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OPTIMIZATION OF ITACONIC ACID (IA) PRODUCTION USING ORGANIC ACID PRODUCING FUNGI ISOLATED FROM SOIL

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Abstract

Microbial production of organic acids has been incessantly escalating over conventional chemical methods due to several advantages including enantio-selectivity, high purity, less environmental pollution and cost effectiveness. Itaconic acid is used as a platform chemical for the production of various value-added chemicals such as poly-itaconic acid, resins biofuel components, ionomer cements. This research work aimed to isolate *Aspergillus terreus* producing itaconic acid from soil and optimize the maximum yield conditions. *Aspergillus terreus* was isolated, screened and identified morphologically and molecularly and for itaconic acid production. The itaconic acid produced was confirmed by bromocresol purple and measured by UV spectrophotometer in which the optical density was taken at 385nm. Itaconic acid was optimized initially by one factor at a time (OFAT) and then response surface methodology (RSM) using Central Composite Design (CCD). Incubation time, substrate concentration, pH and temperature were optimized and obtained the maximum yield of 22.601mg/ml itaconic acid at incubation time of 96hrs, substrate concentration 10% of molasses, pH of 4.0 and temperature of 34.5ºC with a significant quadratic model of P- value less than 0.05. The response surface plots (3D and contour) presented that the interactions between the parameters were significant to itaconic acid production. Based on this research *Aspergillus terreus* has a good potential influence for microbial production of itaconic acid using molasses as substrate.

Key words: Fermentation, itaconic acid, *Aspergillus terreus*, optimization, molasses

INTRODUCTION

Increasing environmental concern and depletion of limited resources have led to the development of microbial approaches for the production of commodity chemicals for sustainable development (Aliyu *et al*. 2022). The advances in microbiology and fermentation technology have led to the development of eco-friendly processes replacing some of the conventional chemical methods. Microbial production of organic acids has been incessantly escalating over conventional chemical methods due to several advantages including enantioselectivity, high purity, less environmental pollution and cost effectiveness (Aliyu *et al*. 2022; Mari *et al.* 2022). The increasing knowledge on metabolism and pathway regulation of industrially relevant organisms has already proven to be invaluable for generating rational strain development with primarily industrial performances. In recent times, microbial production of building block chemicals is progressively expanding its market for the production of succinic, lactic, citric, itaconic, gluconic, lacto bionic acids, etc. Organic acids are utilized directly or indirectly in a wide range of applications including food, healthcare, cosmetics, textile, solvents, and construction industries as well as for the manufacturing of biodegradable packaging materials. Organic acids with wide applications in various fields are made from living cells commercially (Rajendra *et al.* 2017).

Itaconic acid is an important component in the chemical industry. Itaconic acid (IA) and its derivatives have broad application spectrum in textile, chemicals, and pharmaceutical industries. It is used as building block for acrylic plastics, acrylic latexes, anti-scaling agents and super absorbents (Steiger *et al.* 2013). It also has other applications in food packaging, detergents, paints and coatings, pharmaceuticals, agriculture, emulsifiers, herbicides and printing chemicals. Many microorganisms, such as *Ustilago zeae*, *U. maydis*, *Candida sp*., and *Rhodotorula sp*. have been reported to produce itaconic acid (Kawamura *et al.* 1981). However, *Aspergillus terreus* is a preferred source in commercial production of itaconic acid up to 80 g/L (Steiger *et al.* 2013). The annual market of about 80,000 tons is currently met by fungal fermentation relying on natural *Aspergillus spp*. producers (Becker *et al.* 2015).

Itaconic acid is used as a platform chemical for the production of various value-added chemicals such as poly-itaconic acid, resins biofuel components, ionomer cements etc. Itaconic acid and its derivatives have wide applications in the textile, chemical and pharmaceutical industries. The depletion of fossil fuels and the need for sustainable development require that fermentative itaconic acid production replace petroleum-based methods of itaconic acid production. Various microorganisms have been employed in itaconic acid fermentations, with the most prolific producer being *Aspergillus terreus*. Over 80 g/L itaconic acid has been produced in fermentations using glucose. However, there is an increasing interest in the utilization of lignocellulosic materials for itaconic acid production due to the concern of food security. The current industrial applications of itaconic acid and its potential use as a drop-in or novel substitute monomer to replace petroleum-based chemicals were also extensively explored. Recent trends in itaconic acid research summarized that itaconic acid can be produced cost effectively from sustainable raw materials and have the potential to replace petro-based chemicals in various applications (El-Imam *et al.* 2014).

In contrast with citric, gluconic, and lactic acids, itaconic acid is used exclusively in non-food applications. Its primary application is in the polymer industry where it is employed as a co-monomer at a level of 1–5% for certain products. Itaconic acid is also important as an ingredient for the manufacture of synthetic fibers, coatings, adhesives, thickeners, and binders. The market volume has been estimated to be about 15,000 metric tons per year and is expected to grow if the selling price (estimated to be about US$4 per kg) can be reduced (Willke and Vorlop 2001). To date very little research has been directed at the improvement of itaconic acid production. In contrast, there has been a larger research effort directed at lactic acid production to feed the market for biodegradable plastic.

Itaconic acid was historically produced by various chemical methods which includes Destructive distillation of citric acid, and this was the main method of producing it prior to the 1960s; Oxidation of isoprene etc. None of these (or other) processes compete favourably with the fermentative production process (Willke and Vorlop 2001), and itaconic acid (IA) is now almost entirely produced by fermentation of sugars by *Aspergillus terreus* (Tsao 1990). The Northern Regional Research Laboratory (NRRL) of the United States Department of Agriculture (USDA) screened several wild type strains and identified *Aspergillus terreus* NRRL 1960, as the most prolific itaconic acid (IA) producing strain (Lockwood and Reeves 1945) which then went on to become the most published strain.

The initial industrial production of itaconic acid used a chemical approach that is the pyrolysis of citric acid to itaconic anhydride, followed by the hydrolysis of the anhydride which leads to the production of nonrenewable chemicals. The chemical synthesis of itaconic acid (IA) is not very effective. Thus, it is estimated that the market volume for itaconic acid (IA) is about 80,000 tons per year in 2005 and the selling price is $2.00 per kg (Okabe *et al.*, 2009), and there is an expectation for a market demand increase if the selling price can be reduced. Global carbon emissions as a result of petroleum-based processing and products are forcing industries to look for alternative processing and production methods.

Production of itaconic acid by fermentation needs significant improvements which will increase the productivity and reduction costs of the production. Bio-based itaconic acid production promises to be an attractive alternative for the chemicals industry to replace petroleum-based chemicals synthesis. Many chemicals such as succinic acid, 1, 3-propanediol and ethanol which were hitherto made from petroleum refining are now being successfully produced from renewable biomass. The high request for bio-based materials anticipates a further growth of 60% of the itaconic acid (IA) world market, which is predicted to surpass 216 million USD in the year 2020. According to the annual forecast, market is predicted to exceed 410,000 tons of itaconic acid by the year 2020 (Choi *et al*. 2015). Itaconic Acid (IA) is an important platform chemical which has a wide range of actual and potential applications. It can be used to replace a wide range of petroleum based chemicals, e.g. acrylic acid, which will reduce dependence on petroleum and the attendant deleterious environmental effects.

MATERIALS AND METHOD

Sampling of the Soil

The soil sample was collected at Bayero University Kano, old campus (Latitude of 11.9742ºN and Longitude of 8.4684ºE) Gwale LGA, Kano Nigeria and collected from a dark loamy soil and transferred safely to the laboratory. Exactly 1 g of the soil sample was weighted and diluted with 9ml distilled water using test tubes.

Medium Preparation

The primary medium used for isolation of fungi was potato dextrose agar (3.9 g of agar was dissolved in 100 ml of distilled water). The medium was autoclaved at 121℃ and 15 lb pressure for 15 min. The medium was poured on Petri plates in sterilized laminar flow to avoid contamination for the growth of fungi.

Isolation of *Aspergillus terreus* from the Soil Sample

From the collected soil sample (Soil dilution method: Hawaz *et al.*, 2023) diluted with 1 g soil is in 10ml of sterile distilled water. Exactly 9 ml of distilled water was taken to 5 different test tubes and 1 ml of the soil liquid suspension was serially transferred to each 9 ml distilled water containing test tubes. 1 ml of suspension from each 5 different test tubes after dilution was added to sterile Petri plates in duplicates containing sterile Potato Dextrose Agar (PDA) medium. The medium was autoclaved at 121℃ and 15 lb pressure for 15 min. The plates were incubated at 28ºC for 5- 7 days. The growth of separate colonies was observed. A greater number of species were isolated most of the fungus speculates heavily. Pure culture was done using Petri plates in duplicates for each colony containing fresh agar of PDA medium.

Morphological Identification of *Aspergillus terreus*

Staining Reaction

The pure fungus of *Aspergillus terreus* grown as metallic brown colonies or different colours. From the bottom, the colonies confer on the medium a dark brown color. The isolated fungi were identified to the genus level and to the species when possible on the basis of macro morphology and micro morphology (Shalini *et al*. 2014).

The macro morphological is by observing the colony features (color, shape, size and hyphae). The colonies were examined for slow or for rapid growth, topography (flat, heaped, regularly or irregularly folded), texture (yeast like, powdery, granular, velvety or cottony), surface (pigmentation and reverse pigmentation).

The micro morphological is by a compound microscope with a digital camera using a lacto phenol cotton blue-stained slide mounted with a small portion of the mycelium (Gaddeyya *et al*. 2012). The Hyphae, macro conidia, micro conidia, chlamydospores and other special fungal structure, characteristics using suitable media, slide cultures and the most updated keys for identifications. The identified fungi confirmed with microbial expert. The fungal propagules were coloured. The coloured mycelia /spores/conidia and cytoplasm were stained by using Lacto phenol and cotton blue. Cotton blue were stained cytoplasm and results in light blue background. Lacto phenol acts as a cleaning agent. The stained specimen (*Aspergillus terreus*) was observed under the light microscope (Magnus MLXi plus) for identification and microphotograph was taken under 10X × 40X magnification.

Pretreatment of Molasses

Molasses are thick brown sweet liquid that is made from raw sugar. Cane molasses was pretreated with acid (H_2SO_4). 2.0 N of the acid was used for molasses pretreatment. 15ml of the cane molasses was diluted upto 100ml with distilled water. Then 5ml of the acid was added and placed in a water bath at 90±2ºC for 1 hour. After cooling at room temperature, the medium was neutralized with lime (CaO) and left to stand overnight. Two layers were formed, the upper shiny black and lower yellowish brown due to the presence of trace metals. The clear supernatants were diluted to desired sugar level (Shazia *et al*., 2015).

Determination of Optimum Growth Conditions for Itaconic Acid (IA) Production

Four parameters (Incubation time, Substrate concentration, pH and Temperature) were considered for optimum growth of the fungal isolates for the production of itaconic acid (IA). One factor at a time (OFAT) method of optimization was performed first before using response surface methodology (RSM) by using Design Expert version 6.6.0 optimization software (Aliyu *et al*. 2022).

One Factor at a Time (OFAT) for Itaconic Acid (IA) Yield

For optimum incubation time determination, six Erlenmeyer flasks having 100 ml of Czapec Dox broth were prepared in duplicate, and their incubation time were adjusted at 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs and 144 hrs which were then autoclaved. The Erlenmeyer flasks were properly inoculated with freshly prepared spores suspension (inoculum size) of fungal isolates and incubated under proper conditions at 35ºC, pH of 5.0 and substrate concentration (molasses) of 3% in an incubator shaker at constant rpm (200) were used to investigate the influence of itaconic acid (IA) production, their absorbance were taken at 385nm using a UV Spectrophotometer.

For determining the optimum percentage of substrate concentration (molasses), six Erlenmeyer flasks having 100 ml of Czapec Dox broth were prepared in duplicate, and their percentage substrate concentration (molasses) were adjusted to 2%, 4%, 6%, 8%, 10% and 12% which were then autoclaved. The Erlenmeyer flasks were properly inoculated with freshly prepared spores suspension (inoculum size) of fungal isolates and incubated under proper conditions at temperature of 35ºC, pH of 5.0 and incubation time of 120 hrs in an incubator shaker at constant rpm (200) were used to investigate the influence of itaconic acid (IA) production, their absorbance were taken at 385 nm using a UV Spectrophotometer.

For optimum pH determination, six Erlenmeyer flasks having 100 ml of Czapec Dox broth were prepared in duplicate, and their pH were adjusted to 3.0, 3.5, 4.0, 5.0, 6.0 and 7.0 which were then autoclaved. The Erlenmeyer flasks were inoculated with freshly prepared spores suspension (inoculum size) of fungal isolates and incubated under proper conditions at temperature of 35ºC, incubation time of 120hrs and substrate concentration (molasses) of 3% in an incubator shaker at constant rpm (200) were used to investigate the influence of itaconic acid (IA) production, their absorbance were taken at 385nm using a UV Spectrophotometer.

For optimum temperature determination, six Erlenmeyer flasks having 100ml of Czapec Dox broth were prepared in duplicate, and their temperature were adjusted to 30ºC, 32ºC, 35ºC, 37ºC, 40ºC and 42ºC which were then autoclaved. The Erlenmeyer flasks were properly inoculated with freshly prepared spores suspension (inoculum size) of fungal isolates and incubated under proper conditions at incubation time of 120hrs, substrate concentration (molasses) of 3% and pH of 5.0 in an incubator shaker at constant rpm (200) were used to investigate the influence of itaconic acid (IA) production, their absorbance were taken at 385nm using a UV Spectrophotometer (Meena *et al*., 2010).

Response Surface Methodology (RSM) for Itaconic Acid (IA) Yield

In general, the change in culture conditions greatly influenced the production ability of itaconic acid (IA) synthesis. The statistical base optimization was used to study the influential effect of incubation time, substrate concentration (molasses), pH and temperature on itaconic acid (IA) yield using Central Composite Design (CCD). The optimized conditions of parameters were taken as independent variables and the itaconic acid (IA) yield was chosen as the dependent variables (Table 1), this resulted to thirty experimental runs from the software Design expert, version 6.0.6. The modeling and data analysis were performed sing Design expert software, version 6.0.6 (Aliyu *et al*. 2022).

Table 1: Factors for RSM Experimental Design.

Indicator	Factor	Low level	High level
A	Incubation time	72	120
B	Substrate conc.	8	12
C	pH	3	5
D	Temperature	32	37

Validation of the Second Order Polynomial Model

The second order polynomial model obtained from RSM was validated by conducting a series of experiments selected at random from the design in Table 5. The experiments were done by choosing random values of parameters within the optimized levels as presented in Table 5 below. The experimental output was then compared to the values predicted by the second order model obtained from CCD, to estimate the fitness and goodness of the model.

Data Analysis

The average data and standard deviations were obtained from the duplicate of experiments for each run using Microsoft Excel (Office, 2019). The standard deviation for each value was 5% analysis of variance (ANOVA) was done sing Design-Expert software 6.0.6. A confidence level of 95% was used in this study. Any p-value less than 0.05 was considered significant and vice versa.

RESULTS AND DISCUSSION

Morphological Identification of *Aspergillus terreus*

The results obtained from the morphological (macroscopic and microscopic) analysis of isolated organisms (AT2, AT4, AT6 and AT8) have shown variation in colony colours, reverse colours, margin covering and growth level. The macroscopic and microscopic characteristics of isolates show that AT2, AT4, AT6 and AT8 are *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium crysogenum* and *Aspergillus terreus* respectively. The macroscopic and microscopic characteristics of AT8 (*Aspergillus terreus)* is presented in Table 2 and Table 3

Table 2: Screening of isolates for the production of itaconic acid (IA).

Isolates Code	AT2	AT4	AT6	AT8
Indicator (BCP)	+	−	−	+

KEY: AT2 = *Aspergillus niger*, AT4 = *Aspergillus fumigatus*, AT6 = *Penicillium crysogenum*, AT8 = *Aspergillus terreus*, BCP = Bromocresol purple.

Table 3: Morphological Characteristics of *Aspergillus terreus*

Characteristics	*Aspergillus terreus*
Hyphae	Septate and hyaline
Conidiospore	Smooth and hyaline
Conidia heads	Compact
Vesicle	Biseriate spherical
Shape	Glubose

Table 4: Microscopic Characteristics of *Aspergillus terreus*

Characteristics	*Aspergillus terreus*
Surface colour	Light yellow to metallic dark brown colour.
Growth	Slow.
Margin covering	Half to one-third
Reverse of the colony	Appears metallic brown

Molecular Identification of Isolated Fungi

The results for molecular identification of the fungal isolate are presented in Figure 4.1 and 4.2. Figure 4.1 presented gel electrophoresis of the 18S ribosomal RNA (18SrRNA) gene result for AT8 showing 600 base pairs on the DNA molecular weight ladder, the sequence for the 18S rRNA of the isolate confirmed the identity of the isolate (AT8) as *Aspergillus terreus* with 99% identity with *Aspergillus terreus* MW881456 with accession number of OP8866158 after blasting in NCBI.

Production of Itaconic Acid (IA) using One Factor at a Time (OFAT)

Figure 1, 2, 3, and 4 presented the optimum production of itaconic acid results at different parameters (incubation time, substrate concentration, pH, and temperature) using one factor at a time (OFAT) technique.

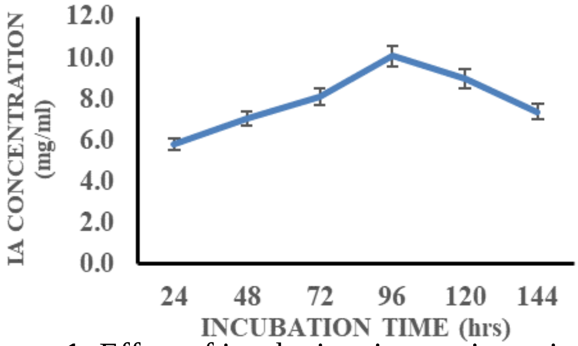

Figure 1: Effect of incubation time on itaconic acid production at constant substrate concentration (3%), pH (5) and temperature (35ºC) using one factor at a time (OFAT).

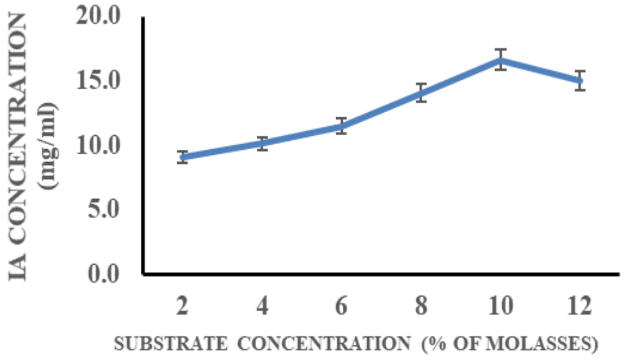

Figure 2: Effect of substrate concentration on itaconic acid production at constant incubation time (96hrs), pH (5) and temperature (35ºC) using one factor at a time (OFAT).

Figure 5: Response surface plots (3D and Contour) presenting the interaction between Incubation time and pH affecting itaconic acid (IA) production

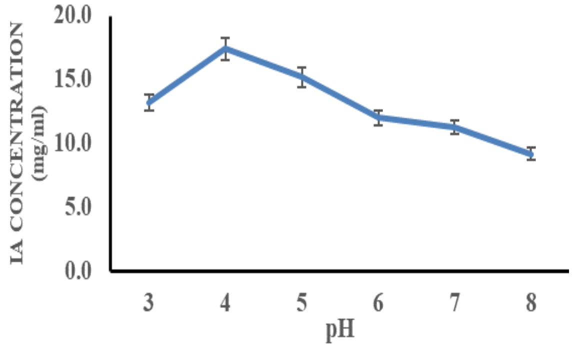

Figure 3: Effect of pH on itaconic acid production at constant incubation time (96hrs), substrate concentration (10%) and temperature (35ºC) using

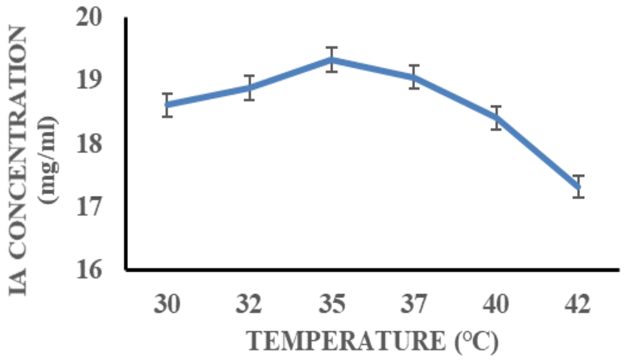

Figure 4: Effect of temperature on itaconic acid (IA) production at constant incubation time (96hrs), substrate concentration (10%) and pH (4.0) using one factor at a time (OFAT).

Incubation Time Optimization

Figure 1 presented the result of gradual increase in itaconic acid (IA) yield with increasing incubation time from 24hrs to 96hrs and later declined at 120hrs and 144hrs. The optimum itaconic acid (IA) yield (10.09mg/ml) was obtained at 96hrs

Substrate Concentration Optimization

Substrate concentration percentage of molasses has effect in itaconic acid (IA) yield in which it was increased with increase in substrate concentration from 2% to 10%. The optimum itaconic acid (IA) yield (16.65mg/ml) was obtained at 10%. In effect, it later declined at 12% of the substrate concentration. Figure 2 presented the result of gradual increase of itaconic acid (IA) yield by effect of substrate concentration percentage.

pH Optimization

Increase in itaconic acid (IA) yield was observed when pH increased from 3.0 to 4.0 and massively

Table 5: Actual and predicted itaconic acid yield at different condition of incubation time, substrate concentration, pH and temperature.

declined from pH 5.0 to pH 8.0. The optimum yield of itaconic acid (IA) was achieved at pH of 4.0 (17.415mg/ml). The result of itaconic acid (IA) yield with effect in pH is presented in Figure 3.

Temperature Optimization

Increase in itaconic acid (IA) yield was observed with increase in temperature from 30°C to 35°C, the optimum itaconic acid (IA) yield (19.05mg/ml) was obtained at the temperature of 35°C and then later declined from 37°C. Figure 4 presented the effect of temperature on itaconic acid (IA) yield.

Optimization using Response Surface Methodology (RSM)

Results obtained for actual itaconic acid (IA) concentration at different conditions of incubation time, substrate concentration, pH and temperature from response surface methodology (RSM) was presented in Table 5, the results of the responses obtained for each experimental run and the predicted responses were closer to each other.

Model F-value of 19.96 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, A^2, B^2, C^2, D^2, AC, BC, BD are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 0.90 implies the Lack of Fit is not significant relative to the pure error. There is a 58.92% chance that a "Lack of Fit F-value" this large could occur due to noise. Regression analysis produced the following second order polynomial fit with a satisfactory coefficient of determination (R^2 = 0.9490).

Itaconic acid concentratin= $+21.91+1.53A+0.36B +0.37C +0.21D -2.30A^2 -1.90B^2 -3.08C^2 -0.65D^2 -0.56AB+0.74AC-0.57AD+0.82BC-1.22BD+0.46CD$ ………..Equation ii

Where A, B, C and D are incubation time, substrate concentration, pH and temperature respectively. AB, AC, AD, BC, BD, CD are the interactions, and A^2, B^2, C^2, D^2 are the quadratic terms.

Table 6: Analysis of Itaconic Acid Yield from Quadratic Model Analysis of Variance.

Run	Incubation time (hrs)	Substrate concentration (ml)	pH	Temperature (degree)	Experimental value (mg/ml)	Predicted value (mg/ml)
1	96.00	6.00	4.00	34.50	14.25	11.18
2	96.00	14.00	4.00	34.50	14.54	15.03
3	120.00	8.00	5.00	37.00	16.25	13.81
4	72.00	12.00	3.00	32.00	13.02	15.41
5	96.00	10.00	6.00	34.50	11.12	7.88
6	96.00	10.00	4.00	34.50	21.35	21.91
7	72.00	12.00	5.00	32.00	14.32	13.80
8	72.00	12.00	3.00	37.00	13.55	18.36
9	120.00	12.00	3.00	32.00	16.75	14.25
10	72.00	8.00	5.00	37.00	13.15	15.81
11	120.00	12.00	5.00	32.00	17.00	12.01
12	120.00	8.00	3.00	37.00	16.99	11.32
13	96.00	10.00	4.00	29.50	19.67	12.79
14	96.00	10.00	4.00	34.50	19.85	17.32
15	72.00	8.00	3.00	32.00	11.75	13.84
16	120.00	12.00	5.00	37.00	17.25	16.12
17	96.00	10.00	4.00	34.50	21.85	19.63
18	96.00	10.00	4.00	34.50	22.55	21.76
19	72.00	12.00	5.00	37.00	13.25	13.60
20	72.00	8.00	3.00	37.00	13.75	15.02
21	96.00	10.00	2.00	34.50	8.23	8.84
22	96.00	10.00	4.00	39.50	19.12	16.34
23	120.00	8.00	5.00	32.00	14.85	18.90
24	96.00	10.00	4.00	34.50	22.45	19.73
25	120.00	8.00	3.00	32.00	13.75	16.91
26	144.00	10.00	4.00	34.50	16.12	19.91
27	96.00	10.00	4.00	34.50	21.27	21.91
28	120.00	12.00	3.00	37.00	10.33	13.91
29	48.00	10.00	4.00	34.50	9.44	12.91
30	72.00	8.00	5.00	32.00	7.00	12.91

Table 6: Analysis of Itaconic Acid Yield from Quadratic Model Analysis of Variance.

Source	Sum of Square	Mean Square	F-Value	P > F	
Model	521.85	37.28	19.96	< 0.0001	Significant
A	56.24	56.24	30.11	< 0.0001	
B	3.05	3.05	1.63	0.2205	
C	3.35	3.35	1.79	0.2008	
D	1.03	1.03	0.55	0.4685	
A2	145.60	145.60	77.95	< 0.0001	
B2	99.04	99.04	53.02	< 0.0001	
C2	260.23	260.23	139.32	< 0.0001	
D2	11.60	11.60	6.21	0.0249	
AB	5.06	5.06	2.71	0.1205	
AC	8.82	8.82	4.72	0.0462	
AD	5.22	5.22	2.80	0.1153	
BC	10.82	10.82	5.79	0.0294	
BD	23.77	23.77	12.72	0.0028	
CD	3.40	3.40	1.82	0.1970	
Lack of Fit	17.98	1.80	0.90	0.5892	Not Significant
R-Squared	0.9490	Adj R-Squared	0.9015	Pred R-Squared	0.7854

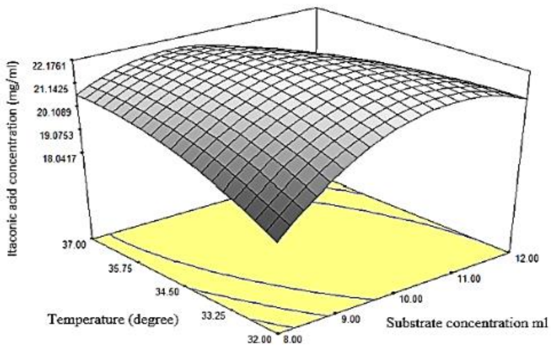

Figure 5: Response surface plots (3D and Contour) presenting the interaction between Incubation time and pH affecting itaconic acid (IA) production.

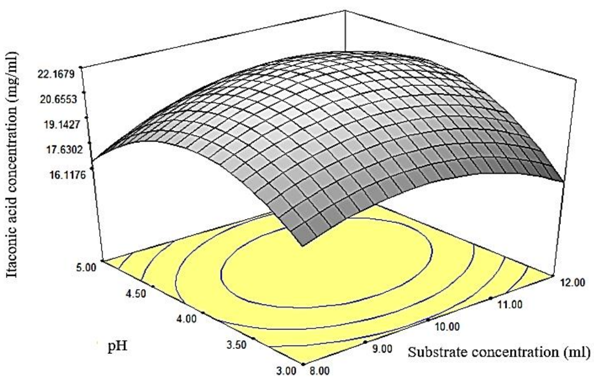

Figure 6: Response surface plots (3D and Contour) presenting the interaction between Substrate concentration and pH affecting itaconic acid (IA) production.

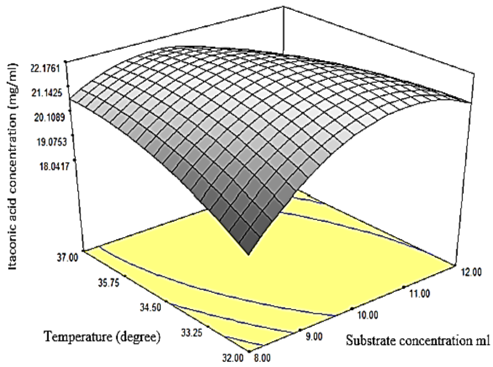

Figure 7: Response surface plots (3D and Contour) presenting the interaction between Substrate concentration and Temperature affecting itaconic acid (IA) production.

3D-Response Surface Plots Representing the Interaction between the Variables

Interaction among the various factors and the determination of optimum condition for maximum itaconic acid (IA) production were studied by plotting three-dimensional (3D) and contour response surface plot, as presented in Figure 5, 6 and 7.

The results obtained shown the parabola shape of the 3D plot and the circular shape of contour plots indicated the interaction between incubation time and pH was significant, keeping substrate concentration and temperature constant. Figure 5 presented the results of response surface (3D and contour) obtained for the interaction between the incubation time and pH.

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Validation of the Second Order Polynomial Model between the Experimental and Predicted Value of Itaconic Acid (IA)

The results obtained indicated that there was very good correlation between experimental and predicted values and in turn proved the validity of the models. The observed values of itaconic acid (IA) yield were compared with the values of predicted by the second order model. Table 7 presented the result of validation runs with observed and predicted values.

Table 7: Validation of the Second Order Polynomial Model between Experimental and Predicted Value of Itaconic Acid (IA) yield.

Run	Incubation time (hrs)	Substrate conc. (%)	pH	Temp (°C)	Experimental value (mg/ml)	Predicted value (mg/ml)
1	103.78	10.11	4.11	34.36	22.481	22.204
2	96.00	10.00	4.00	34.50	22.531	24.000
3	96.00	10.00	4.00	34.50	22.601	22.550

DISCUSSION

In this research study, four different fungal isolates were isolated from soil and identified morphologically and molecularly. AT8 (*Aspergillus terreus*) show the highest itaconic acid production capability among the isolates (Helia and Wan 2015) and (Meena *et al*. 2010). Molasses is used as source of glucose which is a very convenient raw material for itaconic acid production (Lockwood and Reeves 1945). DNA sequencing and molecular identification from Genbank identified isolate AT8 as new strain of *Aspergillus terreus* with closet similarity of 98% identity with *Aspergillus terreus* MW881456. *Aspergillus terreus* assigned the accession number of OP866152. Sequence alignment and evolutionary history was gathered for plotting phylogenetic tree.

Itaconic acid was produced under the influence of physicochemical parameters which were determined to have high yield by optimizing them with conventional technique, one factor at a time (OFAT) and response surface methodology (RSM) (Sadiq *et al*. 2017). The parameters were maintained significantly for better fermentation yield.

Effect of incubation time on itaconic acid (IA) was observed by varying time from 24hrs to 144hrs and keeping all other variables constant. The optimum yield of itaconic acid (IA) was determined at 96hrs of incubation and declined with increase incubation time as presented in Figure 4.3, this was in line with the study done by Linda (2021) by determining the optimum incubation time of the fungal isolates.The itaconic acid yield declined due to the relationship between the *Aspergillus terreus* and sugar contents in the medium and the fungal growth curve. Microbial production of metabolites usually starts after a lag phase of one day and reaches maximum at the onset of stationary phase or late.

Effect of substrate concentration on itaconic acid (IA) was observed and the optimum itaconic acid produced (16.65mg/ml) was obtained at 10% concentration of molasses. The *Aspergillus terreus* cell number increased exponentially which could provide the maximal conversion of substrate to itaconic acid was found at 10% this was related with the work of Meena *et al*. (2010). Low itaconic acid (IA) produced at molasses concentration above 10% and this may occur due to the formation of secondary byproducts that limit itaconic acid (IA) productivity in accordance to the research done by Hawaz *et al*. (2023).

Effect of medium pH was revealed to have the optimum itaconic acid (IA) yield of 17.415mg/ml at pH of 4.0, which was found to be similar with the work of EL Imam *et al*. (2013) and Sudarkodi *et al*. (2012) who reported a maximum yield of itaconic acid (IA) found to be at pH 4. The enzymatic reactions in the utilization of energy are regulated by pH. Basically, the impact of low pH is associated with the activity of enzymes taking part in the biosynthesis of itaconic acid (IA) and subsequent transfer mechanism to the extracellular space/out of the cell. This is related to the finding of Peter *et al*. (2019) reported that itaconic acid (IA) generation by filamentous fungi such as *Aspergillus terreus* favors lower pH conditions and it has been argued that besides enabling the appropriate growth of *Aspergillus terreus*, such a fermentation environment can be useful for suppressing the formation of by-products that would lower the final itaconic acid (IA) yield and productivity.

Effect of temperature on itaconic acid (IA) was also observed and the maximum itaconic acid (IA) production of 19.05mg/ml was obtained at 35ºC. After the optimum temperature the overall growth rate began to fall due to increase in rate of microbial death, as the death rate is also a function of temperature as reported by Meena *et al*. (2010). This high value of cell death increases with increase in temperature, than the growth rate. Hence the overall growth rates rapidly declined above the optimal temperature. Apart from this, the product inhibition effect is also more at higher temperatures than at lower temperatures.

The results obtained from the preliminary optimization using one factor at a time (OFAT) experiment were then applied to response surface methodology (RSM) modelling. 30 experimental runs using central composite design (CCD) were design as presented in Table 5, the experimental yield was recorded and relatively closed to the predicted value presented in Table 5. The statistical analysis for significances of all factors was described by analysis of variance (ANOVA) in Table 6. Based on the result obtained, the model of analysis was confirmed significant and highly reliable at P-value (< 0.0001) less than 0.05, and the no significant of lack of fitness indicated that the model was excellent fitted with no significant noise, the R^2 and adjusted R^2 value were all closed to 1 (0.9490 and 9015 respectively) showing the goodness of the model (Table 6). Additionally, the significant of the interaction between incubation time and pH (AC), substrate concentration and pH (BC) and that of substrate concentration and temperature were presented in ANOVA result (Table 6) with P-value 0.0462, 0.0294 and 0.0028 respectively. Furthermore, the response surface plots (3D and contour) reveal that the interaction between the factors (incubation time, substrate concentration, pH and temperature) were all significant to itaconic acid (IA) production as presented in Figure 5, 6, and 7. This shown that the itaconic acid (IA) production was dependent on the four parameters optimized.

Validation of the second order polynomial model confirmed the optimum itaconic acid (IA) yield of (22.601mg/ml) and indicated linear interaction and quadratic effect of variables on itaconic acid production. Therefore, the developed model is considered reliable.

The experimental and the predicted value were relatively closed indicating the relative fitness of experimental model. Moreover, this shows that extraneous factor terms in a derived model equation will affect in some reduction in the calculation of the error sum of squares (Mohamed *et al*. 2013).

CONCLUSION

According to the results obtained from this study, the isolated fungal species (*Aspergillus terreus*) has the potential ability of utilizing molasses as substrate for itaconic acid (IA) production. The study revealed that itaconic acid (IA) could be effectively yielded by adjusting conditional parameters, such as temperature, pH, incubation duration, and substrate concentration.

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