and NaH_2PO_4 for sugar utilization and production of itaconic acid by *Aspergillus terreus* NRRL 1972.

Expt #	KCl (mg L^{-1})	NaH_2PO_4 (mg L^{-1})	Mn^{2+} (μg L^{-1})	Sugar utilized (g L^{-1})	Itaconic acid (g L^{-1})
1	0	63	0	24.9 ± 4.0	12.6 ± 2.3
		(50 mg PO_4^{-3})	50	17.2 ± 1.8	0.8 ± 0.1
2	42	0	0	5.2 ± 0.6	0.7 ± 0.1
	(22 mg K^+)		50	6.3 ± 2.5	0.0 ± 0.0
3	42	63	0	79.6 ± 0.5	40.7 ± 1.9
	(22 mg K^+)	(50 mg PO_4^{-3})	50	57.6 ± 0.2	31.5 ± 1.1
4	168	63	0	62.9 ± 8.6	39.7 ± 0.0
	(88 mg K^+)	(50 mg PO_4^{-3})	50	80.0 ± 0.0	39.9 ± 0.6
5	336	63	0	60.8 ± 0.5	35.5 ± 0.9
	(176 mg K^+)	(50 mg PO_4^{-3})	50	71.8 ± 11.6	40.5 ± 0.9
6	42	254	0	70.2 ± 2.2	38.7 ± 1.3
	(22 mg K^+)	(200 mg PO_4^{-3})	50	45.1 ± 0.8	6.7 ± 0.6
7	42	507	0	73.3 ± 4.3	38.9 ± 4.0
	(22 mg K^+)	(401 mg PO_4^{-3})	50	45.3 ± 6.8	1.7 ± 0.3

The data presented are means ± standard deviations for triplicate experiments. Sugar used, 80 g L^{-1}.

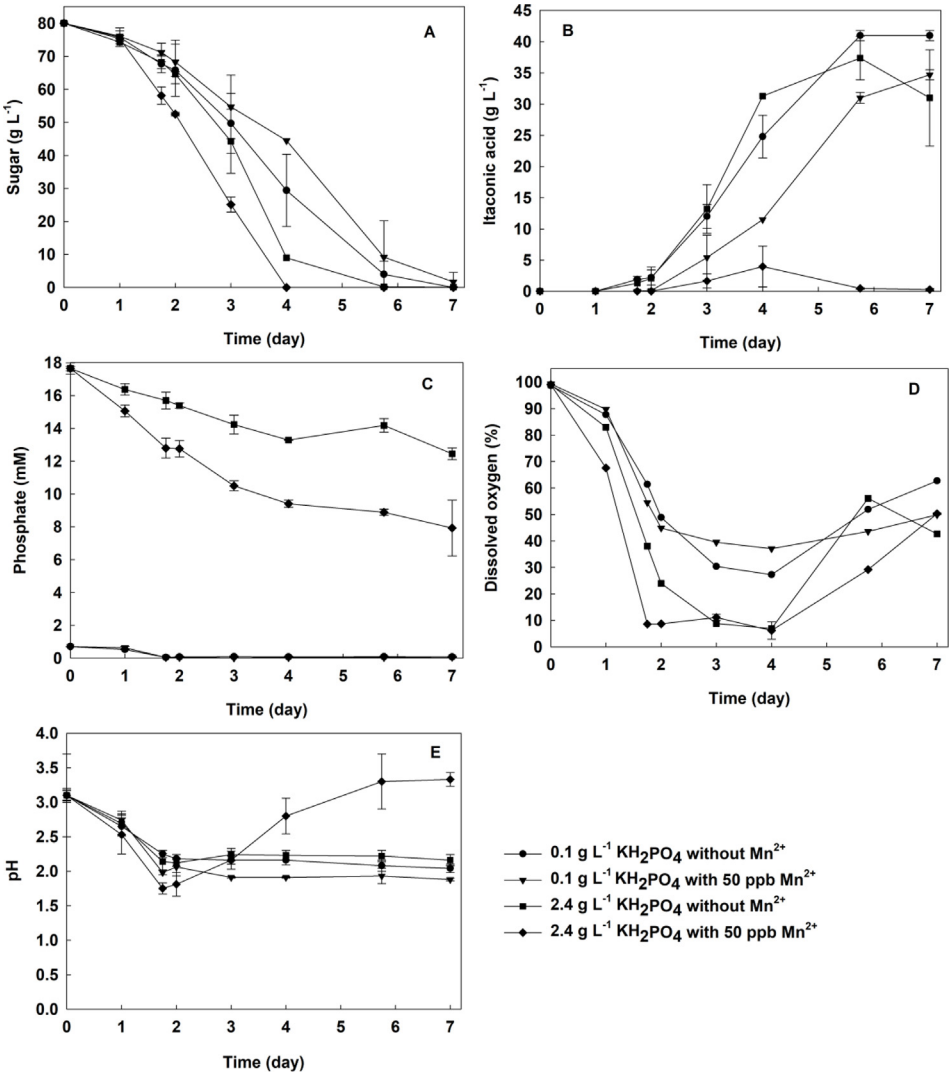

Fig. 3. Effects of low (0.1 g L^{-1}) and high concentrations (2.4 g L^{-1}) of K_2HPO_4 with or without Mn^{2+} (50 μg L^{-1}) on sugar (80 g L^{-1}) utilization (A), IA production (B), phosphate utilization (C), dissolved oxygen content (D), and pH change (E) during growth of *A. terreus* NRRL 1972 at pH 3.1, 33 °C and 200 rpm. The data presented are means ± standard deviations for triplicate experiments. ppb is equivalent to μg L^{-1}.

Table 2
Summary of final pH, PO_4 utilized, minimum dissolved oxygen content, sugar utilized and maximum itaconic acid produced by *A. terreus* NRRL 1972 grown at initial pH 3.1, 33 °C and 200 rpm for 7 days.

KH_2PO_4 (g/L)	Final pH	Cell mass (g L^{-1})	PO_4^{-3} utilized (mM)	Dissolved oxygen (%)	Sugar utilized (g L^{-1})	Itaconic acid (g L^{-1})
0.1 (0 μg Mn^{2+} per L)	2.04 ± 0.06	4.3 ± 0.5	0.63 ± 0.04 (90.0%)	27 (92 h)	80.0 ± 0.0	41.0 ± 4.1 (6 days)
0.1 (50 μg Mn^{2+} per L)	1.88 ± 0.02	9.5 ± 0.6	0.62 ± 0.02 (91.4%)	35 (119 h)	78.3 ± 2.9	34.7 ± 0.8 (7 days)
2.4 (0 μg Mn^{2+} per L)	2.04 ± 0.06	9.1 ± 2.1	5.19 ± 0.36 (29.4%)	6 (91 h)	79.9 ± 0.2	37.4 ± 3.5 (6 days)
2.4 (50 μg Mn^{2+} per L)	3.33 ± 0.10	18.0 ± 1.0	9.71 ± 0.25 (55.0%)	9 (37 h)	80.0 ± 0.0	3.96 ± 0.0 (4 days)

The data presented are means ± standard deviations for triplicate experiments. Sugar used, 80 g L^{-1}.

first 24 h was accompanied by a dramatic fall in DO from 100 to 10–15% of the saturation value. After that DO remained almost constant up to the overall exhaustion of the nitrogen source, then started to increase again. Park et al. (1993) reported that dissolved oxygen level only slightly affected the IA production by *A. terreus*. The final cell masses of the fungus after 7 days were 4.3 ± 0.5, 9.5 ± 0.6 g L^{-1} in the cases of low KH_2PO_4 (0.1 g L^{-1}) without and with Mn^{2+} (50 ppb), respectively. The data indicate that cell growth was increased by 2.2-fold with Mn^{2+}. For high KH_2PO_4 (2.4 g L^{-1}) without and with Mn^{2+} (50 μg L^{-1}), these values were 9.1 ± 2.1 and 18.0 ± 1.0 g L^{-1}, respectively. The data also indicate that cell growth of the fungal strain was almost doubled with Mn^{2+}. These data clearly indicate that PO_4^{-3} and Mn^{2+} enhanced the growth of the fungus separately and both of them helped synergistically to increase the cell mass of the fungus. A summary of the final pH, cell mass, PO_4^{-3} consumption, dissolved oxygen content, sugar utilization and maximum IA production by the fungal strain is given in Table 2.

Five heteroacids (malic acid, α-keto glutaric acid, succinic acid, *cis*-acotinic acid and *trans*-acotinic acid) were identified and quantified by HPLC that were formed during each fermentation. There was very little or no change in the patterns of these minor byproducts formations due to the change of Mn^{2+} content (data not shown).

4. Conclusions

This is the first report on the relationship of Mn^{2+} with the medium components for utilization of sugar and production of IA by an *A. terreus* strain. Low KH_2PO_4 level was necessary to alleviate the inhibitory effect of Mn^{2+} on IA production. Both K^+ and PO_4^{-3} were needed for IA production. However, high PO_4^{-3} was detrimental for IA production in the presence of Mn^{2+}. The inhibitory effect of Mn^{2+} on IA production was partly removed by increasing the $CuSO_4{\cdot}7H_2O$ level in the medium.

Conflicts of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.01.054.

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