

Microbial Itaconic acid Production from Starchy Food Waste by Newly Isolated Thermotolerant *Aspergillus terreus* Strain

Vivek Narisetty ^{a*}, Ashish A Prabhu ^{a*}, Khalid Al-Jaradah ^a, Deeksha Gopaliya ^b, Abeer H Hossain ^c, Sunil Kumar Khare ^b, Peter J Punt ^c, Vinod Kumar ^{a**}

^a School of Water, Energy and Environment, Cranfield University, Cranfield MK43 0AL, UK

^b Department of Chemistry, Indian Institute of Technology Delhi, Hauz Khas, New Delhi – 110016, India

^c Dutch DNA Biotech B.V., Padualaan 8, 3584 CH Utrecht, The Netherlands

* Contributed equally.

**Corresponding author

Dr Vinod Kumar; Phone: +44(0)1234754786; E-mail: Vinod.Kumar@cranfield.ac.uk

Abstract

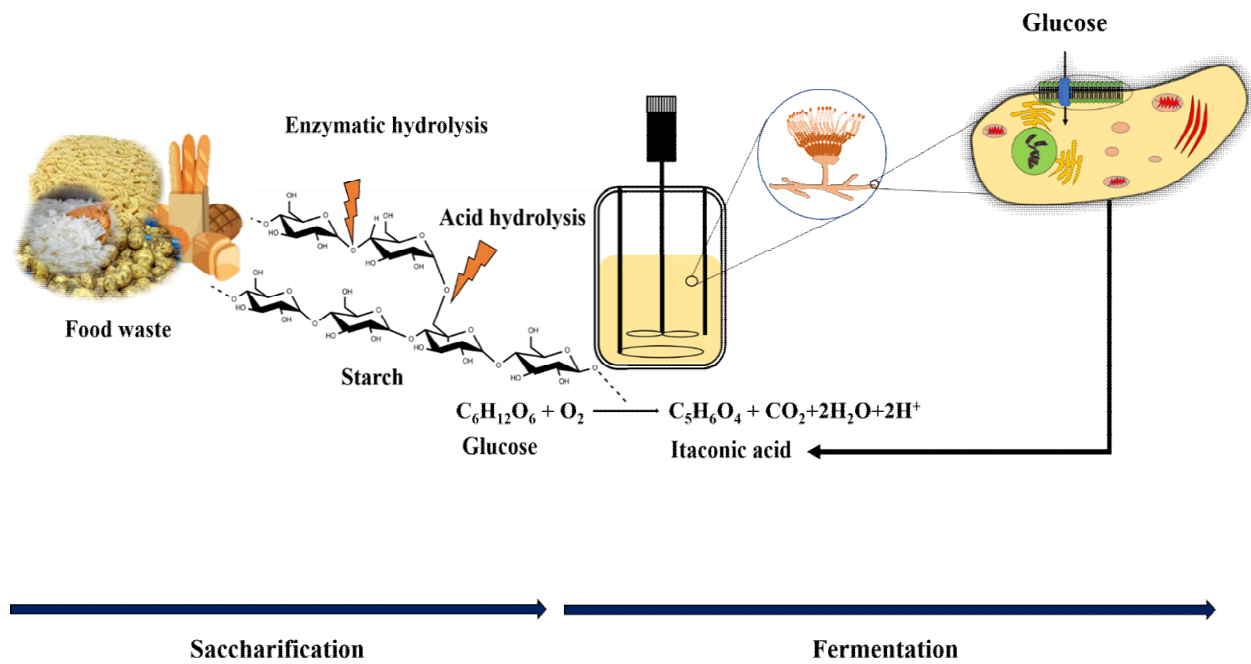
In the present study, we have explored the potential of newly isolated *Aspergillus terreus* BD strain, which can accumulate itaconic acid (IA) at higher temperature. The shake flask cultivation of thermotolerant strain with medium optimized using Box-Behnken Design at 45°C resulted in IA accumulation of 28.9 g/L with yield of 0.27 g/g. The enzymatic saccharification of the synthetic food waste (SFW) consisting of potatoes, rice & noodles were optimized using Taguchi method of orthogonal array to maximize the release of fermentable sugar. The maximum glucose release of 0.60 g/g was achieved with 10% biomass loading, 5% enzyme concentration, pH 5.5 and temperature 60 °C. The sugars obtained from SFW was integrated with IA production and maximum IA titer achieved with SFW hydrolysate during bioreactor cultivation was 41.1 g/L with conversion yield of 0.27 g/g while with pure glucose IA titer and yield were 44.7 g/L and 0.30 g/g, respectively.

Key words: *Aspergillus terreus*; Itaconic acid; Thermotolerant; Synthetic Food waste; Enzymatic hydrolysis.

Highlights

- *Aspergillus terreus* BD, a new thermotolerant IA accumulating strain was isolated.
- Optimization of enzymatic hydrolysis of food waste yielded 0.60 g/g glucose release.
- Optimization of media composition caused 61% increment in IA titers.
- Bioreactor cultivation of BD strain resulted in 41.1 g/L IA with 0.27 g/g yield from SFW.

Graphical abstract



1. Introduction

Food waste is a major global concern as it impacts the social, economic, and environmental status of the region or the country. Over last few decades the constant rise in human population demands and increased supply have ushered the alarming effects on the amount of unutilized food and food byproducts (Sharma et al., 2021). Around one-third of the food produced in the world is wasted (~1.3 billion tonnes) or lost through the food chain every year and cost the world economy about \$750 billion (Gustavsson et al., 2011; Paritosh et al., 2017). These wasted food materials in the landfills could generate 4.4 gigaton (Gt) of CO₂ annually which is equivalent to 8% of global greenhouse gas emissions (FAO, 2015). It has been estimated that about 10 million tonnes of food and drink are wasted in UK on annual basis, worth around £20 billion (Our waste, our resources: A strategy for England, 2018). For example, potatoes and bread are the two major food waste in UK and 24 million slices of bread and 5.8 million potatoes are wasted on daily basis (Jagtap et al., 2019; O'Donovan, 2013). The food should not be wasted in the first place, but the fact is that it is inevitable within the supply chain. In other words, food wastage can be minimized but cannot be eliminated.

Chemical and allied industries are heavily reliant on crude oil, a finite and non-sustainable resource. Microorganisms have been exploited from many years to produce value-added products using renewable carbon sources (Ahn et al., 2016; Kim et al., 2021). The microbial cell factories overproducing metabolites through fermentative route is a potential alternative to fossil-based production of chemicals. The chemical building blocks can be amassed through microbial routes using edible as well as non-edible feedstocks. Further, the bio-based products offer added advantages such as biodegradability, reusability, sustainability and contribute to a carbon neutral society (Choi et al., 2015). Food waste (FW) is attractive in terms of its nutrient content, i.e. 30–60% starch/sugars, 5–10% proteins and 10–40% lipids (Kumar and Longhurst, 2018). The conventional methods employed for treating food wastes are composting, anaerobic digestion and/or landfills. In UK, most of the food waste goes into anaerobic digestion (AD) processing and to some extent to incineration. In fact, the number of AD

plants treating food/farm waste in UK has increased from 63 to 420 since 2011 (Our waste, our resources: A strategy for England, 2018). These methods do not harness the full potential of nutrient rich food waste that is much higher, and the scope of opportunity is enormous. There is high demand to adopt low carbon climate resilient pathways that can not only effectively manage waste but create wealth out of it. The microbial conversion of renewable biomass into fuels and chemicals is a green, clean and low carbon manufacturing approach (Uçkun Kiran et al., 2015). The high quantity of FW has the potential feedstock for global bioproduction of large quantities of chemicals with high market value. A more profitable way of channelizing FW could be the efficient transformation of this renewable organic carbon source to spectrum of industrially important chemicals and fuels via greener route (Tuck et al., 2012; Kumar and Longhurst, 2018).

Itaconic acid (IA) (2-methylidenebutanedioic acid), an unsaturated dicarboxylic acid, is a highly desired platform chemical, which is used as a building block or additive in manufacturing fiber, resins, lattices, plastic, detergents, rubber, paint, surfactants, lubricants and bioactive compounds (Bafana and Pandey, 2018). The presence of the unsaturated bond, and dicarboxylic acid functional groups in IA make it an effective intermediate in the preparation of complex organic compounds and various reactions, like salt formation, esterification, anhydride formation, addition, and polymerization reactions. It has a modest market worldwide and the global production of IA in 2011 was over 41,400 tonnes worth \$74.5 million. It has been predicted that the annual production capacity will improve by 5.5% between 2016 and 2023 and reach by \$204.6 million (Weastra, 2013; Kumar et al. 2017). It has potential to replace valuable chemicals derived from fossil fuels, such as acrylic acid, acetone, cyanohydrin, maleic anhydride, and sodium tripolyphosphate in cleaning products. The total addressable market volume for IA was estimated to be approximately 6,163,409 tonnes with a value of \$11.1 billion (Market Report, 2015). Currently, IA is produced from biological routes. Plethora of literature is available on the bioproduction of IA using different microorganisms. Among them *Aspergillus terreus* strain is the most investigated one and prevalent host for bioproduction of IA, reaching titers up to 129 g/L. The biosynthesis of IA in *A. terreus* takes place through decarboxylation of cis-aconitate by enzyme cis-aconitate decarboxylase (CAD). Cis-aconitate is an intermediate of

the aconitase reaction in the TCA cycle during conversion of citrate into isocitrate (Saha et al., 2017; Bafana and Pandey, 2018) (see supplementary material).

In present study, we have isolated a new thermotolerant strain of *A. terreus* BD, which can grow and accumulate IA at high temperature (45°C). The IA production capability of BD strain was investigated and compared with well-studied native IA producing *A. terreus* DSMZ 23081 strain. To enhance the IA production, process optimization was carried out using Box-Behnken statistical method followed by validation in the shake flask and bioreactor. Further, enzymatic saccharification of synthetic food waste (SFW) consisting of potatoes, rice & noodles were optimized using Taguchi method of Orthogonal array to maximize the release of fermentable sugar. As a case study, the sugar obtained from SFW was integrated with IA production by BD strain and compared with pure glucose. Overall, this is the first study on production of IA by a thermotolerant *A. terreus* strain using food waste as a feedstock.

2. Materials and Methods

2.1. Materials

All the chemicals used in this study were purchased from Sigma Aldrich (USA) and Fischer scientific and are of analytical grade quality. The enzyme amyloglucosidase from *Aspergillus niger* was purchased from Sigma-Aldrich, St Louis, MO with 260 U/ml activity. SFW was comprised of potato, rice, and noodles in 1:1:1 ratio on dry weight basis. One hundred grams of SFW was washed thoroughly under tap water and mixed with 900 ml distilled water and autoclaved at 121 °C for 15 min. The resulting mash was used for enzymatic hydrolysis.

2.2. Strain isolation and identification

The thermotolerant fungal strain used in this study was isolated from soil samples collected from various locations in the Dhaka region of Bangladesh. Through serial dilutions and colony plating, various fungal species were isolated. After single colony purification, the isolates were plated on MacConkey Agar plates to identify isolates with significant acidification at elevated growth temperatures. One isolate from agricultural soil, showed a particularly strong acidification at 45°C. Genomic DNA was isolated from mycelium using a mag kit from LGC (LGC, Queens Road,

Teddington, Middlesex, TW11, 0LY, UK). Further ITS (Internal transcribing spacers) sequence analysis of the acid producing strain was performed using following forward primer (ITS1 5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer (ITS4 5'-TCCTCCGCTTATTGATATGC-3'). PCR was performed in an Alpha Cyclor 4 (PCRmax) using the isolated genomic DNA as template and Phusion Hot Start II DNA Polymerase (Thermo Scientific). The obtained PCR product was purified using QIAquick® PCR Purification Kit (QIAGEN) and sequence analysis was performed at Baseclear (The Netherlands). The ITS sequence obtained was aligned with sequences available in the NCBI database through nBLAST, and the results showed complete identity to *Aspergillus terreus* strains (e.g., accession MH856139.1), hence defined the isolate as *A. terreus*. The strain was subsequently named *A. terreus* BD. Based on this identification it was likely that the acidification caused by this strain was due to the production of IA, as this is the major organic acid produced by this species. According to the literature, IA production by *A. terreus* strains has been shown to be optimal between 30-35°C (Bafana and Pandey, 2018). Since the thermotolerant growth characteristics of the newly isolated *A. terreus* are unusual, it was interesting to explore if the new strain was also able to grow and accumulate IA at higher temperature which has several benefits. Initial flask experiments were performed using the isolated strain *A. terreus* BD provided by Dutch DNA Biotech BV, Netherland (<https://www.ddna-biotech.com/>) and results were compared with *A. terreus* NRRL1960 showed that the newly isolated strain had better growth and more Itaconic acid production at 45°C then at 33°C, while the reverse was true for NRRL 1960 strain (See supplementary material).

2.3. Inoculum preparation and submerged cultivations in shake flask

The *A. terreus* strains (BD and DSMZ) used in the present study were preserved in 20 % glycerol (v/v) at – 80°C and maintained on potato dextrose agar (PDA) plates containing 4 g/L potato extract, 20 g/L glucose and 15 g/L agar. *A. terreus* DSMZ 23081 strain was procured from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Conidia were used for the pre-inoculum preparation and fermentation experiments and derived either from surface or submerged cultivation. For spore formation, the two strains were plated on PDA, incubated for 120 h, harvested using 0.9% w/v NaCl solution containing 0.01% tween 20. The

concentration of the spore suspension was determined using a Thoma counting chamber (BLAUBRAND®, Brand, Germany) with Olympus microscope (Olympus BX40, UK). The culture medium composition used for preparing seed culture and IA production was as follows: 100 g/L glucose, 0.8 g/L KH_2PO_4 , 3.0 g/L NH_4NO_3 , 1.0 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 5 ml/L trace elements (5.0 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.67 mg/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 8.0 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 15.0 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (Krull et al., 2017). Individual components were autoclaved separately except trace elements solution, which was filter sterilized. The final pH of the medium prior to autoclaving was adjusted to 3.4 using 1N H_2SO_4 . The seed culture preparation and submerged fermentation for IA production was conducted in 500 ml Erlenmeyer flask with 100 ml working volume. Cultivation was carried out at specified temperature on a rotary shaker (Excella 24, New Brunswick) at an agitation speed of 250 rpm. The pre-culture and IA production medium was inoculated at spore concentration of 1×10^6 and 1×10^8 spores/ml, respectively. The pre-culture for inoculum preparation was cultivated for 96 h, and the spores formed were separated from the mycelia using a sieve. The spore suspension was centrifuged at 10,000g for 10 min and washed twice using 0.9% w/v NaCl solution containing 0.01% tween 20 solution.

The experiments comparing IA production by isolated *A. terreus* BD and DSMZ strain was performed in shake flask using the culture conditions described above. Each strain was cultured at two temperatures, 33 and 45°C, and the results were compared.

2.4. Taguchi method of orthogonal array for optimization of enzymatic hydrolysis of SFW

A standard Taguchi orthogonal array $\text{L}_9(3^4)$ fractional factorial experimental design matrix was implemented for the optimization of four parameters viz., biomass loading (% w/v), enzyme loading (% v/v), pH and temperature (°C) for enzymatic hydrolysis of SFW. The letter (L) with subscript 9 symbolizes the number of experimental runs. The parameters levels taken into consideration along with L_9 experimental design are shown in Table 1 and 2. All the experimental runs were carried out in a 2L glass bottle with 1L working volume and incubated at 45°C with shaking at 150 rpm for 24h. Designated amount of SFW with amyloglucosidase (260 U/ml) was diluted in 0.05M acetate

buffer. After the completion of reaction, hydrolysate was filtered, and the glucose content was quantified by high performance liquid chromatography (HPLC) method.

Independent variable	Levels		
	Level 1	Level 2	Level 3
Biomass loading (w/v %)	10	30	50
Enzyme loading (v/v %)	0.5	2	5
pH	3.5	4	5.5
Temperature (°C)	45	55	60

Table 1: Parameters and levels used for Taguchi (OA) optimization method

Biomass (w/v %)	Temperature (°C)	pH	Enzyme conc (v/v %)	Glucose (g/g)	SNRA
10	45	3.5	0.5	0.31 ± 0.02	-10.17
10	55	4	2	0.42 ± 0.02	-7.46
10	60	5.5	5	0.60 ± 0.04	-4.47
30	45	4	5	0.46 ± 0.02	-6.74
30	55	5.5	0.5	0.29 ± 0.01	-10.68
30	60	3.5	2	0.25 ± 0.01	-12.18
50	45	5.5	2	0.12 ± 0.005	-18.60
50	55	3.5	5	0.13 ± 0.008	-17.48
50	60	4	0.5	0.09 ± 0.005	-20.85

Table 2: L₉ (3⁴) orthogonal array of Taguchi experimental design for optimization of synthetic food waste hydrolysis

The optimized parameters were analyzed by providing the results from the HPLC as the input to MINITAB statistical software (version 16, PA, USA). The objective function for the experimental design was provided by signal to noise ratio (S/N) to evaluate the interaction among the control and noise terms. The quality characteristics of “Bigger is better” are used as the measure of S/N ratio. The S/N ratio is represented as Equation 1.

$$\frac{S}{N} = 10 \log_{10} \left(\frac{\beta^2}{\sigma^2} \right) \quad (1)$$

Where signal represents the mean value (β) and noise represents the variance (σ) present in the system. To determine the influence of parameters involved in the Taguchi experimental design, glucose yield (g/g) obtained from the food waste and S/N ratios of 9 runs were investigated. The statistical significance of individual parameters

and in combination were determined by analysis of variance (ANOVA), and the performance statistics that measure deviation from the target, called as mean square deviation (MSD) was calculated using Equation 2.

$$\eta = -10 \log \left[\frac{1}{n} \sum_{i=1}^n \frac{1}{Y_i^2} \right] \quad (2)$$

n: number of replication, Y_i : response (objective function).

Accordingly, the optimum parameters for the efficient hydrolysis of food waste were determined by uniting the levels of parameters from which the highest output (g/g glucose yield) was observed.

2.5. Optimization of medium components using Box-Behnken Design (BBD) and validation in shake flask conditions

The Box-Behnken design (BBD) matrix was used to evaluate the influence of concentrations of media components like glucose (g/L), NH_4NO_3 (g/L), KH_2PO_4 (g/L), and trace elements (ml/L) on IA production and to find the optimal level of each individual parameter resulting in maximum IA production. The design comprises of replicated center points and the set of points lying at the midpoints of each edge of the multidimensional cube, which is used to evaluate the interaction between the parameters and defines the region of interest forming a second order polynomial equation. The four variables were designated as X_1 , X_2 , X_3 , X_4 (independent variables) and IA was designated as Y , which is a predicted response (dependent variable). The low, middle and high levels of each variable are coded as -1, 0 and +1 (Table 3).

Independent Variables	Units	Symbol code	coded value		
			-1	0	1
Glucose	g/L	X_1	75	112.5	150
KH_2PO_4	g/L	X_2	0.1	0.55	1
NH_4NO_3	g/L	X_3	1	3	5
Trace elements	ml/L	X_4	1	5.5	10

Table 3: Experimental codes, range and levels of the independent variables used for Box Behnken design (BBD) experiments

The total number of experiments was calculated from the Equation (3).

$$N = 2K(K - 1) + C_0 \quad (3)$$

The following empirical second order polynomial equation (Equation 4) was adopted that predicts the effect of individual parameters on the response.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (4)$$

Where, Y is the measured response variable; β_0 is the constant term, β_i is the co-efficient of linear terms, β_{ij} is the co-efficient for cross product terms, β_{ii} is the co-efficient for quadratic terms of the model, and x_i, x_j represent the independent variables in coded values. The goodness of fit of the polynomial equation produced was articulated as co-efficient of determination R^2 and its statistical significance was evaluated using F-test. MINITAB statistical software (version 16, PA, USA) was used for the regression analysis and the graphical representation of the data obtained from the optimization experiments. With the second order polynomial Equation 4, generated based on the experimental results and the predicted values, the derived optimum media composition was as follows: 150 g/L, glucose; 3 g/L, NH_4NO_3 ; 1.0 g/L, KH_2PO_4 ; 1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; and 5.5 ml/L Trace elements. Using this optimized media, the BBD model was validated in the shake flask cultivation using *A. terreus* BD strain at 45°C.

2.6. Batch bioreactor cultivation

After the validation in the shake flask, the process was scaled-up in 2.5 L bioreactor (Electro Lab Bioreactors, UK) with 1.0 L working volume. The batch bioreactor experiments were performed using optimized media components as mentioned above. The sterile fermentation medium was inoculated with 1.0×10^8 /ml spores from 96 h old pre-inoculum, prepared from submerged cultivation. The initial pH was set at 3.4 before inoculation, medium was allowed to acidify naturally till pH 2.3 and thereafter, pH was maintained at 2.3 by addition of 5M KOH. The temperature and agitation speed were controlled at 45°C and 600 rpm, respectively, while the aeration rate was maintained at 2.0 vvm (volume flow per unit of liquid volume per minute).

2.7. Analytical methods

The samples were withdrawn periodically and analyzed for cell dry weight, pH, residual glucose, and IA. Biomass from 2.0 ml of the fermented broth was harvested and dried to determine the mycelium dry weight (CDW). The concentrations of glucose and IA were measured by HPLC (Agilent Technologies 1200 series, USA). The

supernatants, obtained by centrifugation of the culture samples at 10,000 rpm for 10 min, were filtered through a 0.22 μm PVDF membrane (Sartorius, Germany). The glucose concentration was determined using Rezex ROA-Organic Acid H⁺ (Phenomenex, USA) column (300 mm $\times$ 7.8 mm) maintained at 60°C connected to a refractive index detector (RID). The mobile phase and flow rate were 5.0 mM H₂SO₄ and 0.4 ml/min, respectively. While the IA concentration was determined using ACE excel 3 C18-amide columns (150 $\times$ 2.1 mm) coupled with Diode – Array Detection (DAD) detector (210 nm). The mobile phase and flow rate were 40 mM (NH₄)₃PO₄ (pH=2.48) and 0.2 ml/min, respectively. All the shake flask, and the bioreactor experiments were conducted in triplicates and the values were averaged. The standard deviation was not more than 10 %, and the same is depicted as the error bars in time series plots.

3. Results and Discussion

3.1. Comparative evaluation of *A. terreus* BD and DSMZ 23081 strains

A. terreus is a well-known IA producer, specifically the highest titers have been reported using *A. terreus* NRRL1960 and DSMZ 23081 strains (Bafana and Pandey, 2018). In the current study, initially shake flask experiments were performed to determine the IA production efficiency of newly isolated *A. terreus* BD strain and compared with well-established *A. terreus* DSMZ 23081. Both the strains were cultured in synthetic medium using glucose as a sole carbon source and the time course profile are shown in Figure 1. Initially, the DSMZ strain was grown at 33°C while a temperature of 45°C was employed for cultivating BD strain. Though initial glucose level of 100 g/L was nearly exhausted in both the strains at 216 h, the glucose assimilation rate was faster in DSMZ strain. The higher rate of substrate consumption was also reflected in biomass growth with maximum cell dry weight (CDW) of 7.6 ± 0.53 g/L observed on 7th day (168 h). Further decrease in physiological pH (1.5) and depletion of glucose concentration reduced the biomass concentration. Similar behavior was observed in BD strain with maximum CDW of 4.2 ± 0.25 g/L. In case of DSMZ strain, the active IA production took place between 3-8 days (72-192 h) and thereafter, it was slowed down. The final concentration IA after 10 days (240 h) was 61.2 ± 3.67 g/L with conversion yield of 0.61 g/g. On the other hand, IA accumulation was slow in BD strain and highest amount was observed on 8th day (192 h) and

thereafter, it was almost constant. The maximum IA obtained with BD strain was 17.9 ± 1.04 g/L with yield of 0.18 g/g.

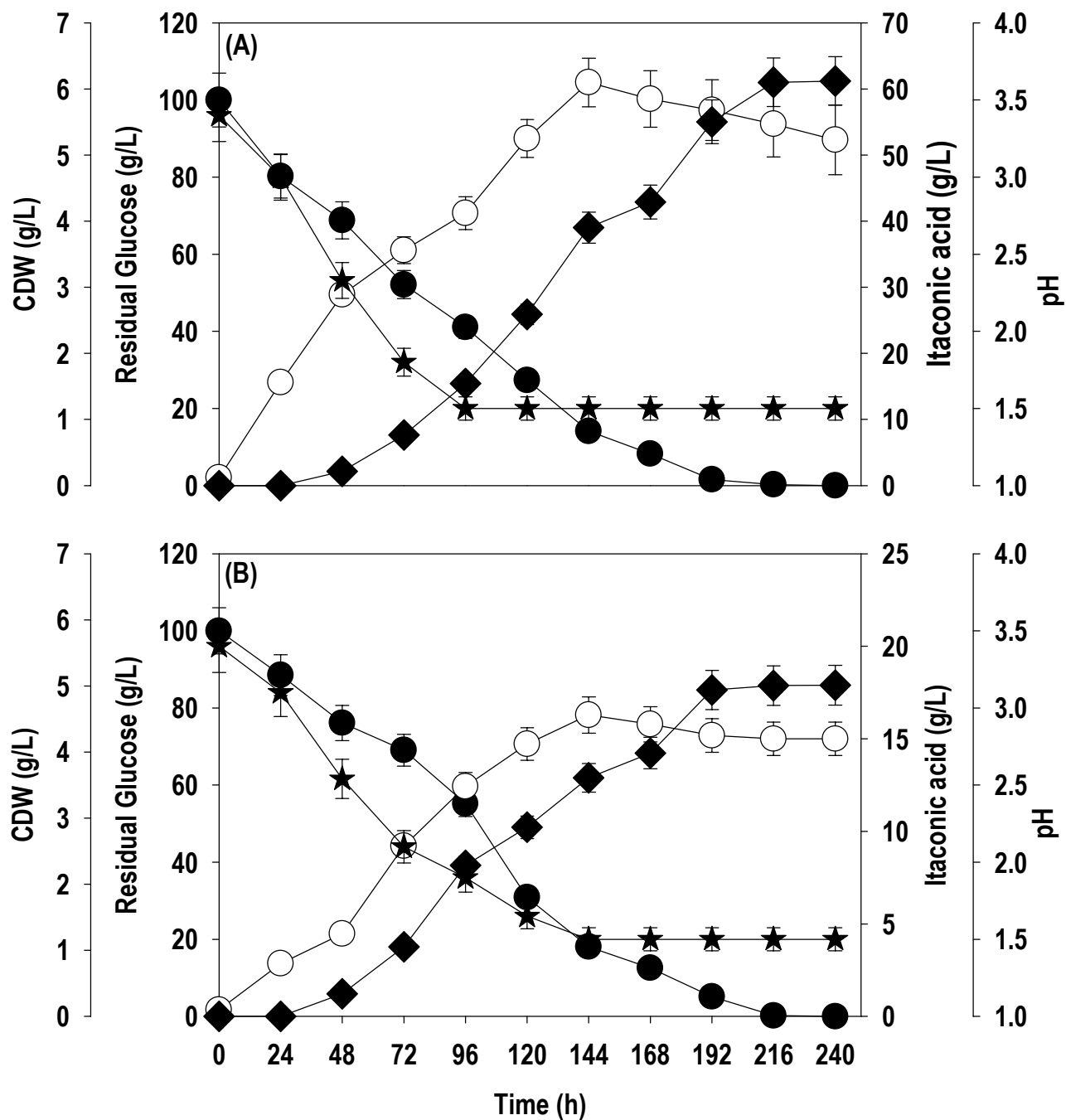


Figure 1: Time course profiles of glucose consumption, cell dry weight (CDW), IA production and pH; (A) *Aspergillus terreus* DSMZ23081, (B) *Aspergillus terreus* BD. Symbols: filled circle (glucose), empty circle (CDW), filled diamond (IA) and filled star (pH).

We also cultivated BD and DSMZ strains at 33°C and 45°C, respectively. The glucose remained almost unconsumed with no production of IA by DSMZ strain while little IA synthesis (< 5 g/L) was exhibited by BD strain (data not shown), indicating the both the strains are temperature sensitive. Thermotolerance of the BD strain is quite unusual and confers a significant advantage over other well-known *A. terreus* strains hyper-accumulating IA under moderate temperature range of 28-33°C. The high growth temperature significantly improves the rate of feed conversion by conferring some desirable properties to the growth medium such as reduced viscosity, reduced energy requirements for mixing, increased diffusion rates and improved substrate solubility. Combined, these effects would make the process more viable and would also reduce cooling costs during fermentation. IA has a solubility of ~80 g/L in water and another benefit of high temperature is enhanced solubility (Klement and Büchs, 2013). A further advantage of high growth temperatures is the reduced risk of contamination by other mesophilic microorganisms. Fermentation at the commercial level under conditions that aren't necessary to be entirely sterile would greatly reduce the process costs. The other important process intensification benefits are the strain can be channelized for simultaneous saccharification fermentation process as most of the starch and cellulose hydrolyzing enzymes exhibit maximal activity in temperature range of 45-55°C (Ghosh et al., 2015; Pietrzak and Kawa-Rygielska, 2015; Riaukaite et al., 2019). Thus, we envisage that *A. terreus* BD would be a powerful microbial cell factory when coupled with proper process and evolutionary engineering approaches.

3.2. Optimization of enzymatic hydrolysis of SFW using Taguchi method of orthogonal array

One of the key factors contributing to cost of bioproduction of IA is the high price of the substrate, and specifically the carbon source. The current price of IA is \$1.5-2.0/kg and when using glucose (\$0.30-0.60/kg) or sucrose (\$0.45–0.72/kg), the substrate price significantly contributes to the cost of production. The high current production cost of IA is commercially prohibitory and the potential of IA as a platform chemical can only be realized if this cost can be considerably reduced. Wider use of IA would be attractive if the selling price fell to below \$1.5/kg. In this case, complete replacement of petroleum-based polyacrylic acid would be possible, accessing an annual market worth over \$11 billion (Steiger et al., 2013; Jeon et al., 2016; Bafana and Pandey, 2018). Therefore, sourcing inexpensive

non-edible and waste biomass as a feedstock to replace high-cost carbon sources is a key strategy to curb the production cost. Since *A. terreus* has a high degree of sensitivity to inhibiting compounds in a culture medium, it is important that sugar extraction from feedstock generate little or no fermentation inhibitors, such as organic acids, furan derivatives and phenolic compounds. These compounds are commonly generated during processing of lignocellulosic feedstocks. Starch is considered one of the best alternatives to glucose as extraction of glucose from starch is quite a clean process without release of any fermentation inhibitors. Thus, the cultivation of *A. terreus* on starch hydrolysates from bread waste is likely to be significantly more successful than on lignocellulosic feedstocks (Kuenz and Krull, 2018; Yang et al., 2020).

To this end, the present study investigated and optimized sugar release from food waste. For this purpose, synthetic food waste (SFW) consisting of rice, potatoes and noodles was prepared. The process parameters such as biomass, enzyme loading, pH and temperature plays a decisive role in enzymatic saccharification of food waste. To get the overview of key parameters influencing the hydrolysis, we have adapted Taguchi method of orthogonal array. In this method the variation caused due to the individual factor was determined using Analysis of variance (ANOVA). Delta Signal by Noise (S/N) ratio was calculated to access the major factors by calculating the difference between the highest and lowest S/N ratio for each factor and further ranking is assigned. High delta S/N ratio indicates the maximum effect of individual medium components, biomass loading (11.61), and enzyme loading (4.33) has the highest values followed by pH (2.02) and temperature (0.65) (Table 4). The delta S/N ratio for each parameter maximizing the glucose release is shown (Figure 2). It was observed that biomass loading displayed the higher ranking followed by enzyme loading, pH, and temperature, indicating the amount of glucose release is dependent on the quantity of biomass and enzyme used. The ANOVA and regression coefficients for the response of glucose release from SFW are shown in supplementary material. It was evident from the P-value that all factors are statistically significant at 95 % confidence limit. The model displayed a coefficient of determination of 0.93 for glucose release, which suggest that more than 93% errors/ variation in the model can be explained. The general trends of the influencing factors on the response (process) can be characterized by studying the main effect of

individual factors. In order to achieve a preferred result, the characteristics of the system can be controlled by lowering or increasing the value of a particular influencing factor (Prabhu et al., 2016). Thus, the level of factors to produce the best results could be predicted. The regression equation for glucose release is shown below (Equation 5):

$$\text{Glucose} \left(\frac{g}{g} \right) = 0.23 - 0.008 \text{ Biomass loading} + 0.001 \text{ Temperature} + 0.042 \text{ pH} + 0.038 \text{ Enzyme loading} \quad (5)$$

Level	Biomass loading (w/v %)	Temperature (°C)	pH	Enzyme loading (v/v %)
1	-7.367	-11.84	-13.276	-13.902
2	-9.868	-11.873	-11.685	-12.747
3	-18.977	-12.498	-11.251	-9.562
Delta	11.61	0.657	2.025	4.339
Rank	1	4	3	2

Table 4: Response table for S/N ratio (in decibels) and relative ranking of variables for the synthetic food waste hydrolysis

The main effect plot of S/N ratio for glucose release by food waste hydrolysate is shown in supplementary material. It was clear that with increase in biomass concentration there was a significant drop in the glucose release indication that the amount of enzyme is not sufficient to hydrolyze higher biomass concentration. Further optimum temperature and high pH values supported significant sugar release. Hydrolysis of food waste is a crucial step as the starchy polymer should be completely degraded to liberate monomers, which can be readily utilized by microbes as carbon source. We achieved maximum of 0.60 ± 0.04 g/g glucose release with 10% w/v biomass loading and 5% v/v enzyme concentration at pH 5.5 and temperature of 60°C. Further the hydrolysate was concentrated using rotary vacuum evaporator (BUCHI, UK) maintained at 50 – 70°C to obtain the total concentration of 600 g/L glucose. The process of enzymatic starch hydrolysis usually requires participation of two enzymes, α -amylase for initial liquefaction and amyloglucosidase for saccharification. However, current study did not make use of α -amylase and saccharification of SFW after autoclaving was carried out using amyloglucosidase only. The saccharification yield

was reduced above biomass loading of 10% w/v (Table 2). In a study conducted by Bafana et al., 2019, combined action of α -amylase and amyloglucosidase with 20% biomass loading resulted in maximum glucose release of 230, 200 and 180 g/L from potato, corn, and rice starch, respectively. In our study, we found that after biomass loading, enzyme loading is next important parameter for efficient saccharification, which was in good agreement with Dwiarti et al., 2007 where increment in enzyme (amyloglucosidase) loading from 0.1 to 0.5 % w/v resulted in glucose yield of 0.4 g/g glucose from sago starch, and subsequent fermentation with a wild type strain *A. terreus* TN484-M1 strain accumulated IA with yield of 0.34 g/g.

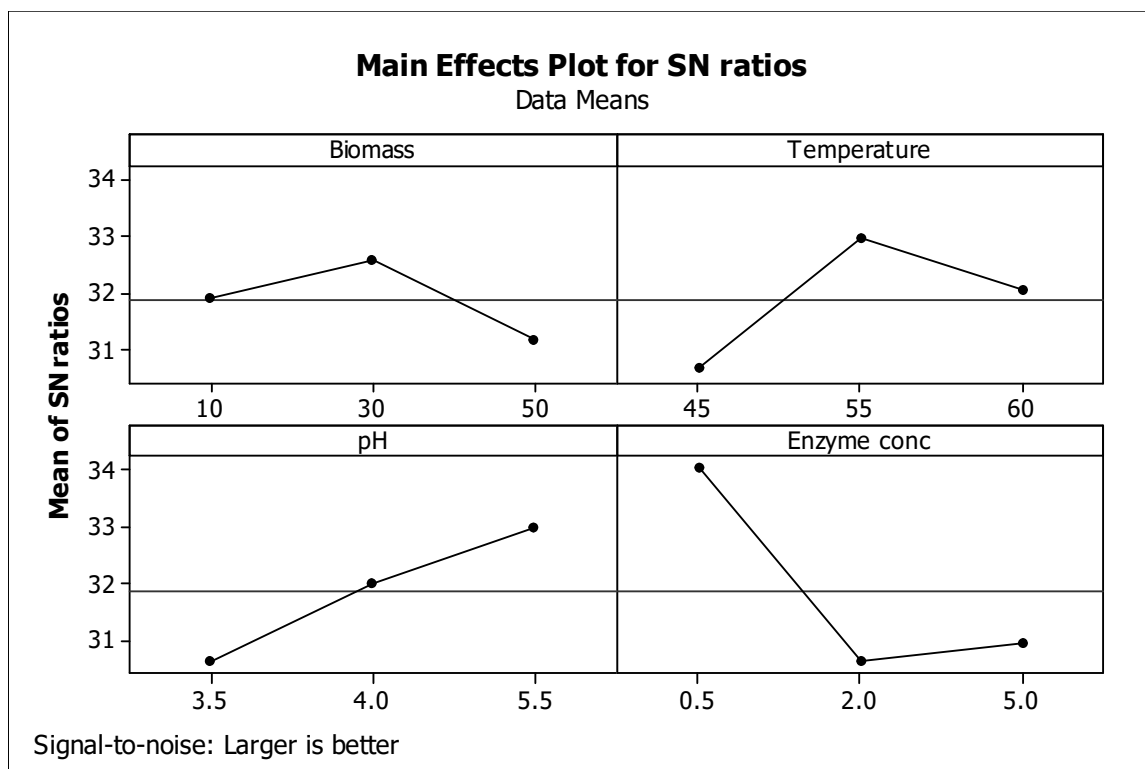


Figure 2: Variation of S/N ratio with different level of temperature, pH, biomass, and enzyme loading.

3.3. Optimization of medium components using Box-Behnken design

IA production is influenced by carbon and nitrogen (C/N) ratio, phosphates, trace elements, dissolved oxygen, carbon dioxide, pH, and temperature (Kuenz et al., 2012). Previously statistical based optimization such as central composite design were used to optimize the process and media parameters for increased IA production (Hajian and Yusoff, 2015). Statistical method offers more reliable results as compared with one factor at a time optimization experiments as the latter overlooks the interaction among the variables and fails to give the combined effect over

the process. Further, with statistical optimization the maximizing value can be achieved with minimum number of experiments (Narisetty et al., 2017). To understand the effect of medium components of the IA production, we have adopted Box-Behnken design (BBD). The design matrix of the parameters along with the observed and predicted responses for IA bioproduction is shown in supplementary material. Multiple regression analysis was carried out to study the model accuracy and based on the evaluation, the second-order polynomial model was fitted to equation. The IA production in the range of 5.59 to 29.3 g/L was observed with changes in the parameters.

$$Y_{IA} = 43.34 - 0.41X_1 - 54.3X_2 + 7.92X_3 - 3.74X_4 + 0.001X_1^2 + 10.27X_2^2 - 1.58X_3^2 - 0.10X_4^2 + 0.27X_1X_2 + 0.005X_1X_3 + 0.038X_1X_4 + 0.78X_2X_3 + 1.78X_2X_4 + 0.13X_3X_4 \quad (6)$$

The interaction between parameters were interpreted using ANOVA and the results are listed (see supplementary material). Fisher *F* test of the model showed high significance with the value of 145.07 ($P < 0.05$). The model coefficient of determination (R^2) was 0.99, explaining 99.4% of the model variation can be explained and there was a significant correlation between the experimental and predicted values. In general, the R^2 varies between 0 to 1 and the value close to 1 is considered as reliable as most of the data points are fitting the model. The model lack of fit was found to be insignificant ($p > 0.05$) with *P* value of 0.26, which implies that the represented data in experimental domains at points not mentioned in the model. Further the interaction among the variables and its effect on IA production was explained with the aid of 3D surface plots. The surface plots represent an optimum level of the independent variables, and also envisage the interaction between the response and the test variable. The plot was so built against any two independent variables that the response (IA) was plotted on z-axis while maintaining other variables at their mid-levels, as shown in the Figure 3 (A - F). The effect of glucose and KH_2PO_4 concentrations on IA production is shown in Figure 3A, 150 g/L glucose, and 1 g/L KH_2PO_4 showed >25 g/L IA, i.e., a higher concentration of glucose and KH_2PO_4 turned out to be more helpful in enhancing the IA production. It is evidenced from the graph that there is a direct correlation between IA production and glucose concentration.

A steep increment in IA production was witnessed with growing amount of glucose concentration inferring the importance of glucose assimilation in IA production. Similarly, in Figure 3B, 3D and 3E the increase in NH_4NO_3

concentration up to 3 g/L had positive impact on IA accumulation, whereas further increase >3 g/L resulted in decrease of IA production. From the Figure 3C, 3E and 3F, effect of trace elements was insignificant with other three independent variables, it was observed to be in positive correlation with glucose, but increase in KH_2PO_4 reduced IA accumulation, similarly around 3 g/L of NH_4NO_3 and 5.5 ml of trace element solution provided maximum IA concentration. Hence mid-level concentration i.e., 5.5 ml of trace element solution can be considered as optimum value. On the contrary, multiple regression analysis generated by adapting BBD revealed that the majority of the linear factors have a negative effect on IA production, but the overall interaction effects exhibited a positive impact, indicating that medium components such as glucose, NH_4NO_3 , KH_2PO_4 and trace elements had a significant positive influence on IA accumulation. We were able to achieve 29.3 ± 1.55 g/L of IA with the optimized media composition: 150 g/L, glucose; 3 g/L, NH_4NO_3 ; 1.0 g/L, KH_2PO_4 ; 1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; and 5.5 ml/L trace elements.

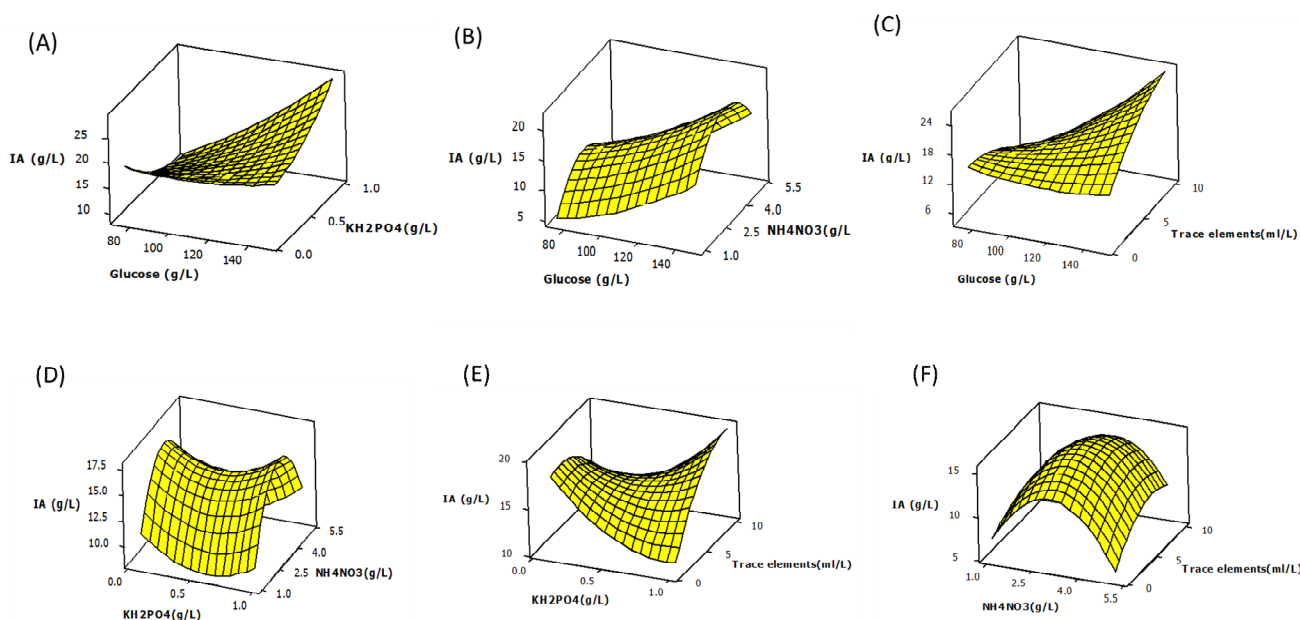


Figure 3: Three-dimensional response surface plot for IA production showing the interactive effects of (A) Glucose and KH_2PO_4 (B) Glucose and NH_4NO_3 (C) Glucose and Trace elements, (D) KH_2PO_4 and NH_4NO_3 , (E) KH_2PO_4 and Trace elements, (F) NH_4NO_3 and trace elements with the remaining factors kept constant at the middle level of the central composite experimental design.

Finally, the model validation was carried out by performing the experiment under the optimized conditions using BD strain in shake flask. Figure 4 shows the batch fermentation kinetics of glucose consumption, CDW, IA production and change in pH. The glucose consumption rate was fast in initial 120 h and was slowed down in later phase of fermentation. The biomass concentration increased gradually and maximum CDW (4.2 ± 0.075 g/L) was recorded on 6th day (144 h). The pH reduced during the course of fermentation and dropped to 2.8 after 24 h, possibly due to germination of spores. The IA production commenced at 48 h when initial pH was reduced to 2.1.

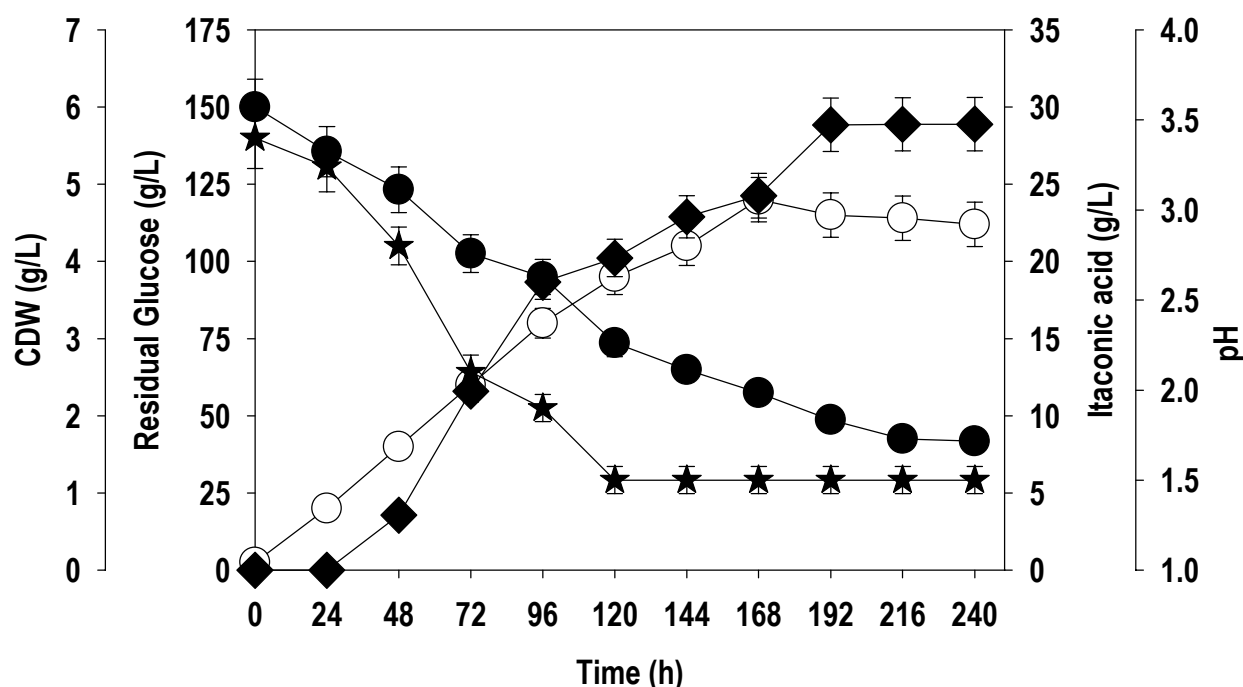


Figure 4: Shake flask cultivation of *Aspergillus terreus* BD with optimized media composition. Symbols: filled circle (glucose), empty circle (CDW), filled diamond (IA) and filled star (pH).

The pH further declined to 1.5 on 5th day (120 h) and thereafter, remained constant throughout the fermentation. The maximum IA titer of 28.9 ± 1.74 g/L with conversion yield of 0.26 g/g was observed on 8th day (192 h) which is 61% higher in comparison to unoptimized media composition. The IA production was active in initial 96 h (18.7 g/L) and comparatively slow in next 96 h. As a result of it, 41.8 ± 2.5 g/L of residual glucose was present at the end of fermentation. We suspect that this may have caused by drop in pH of the suspended media to 1.5 as glucose was completely consumed when pH was controlled in case of bioreactor cultivation (section 3.4). Our

results are in good agreement with Li et al., 2012, where they optimized five different medium for genetically modified *A. terreus* strain and found that high concentration of glucose and phosphate source influenced IA production positively. Similarly, Hevekerl et al., 2014b, optimized the media composition, and described the effect of individual components on IA titer and yield. They found that enhanced phosphate (0.1 to 0.8 g/L), and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (5 to 15 mg/L) levels caused improvement in biomass and IA production. With the optimized conditions *A. terreus* DSMZ 23081 strain accumulated 86.5 g/L IA, with 0.54 g/g and 0.62 g/L/h, yield and productivity, respectively.

3.4. Batch bioreactor cultivation of *A. terreus* BD strain

After shake flask cultivation, BD strain was cultured in bioreactor (2.5 L scale bench bioreactor with 1L working volume) using optimized medium under controlled conditions. The aeration, agitation and temperature were maintained at 2.0 vvm, 600 rpm and 45°C, respectively. The initial pH was set at 3.4 and allowed to drop till 2.3, thereafter, it was maintained at 2.3 by using 5M NaOH solution. The batch fermentations with pure glucose and glucose rich SFW hydrolysate respectively, were run in triplicates with the optimized medium composition. The time course profiles for both the fermentations are shown in Figure 5A and 5B. The glucose consumption rate was similar with pure glucose and SFW hydrolysate. About two-third of supplied glucose was assimilated within 4 days (96 h) and remaining one-third was exhausted in next 4 days (96 h). Due to higher aeration rate, and pH maintenance, higher mycelial growth was observed on 8th day (192 h) with maximum CDW of 11.6 ± 0.69 and 17.4 ± 1.04 g/L with pure glucose and SFW hydrolysate respectively, which is 2.3 times higher in comparison to shake flask experiments. IA production was faster and higher in comparison to flask culture and started within 24 h and increased steadily till the exhaustion of carbon source. The complete consumption of glucose was observed on 9th day (216 h) of fermentation concomitant with maximum IA accumulation. The maximum IA titer achieved with pure glucose and SFW hydrolysate was 44.7 ± 2.69 and 41.1 ± 2.46 g/L with a conversion yield of 0.30 and 0.27 g/g. Unlike flask culture, complete utilization of glucose and faster IA accumulation in bioreactor can be attributed to controlled pH

and better aeration as it is well documented in literature that pH and oxygen strongly influences growth and IA production by *A. terreus*.

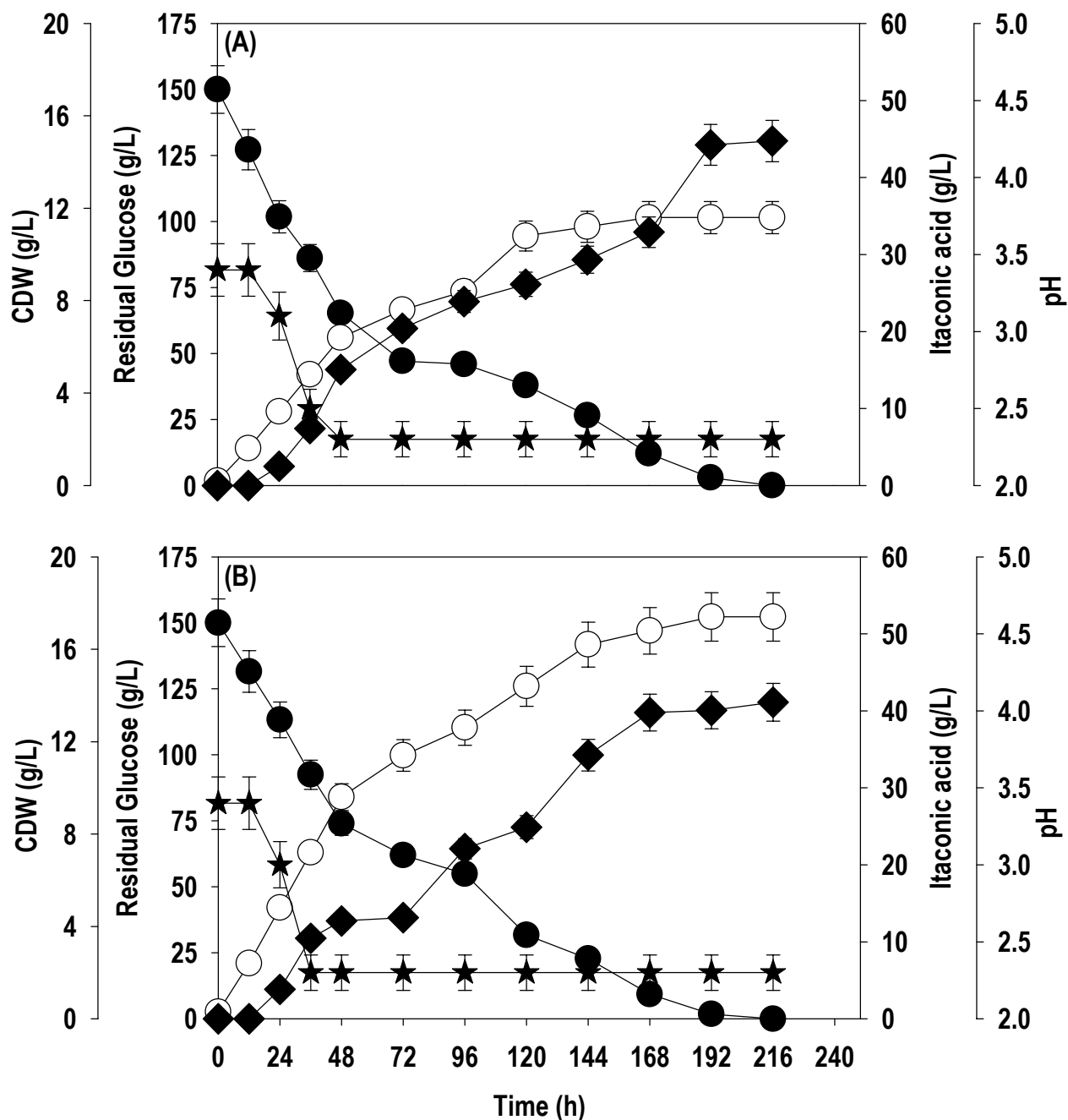


Figure 5: Bioreactor culture of *Aspergillus terreus* BD on (A) pure glucose, (B) synthetic food waste hydrolysate.

Symbols: filled circle (glucose), empty circle (CDW), filled diamond (IA) and filled star (pH).

The metabolic pathway for IA production is a non-fermentative one and require sufficient oxygen levels to maintain redox homeostasis (Bafana and Pandey, 2018). There is a very interesting report by Hevekerl et al., 2014a on impact of pH on IA production and observed that similar results were obtained with initial pH in range of 2.9-4.9. The IA fermentation consisted of two phases; spore germination followed by IA accumulation. The spore germination causes drop in pH (~2.1) due to uptake of ammonium ions and if pH is not maintained thereafter, it affects IA production as it exposes the cells to stress caused by pH shift. Low initial pH (<2.4) delays the germination of spores resulting in slow growth and IA synthesis and at pH>2.4, IA production started soon after 24 h of germination phase. The high IA titers can be achieved if pH control is started from the production phase but not from the beginning of fermentation which has a negative influence on IA accumulation.

The usage of glucose severely impacts the economical production of IA at industrial scale. Hence, it is imperative to replace with cost-effective renewable feed stock for economical production of IA. Food waste contains considerable amount of starch and protein making it a promising feedstock for the production of value added compounds (Lin et al., 2014). Further, the enzymatic hydrolysis of food waste does not produce any potential growth inhibitors. However, report on using food waste as feedstock for the production of IA are scarce in literature. Various renewable feedstocks like corn starch , sago starch, and agro-residual hydrolysates, have been utilized as the feedstock for IA production (Dwiarti et al., 2007; Maassen et al., 2014; Petruccioli et al., 1999; Reddy and Singh, 2002). In a study by Yahiro et al. (1997), *A. terreus* TN-484 strain was able to accumulate 60 g/L IA with a yield of 0.42 g/g using nitric acid hydrolyzed corn starch as sole carbon and nitrogen source during shake flask cultivation. The results were replicated when fermentation was performed in a 2.5L air-lift bioreactor with 0.5 vvm aeration rate (titer: 58 g/L IA; yield: 0.41 g/g). Low shear stress, no generation of heat due to agitation, and promotion of globular fungal growth are the advantages of air-lift bioreactors. Dwiarti et al. (2007) evaluated *A. terreus* TN484-M1 strain for IA production from sago starch as feedstock containing 73.7% starch, 4.0% dietary fibers, 2.4% protein, 16.1% water, and 0.3% crude lipid. The IA yield using nitric acid and glucoamylase hydrolyzed sago starch were 0.35 g/g and 0.36 g/g sago starch, respectively. Due to its low cost, nitric acid was preferred over expensive enzyme. The

fermentation in bioreactor using nitric acid hydrolyzed sago starch and optimized medium composition using RSM design produced 48.2 g/L IA with yield of 0.34 g/g. Downstream processing of fermented broth resulted in recovery of 37.1 g IA with purity of 97.2%. In a recent report, Bafana et al., 2019 isolated a strain *A. terreus* C1 from mangrove soils, and obtained 29.7 g/L IA with 0.18 g/g yield using enzyme hydrolyzed potato starch and statistically optimized medium composition. There have been reports where random mutagenesis and rational engineering have been employed for IA production on starchy feedstocks. In 2002, Reddy and Singh, isolated a strain *A. terreus* SKR10 from horticulture waste which accumulated 28.5 and 31.0 g/L IA from acid and enzyme (α -amylase) hydrolyzed corn starch. To further improve the production, the strain was subjected to ultraviolet (UV) and chemical N-methyl-N'-nitro-N-nitrosoguanidine resulting in two potent strains, N45 and UNCS1, respectively. The N45 strain was able to accumulate 46 and 50 g/L IA with acid and enzyme hydrolyzed corn starch while in case of UNCS1, IA titers were 43.6 and 48 g/L, respectively. Thus, an improvement of more than 50% enhancement in IA titer was achieved after mutagenesis (Reddy, and Singh, 2002). Wei et al., 2013, developed an unequivocal method for screening of chemically mutates strains, where the strains with thick hyphae and light-colored spores accompanied high titers of IA. The mutant strain Ast165 isolated after LiCl treatment produced 53.8 g/L IA with 0.53 g/g yield, using hydrolyzed corn starch. To integrate the saccharification and fermentation, Huang et al., 2014 overexpressed the glucoamylase gene in *A. terreus* from *A. niger* under the control of native citrate synthase promoter, the resultant strain could accumulate 77.6 g/L IA using liquified starch, whereas highest IA titers of 80 g/L was observed when saccharified starch hydrolysate was supplemented for wild type *A. terreus* strain.

In last two decades, a very few reports have been published on IA synthesis from starchy feedstocks and most of the studies made use of edible starch. The range of IA titer and yield obtained in these studies broadly varies from 15 - 80 g/L and 0.20 to 0.65 g/g. The results obtained in this study with thermophilic *A. terreus* BD strain signifies the efficiency and potential for IA production using pure glucose and sugars derived from hydrolyzed food waste in comparison with other *Aspergillus* strains and making it competitive. In the current work, IA has been accumulated on starchy food waste and can make significant contribution in recycling of such waste streams.

Although the current IA production would not be practical for commercial use, these results still suggest great potential for this thermotolerant BD strain in the production of IA from waste starchy substrates. As the strain is newly isolated, further rational strain engineering, adaptive evolution and process development approaches will be employed to improve the IA titers, yield and productivity, so that the strain attains industrial significance.

4. Conclusions

The IA accumulation at higher temperature is an advantageous feature and can lead to several benefits at large scale production. The IA production (>40 g/L) by BD strain was integrated with fermentable sugars from food waste, a global problem and the performance was similar in comparison with pure glucose as feedstock. More work is required to commercialize the bioprocess and product formation can be improved further through proper strain engineering and bioprocess optimization. Overall, the work resulted in demonstration of a promising cell factory for IA production from food waste.

E- supplementary data for this work can be found in e-version of the paper online.

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Microbial itaconic acid production from starchy food waste by newly isolated thermotolerant *Aspergillus terreus* strain

Narisetty, Vivek; Prabhu, Ashish A.; Al-Jaradah, Khalid; Gopaliya, Deeksha; Hossain, Abeer H. ...

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