Itaconic Acid Production by Filamentous Fungi in Starch-Rich Industrial Residues

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Abstract Several fungi and starch-rich industrial residues were screened for itaconic acid (IA) production. Out of 15 strains, only three fungal strains were found to produce IA, which was confirmed by HPLC and GC–MS analysis. These strains were identified as *Aspergillus terreus* strains C1 and C2, and *Ustilago maydis* strain C3 by sequencing of 18S rRNA gene and internal transcribed spacer regions. *Cis*-aconitate decarboxylase (*cad*) gene, which encodes a key enzyme in IA production in *A. terreus*, was characterized from strains C1 and C2. C1 and C2 *cad* gene sequences showed about 96% similarity to the only available GenBank sequence of *A. terreus cad* gene. 3-D structure and *cis*-aconitic acid binding pocket of Cad enzyme were predicted by structural modeling. Rice, corn and potato starch wastes were screened for IA production. These materials were enzymatically hydrolyzed under experimentally optimized conditions resulting in the highest glucose production of 230 mg/mL from 20% potato waste. On comparing the production potential of selected strains with different wastes, the best IA production was achieved with strain C1 (255.7 mg/L) using potato waste.

Elemental composition as well as batch-to-batch variation in waste substrates were analyzed. The difference in IA production from two different batches of potato waste was found to inversely correlate with their phosphorus content, which indicated that *A. terreus* produced IA under phosphate limiting condition. The potato waste hydrolysate was deionized to remove inhibitory ions like phosphate, resulting in improved IA production of 4.1 g/L by C1 strain, which is commercially competitive.

Keywords *Aspergillus terreus* · *Ustilago maydis* · *Cad* gene · Potato starch waste · GC–MS · Deionization

Introduction

Itaconic acid (IA) is a bio-based industrial chemical that can play a very important role in sustainable development as it is a substitute of wide range of petroleum-based chemicals like acrylic and methacrylic acids [1]. In 2004 US-Department of Energy (DOE) assigned IA on top 12 list of biotechnologically produced building block chemicals that can be used as precursor in the production of various high-value chemicals [2]. Industries are currently using glucose as the substrate for commercial IA production, which is relatively expensive. Thus, there is a need to use cheaper substrate such as carbohydrate-rich waste to bring down the cost. Few studies have been carried out with jatropha seed cake [3], sorghum bran [4] and sago starch [5] in the past for IA production, but other starch-rich wastes still remain to be explored.

The present study evaluated IA production using different starch-rich wastes (potato, rice and corn), which are much cheaper than glucose, providing an opportunity to recycle the waste material into value-added chemicals. The

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waste materials were characterized for different constituents, which are known to affect IA production profoundly. The IA yield was enhanced by appropriate modification of waste material. The seasonal variation in the waste composition and its effect on IA production was also determined. Similarly, several fungal isolates were screened to tap their natural potential for IA production. *Cis*-aconitate decarboxylase (*cad*) gene involved in IA synthesis has been reported from very few fungal strains so far. Current study demonstrated presence of this gene in the IA producing fungi.

Materials and Methods

Isolation and Evaluation of Fungi for IA Production

Fungi were isolated from coastal and mangrove forest soils using Czapek agar plates. Resulting fungal colonies were repeatedly sub-cultured by streak plate technique to isolate pure cultures. Spores of fungal isolates were collected with a platinum loop and suspended in sterilized water containing 0.1% Tween 80. The spore suspension was inoculated in 50 mL pre-culture medium dispensed in 250-mL flasks to final concentration of 1×10^6 spores/mL (Czapek broth, pH 7.5). The flasks were incubated at 30 °C for 4 days at shaking speed of 150 rpm. 10% inoculum from the pre-culture medium was transferred into 500-mL flasks filled with 100 mL fermentation medium. Two types of fermentation media were used. Medium 1 consisted of (g/L) glucose-180, NH_4NO_3 -3.02, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -1, KH_2PO_4 -0.1, CaCl_2 -5, FeCl_3 -0.0017, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ -0.008 and $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ -0.015 in distilled water with pH of 3 [6]. Medium 2 consisted of (g/L) glucose-120, NH_4Cl -1.6, KH_2PO_4 -0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.2, yeast extract-1, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -0.01, CaCO_3 -33 in distilled water with pH of 6.5 [7]. Flasks were shaken at 150 rpm and 35 °C. After 5–7 days of cultivation, the culture broth was centrifuged and supernatant was used to assay IA production. All the fermentation experiments were carried out in triplicate and statistical significance was determined at the level of 0.05.

IA was assayed by high-pressure liquid chromatography (HPLC; Waters, USA) with a 20 cm Sunfire C18 column (5 μm particle size, 4.6 mm $\times$ 250 mm). 20 mM orthophosphoric acid was used as mobile phase at flow rate of 1 mL/min and metabolites were detected at 210 nm with photodiode array detector (Waters 2998, USA) [8]. Peak of IA was identified using standard IA (Sigma, USA). Identification of IA was further confirmed by gas chromatography–mass spectrometry (GC–MS). The details of sample preparation and GC programming are given in Supplementary Material S1.

Identification and Characterization of IA Producing Isolates

Morphological, microscopic, biochemical and molecular characterization was carried out for the identification of IA producing fungi. Morphological examination was performed after growth on Czapek agar at 28 °C for 7 days. For microscopic examination, slides were stained with lactophenol cotton blue to study asexual and sexual reproductive structures. Isolates were further characterized for amylase and cellulase activities on specific media [9].

Molecular characterization was done by sequencing 18S rRNA gene, internal transcribed spacer (ITS) regions and *cis*-aconitic acid decarboxylase (*cad*) gene. Genomic DNA was isolated using ZR microbe DNA miniprep kit (Zymo research, USA) as per manufacturer instructions [10]. Detail of primer sequences and PCR programming are given in Supplementary Material S1. The complete PCR products were sequenced and searched against the nr database using BLAST program [11]. The identification of strains was further confirmed by searching ITS sequences against ISHAM (International Society for Human and Animal Mycology) ITS database [12]. The nucleotide sequences determined in this study were submitted to GenBank under accession numbers KX892615 to KX892622.

Cad Enzyme Structure Modelling Study

The *cad* gene sequences determined in this study were processed to remove intron and translated to respective protein sequences. The resulting sequences were submitted to Phyre2 web server for structure prediction [13]. The protein structure with the highest score was selected to predict the binding site of substrate *cis*-aconitic acid. SwissDock web server was used for the protein docking study [14].

Characterization and Hydrolysis of Starch-Rich Wastes

Three different types of starch-rich wastes were collected for the experiment. Potato starch waste was collected from food processing industry. Specifically, wash water from potato cutting stage was filtered through a fine sieve, vacuum dried, and finally oven dried to get potato starch waste. Rice waste (damaged grain) was obtained from grain-based ethanol producing industry, and corn waste (damaged kernels) was collected from poultry farm. Rice and corn wastes were ground into fine flour and sieved before use.

All waste materials were characterized for physico-chemical parameters. Moisture content was determined by

ASTM E1756-08 method [15] and ash content was determined by ASTM E1755-01 method [16]. Total carbohydrate content was determined by phenol sulfuric acid method [17]. Carbon, hydrogen, nitrogen and sulphur contents were determined by CHNS elemental analyzer (Vario EL, Elementar Germany). To determine the total phosphorus content (organic as well as inorganic), the samples were first subjected to persulfate digestion and then analyzed by Olsen's method [18].

Hydrolysis of starch-rich wastes was carried out using commercial enzymes, namely α -amylase from *Aspergillus oryzae* (960 U/mL) and amyloglucosidase from *Aspergillus niger* (300 U/mL), both obtained from Sigma, USA. All the experiments were carried out in triplicate and statistical significance was determined at the level of 0.05. Details about optimization of enzymatic starch hydrolysis are given in Supplementary Material S1.

Selection of Starch-Rich Waste and Fungal Strain for IA Production

20% of potato, rice and corn wastes were hydrolyzed under the optimized conditions. Resulting hydrolysates containing approximately 180 g/L of glucose equivalent were used as carbon source in the fermentation medium 1. Fermentation was carried out using all IA producing strains and subsequent IA determination was carried out as described above. All the experiments were carried out in triplicate and statistical significance was determined at the level of 0.05.

To study the effect of seasonal variation in waste, two batches of potato starch waste, designated as P1 and P2, were collected in different seasons; first batch in the month of June and second batch in the month of November. Both were used in fermentation to study the effect of seasonal variation in the waste substrate on IA production. In order to remove undesirable ionic species present in the hydrolysate, deionization was carried out (details are given in Supplementary Material S1) and its effect on IA production was studied.

Results

Isolation and Characterization of Potential Fungal Strains for IA Production

Total 15 fungal cultures were isolated from the soil samples collected from different coastal areas and mangrove forest near Mumbai. Screening for IA production was done through HPLC (Supplementary Fig. S2). Out of the 15 cultures, only 3 (C1, C2 and C3) were found to produce IA. The production of IA by these strains was confirmed by

GC–MS analysis (Supplementary Fig. S2). All 3 potential strains were further characterized and identified. Morphological, microscopic and biochemical characterization is shown in Supplementary Table S3 and Supplementary Fig. S4.

Molecular characterization was carried out by sequencing of 18S rRNA gene, ITS regions and *cad* (*cis*-aconitase decarboxylase) gene. Figure 1 shows the PCR amplification products for above genes. About 500 bp of 18S rRNA gene was amplified in all the three isolates (GenBank accession numbers KX892618, KX892620 and KX892622 for strains C1, C2, and C3 respectively). For *cad* gene, the product length was 1500 bp for C1 and C2 (GenBank accession numbers KX892615 and KX892616 respectively), while C3 did not show any amplification. ITS primers gave amplicons of 600 bp for C1 and C2 (GenBank accession numbers KX892617 and KX892619 respectively), and 800 bp for C3 (GenBank accession number KX892621). BLAST similarity search for 18S rRNA gene and ITS regions identified C1 and C2 as *A. terreus* strains, and C3 as *U. maydis*. Both C1 and C2 *cad* gene sequences showed 96% similarity with the *A. terreus cad1* sequence available in GenBank (accession number AB326105).

In-Silico Characterization of Cad Enzyme

cad gene sequences from strains C1 and C2 were translated to protein sequences and subjected to structural modelling. Protein threading using Phyre2 web server identified iminodisuccinate epimerase (PDB id 2HP0) and 2-methylcitrate dehydratase (PDB id 1SZQ) as the closest templates with 100% confidence from the Protein Data Bank. Both the above templates share very similar 3-D structure. As both Cad sequences showed slightly higher sequence similarity to iminodisuccinate epimerase among the above 2 templates, it was selected for the structural modelling. A 3-D structure model for Cad was successfully built and is shown in Supplementary Fig. S5. The structure clearly shows that Cad enzyme consists of 2 distinct domains, both containing α -helices as well as β -sheets. One domain is larger than the other. In all, the modelled structure was very similar to that of the template iminodisuccinate epimerase [19]. The alignment of Cad sequences with the template along with predicted secondary structure is shown in Supplementary Fig. S6. The predicted 3-D structure of Cad was further used for docking study with *cis*-aconitic acid. Majority of the resulting binding modes predicted a common pocket for binding of *cis*-aconitic acid, which is shown in Supplementary Fig. S5. This pocket was found to be similar to the suggested substrate binding site of template iminodisuccinate epimerase.

Fig. 1 Agarose gel showing amplification of 18S rRNA gene from cultures C1 (lane 1), C2 (lane 2), C3 (lane 3); ITS regions from cultures C1 (lane 5), C2 (lane 6), C3 (lane 7); and *cad* gene from C1 (lane 9), C2 (lane 10). Lanes 4, 8 and 11 represent 100 bp ladder

Characterization of Starch-Rich Wastes

The analytical determination showed no substantial difference in the hydrogen, nitrogen and sulfur contents of corn, rice and potato, while their carbon content was different (Table 1). Total carbohydrate analysis showed the variation in carbohydrate content among different wastes. Amount of total phosphorus was maximum in corn starch waste (1039 ppm) and minimum in potato starch waste (330 ppm).

Hydrolysis of Starch-Rich Wastes

The optimum pH and temperature of α -amylase were determined to be 5 and 55 °C respectively, while those for amyloglucosidase were found to be 4 and 55 °C respectively. Different concentrations of starch-rich wastes were subjected to hydrolysis by a range of concentrations of

these enzymes at their optimum pH and temperature. It was observed that 10% concentration of all wastes were liquefied within 24 h of incubation at all the tested α -amylase concentrations (Fig. 2). However, 20 and 30% concentrations of all wastes did not liquefy even after 96 h of incubation at lower α -amylase concentration. About 48–72 h of incubation was required to liquefy 20 and 30% wastes at higher α -amylase concentration (5 U/g waste).

After liquefaction, the samples were used to optimize saccharification by amyloglucosidase. The highest glucose production was obtained with amyloglucosidase concentration of 1 U/g waste and 48 h incubation for all wastes (Supplementary Fig. S7). The final optimized conditions to obtain maximum glucose production per g of waste with least amount of enzyme were 20% substrate concentration, 5 U/g waste of α -amylase and 1 U/g waste of amyloglucosidase. The maximum glucose production was obtained with potato starch (230 mg/mL) followed by corn (200 mg/

Table 1 Characterization of starch-rich waste

Waste material	Composition (dry weight basis)							
	Moisture content (%)	Ash content (%)	Total carbohydrate (glucose equivalent %)	C (%)	H (%)	N (%)	S (%)	P (%)
Rice	8.52	0.67	96.52	41.98	7.19	1.52	0.16	0.067
Corn	3.56	1.18	72.85	45.08	7.60	1.62	0.05	0.104
Potato (P1)	14.15	0.40	85.60	39	7.08	0.06	0.15	0.033
Potato (P2)	12.8	0.642	106.30	38.45	7.22	0.00	0.14	0.064

Fig. 2 Optimization of enzyme concentration, waste substrate concentration and incubation time for potato starch waste hydrolysis by α -amylase. Error bars represent standard error from triplicate experiment

mL) and rice (180 mg/mL). The highest glucose yield corresponded to 0.92 g/g of potato starch waste.

Literature suggests that sonication increases the surface area of substrate for enzyme to attack [20]. In this study, however, no significant difference in the degree of hydrolysis was detected between sonicated and non-sonicated wastes, when either was used as substrate for α -amylase and amyloglucosidase (Supplementary Fig. S8).

Selection of Appropriate Waste and Fungal Strain for IA Production

Out of the three potential cultures, *A. terreus* strains C1 and C2 produced 30.8 and 23.4 g/L of IA in medium 1 respectively, but both the strains did not produce any IA in medium 2. Similarly, *U. maydis* strain C3 produced 34.52 mg/L IA in medium 2, but showed no production in medium 1. Based on the productivity, strains C1 and C2 were selected for further screening with waste hydrolysates as substrate. *A. terreus* strain C1 was found to give the best IA production of 255.7 mg/L on day 5 with potato starch hydrolysate followed by rice and corn wastes (Supplementary Fig. S9). Although potato starch waste gave the best IA production, it can exhibit seasonal variation, which in turn may affect IA fermentation. Hence, the potato waste was again collected in a different season (P2) and compared to earlier potato waste batch (P1) for physico-chemical characteristics and IA production. No significant difference was observed for C, H, N and S content of P1 and P2 batches, but their total carbohydrate and total phosphorus contents were different (Table 1). Upon hydrolysis and fermentation, P2 showed IA production of 198.32 mg/L, as compared to 255.7 mg/L for P1.

In an attempt to enhance IA yield, potato starch hydrolysate was treated with ion exchange resins, which resulted in 16 fold increase in IA production (final titer of 4.1 g/L) by C1 compared to crude hydrolysate. Based on the consumption of starch waste, the IA yield was found to be 0.02 g/g starch.

Discussion

Recycling, reprocessing and eventual utilization of carbohydrate present in the waste offer potential of returning it to beneficial uses rather than its discharge to the environment. Okabe et al. [21] reported that 25% of the production cost of IA is the cost of carbon source and it is a major factor currently preventing its wider application [22]. Current study was designed to screen the potential of several wild type fungi and starch-rich waste materials for improved IA production.

Three fungal strains (C1, C2 and C3) were found to produce IA by HPLC screening, which was further confirmed by GC–MS analysis. This is very important as fungi can secrete several unknown metabolites and organic acids along with IA [22]. HPLC has limited ability to resolve such complex mixtures, which may result in other compounds co-eluting with or in place of IA in the chromatogram. Hence, there is need to confirm the identity of IA by using advanced techniques such as GC–MS, where the identification is based on mass fragmentation. To the best of our knowledge, this might be the first study to report GC–MS confirmation of fermentative IA production. The potential IA producing strains were identified by sequencing of 18S rRNA gene. The identification was further confirmed by sequencing of ITS regions, which has been recommended as the universal fungal barcode sequence [23]. Based on the results, C1 and C2 were identified as different strains of *A. terreus*, while C3 was identified as *U. maydis*.

The strains C1 and C2 produced IA under phosphate limiting conditions (Medium 1), while C3 produced IA under nitrogen limiting conditions (Medium 2). This is in agreement with earlier reports that *A. terreus* secretes IA in phosphate limiting medium, while *U. maydis* produces IA under nitrogen limiting condition [22]. Similarly *cad* gene which encodes *cis*-aconitate decarboxylase, a key enzyme in IA production, was found to be present in C1 and C2, but was absent in C3. Indeed, *cad* gene is involved in IA

production in *A. terreus* while *U. maydis* uses an alternative pathway involving *trans*-aconitate [24]. The Cad enzyme (EC 4.1.1.6) catalyzes decarboxylation of *cis*-aconitate, a TCA cycle intermediate, to IA. The open reading frame of *cad* gene consists of 1529 bp DNA which encodes a polypeptide of 490 amino acids. It is expressed as a monomeric protein of 55 kDa in the cytoplasm [25]. Only one sequence entry of *A. terreus cad* gene is available in GenBank (accession number AB326105) so far. Current study determined 2 more sequences of *cad* gene, which showed about 96% similarity to the above GenBank sequence. Structural analysis of Cad protein sequences revealed their similarity to iminodisuccinate epimerase and 2-methylcitrate dehydratase enzymes, both of which belong to MmgE/PrpD family of proteins. This is in agreement with Kanamasa et al. [25], who first cloned *A. terreus* Cad1 and showed its high similarity with the MmgE/PrpD family. However, the structure of Cad enzyme is still not available or predicted. Structural analysis can lead to identification of active site residues and understanding of the reaction mechanism. This information can help in generating more efficient Cad variants by recombinant DNA technology. Current study filled this gap by predicting 3-D structure of Cad sequences from the cultures C1 and C2. Further, *cis*-aconitic acid binding pocket was also identified by in silico docking.

In the present study, *A. terreus* strains C1 and C2 produced 30.8 and 23.4 g/L of IA from glucose respectively, which is higher than the earlier reported production of 21.35 g/L from a wild type *A. terreus* strain [26]. As C1 and C2 showed around 1000 fold higher production of IA as compared to C3, they were selected for further study. This is in accordance with previous studies showing higher IA production from *A. terreus* than *U. maydis* [22].

Few studies have targeted starch-rich wastes for IA production. IA yield of 0.35 and 0.34 g per g of corn starch and sago starch respectively, have been reported in the past [5, 27]. However, waste characterization to account for the effect of non-starch constituents present in the waste is still limited. Riscaldati et al. [28] reported that when phosphorous level in the medium was reduced to <10 ppm, the fungal mycelium exhausted its primary growth and started to excrete IA. To address these issues, the elemental composition of waste substrates was determined in the present study. Also, batch-to-batch variation in waste substrates was analyzed. The phosphorous concentration in two different batches of potato waste was found to be 330 (P1) and 640 ppm (P2), which is in the range of potato phosphorous content (434–1087 ppm) reported by Noda et al. [29]. The IA production from P2 was found to be about 78% of that from P1, which is in agreement with their phosphorous contents, as *A. terreus* has been reported to produce IA under phosphate limiting condition.

Although IA production was different from the 2 batches of potato waste, the difference was not large enough to affect the commercial viability.

Both C1 and C2 showed very weak amylase activity on starch agar, and hence, starch wastes were hydrolyzed using commercial enzymes. Sonication of waste materials before addition of enzymes did not enhance the degree of hydrolysis significantly. This is in agreement with Azhar et al. [20] and Apar et al. [30] who studied the effect of sonication on enzymatic hydrolysis of potato and other starchy materials.

On comparing the production potential of C1 and C2 with different waste hydrolysates, the best production was achieved with C1 (255.7 mg/L) followed by C2, using potato starch hydrolysate as carbon source. IA production is highly sensitive to several medium components such as Fe, Mn, Cu, Zn, P, and N [31]. The crude starch hydrolysate used in the study may contain many undesirable ions which may affect IA production. Therefore, deionization of potato starch hydrolysate was carried out using ion exchange resins. The result indicated that IA production by C1 strain was increased 16 fold after removal of ionic impurities present in crude hydrolysate. Thus, the current study established deionization as a simple method to enhance IA productivity. Similar enhancement of citric acid secretion by *Yarrowia lipolytica* upon deionization of starch hydrolysate was reported by Shah et al. [32].

Further optimization of medium and growth conditions such as salinity, nitrogen source, phosphorus, nitrogen, Fe and Ca ion concentration, temperature, pH, shaking speed, inoculum size etc. may enhance the IA yield and improve the techno-economical feasibility of IA production from hydrolyzed potato starch waste. These parameters can be studied by classical one at a time approach, or statistical designs like Plackett-Burman design and response surface designs.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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