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Production of itaconic acid from *Chlorella vulgaris* biomass using *Aspergillus alabamensis* MN907795

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Abstract

The patronage of relatively costly materials in industrial production of products incurs certain degrees of economic implications when the products can be seamlessly produced using materials of insignificant value. The quest to arrest this situation is the drive behind the fermentation of aquatic lignocellulosic waste Chlorella vulgaris biomass using *Aspergillus alabamensis* MN907795. The biomass was analyzed for the possession of some nutrients and it demonstrated appreciable quantity of carbohydrate, protein, lipid, ash, moisture, and fiber which are suitable for itaconic acid (IA) fermentation. Prior to the "one-factor-at-a-time" (OFAT) studies, IA was produced using carbon (10%), nitrogen (5%), pH 3.0, inoculum size (10%) and phosphorus (0.5%) for 10 days, and the best results (17.09g/L) was recorded on day 6. The OFAT studies were carried out to observe the influence(s) of the parameters on both IA yield and cell growth and the best results of IA: 17.8g/L, 18.0g/L, 18.65g/L, 18.9g/L, and 18.55g/L were recorded from carbon 10%, inoculum size 12%, nitrogen 3%, pH 3.0, and phosphorus 0.5% respectively; while the best cell dry masses of 1.73g, 2.24g, 1.38g, 1.47g and 1.88g were observed from 12% carbon, 10% inoculum size, 7% nitrogen, pH 3.0 and1.0% phosphorus respectively. One way analysis of variance was carried out to evaluate their significance using F-test but only phosphorus and inoculum size were considered significant for IA and cell growth respectively with $P \leq 0.05$. This study has shown the feasibility of IA production using A. alabamensis MN907795 as production organism and C. vulgaris biomass as carbon source.

Keywords: *Aspergillus alabamensis*; Itaconic acid; Factors affecting itaconic acid production; Statistical analyses; C. vulgaris; Cell growth

1. Introduction

The environmental, health, and economic impact of humans' patronage of fossil and synthetic products have caused us a significant dent in the world today, therefore credible alternatives should be reasonably practiced to undo the menaces and subsequently conserve and sustain lives. Bio-products have overtime shown its credibility to out-rightly substitute the synthetic products or contribute to its modification. The bio-production of organic acids has been in practice for so many years; they are characterized by their low molecular weight (LMW) and contain few or just one carboxylic group (Sandeep *et al*., 2020) such as malic acid, fumaric acid, lactic acid, itaconic acid, succinic acid *etc*. the natural (bio-based) production of organic acid can best be achieved through fermentation, either submerged and or solid state fermentation using specific and appropriate microorganism(s). Fungi have over the years expressed their unique suitability in the production of litany of lipids comprising free or esterified forms of fatty acids such as phospholipids, esters; and other lipids such as sterols and hydrocarbons (Sudarkodi *et al*., 2012).

Itaconic acid, also called 2-methylenebutanedioc acid, 2-propene-1,2-dicarboxylic acid, 2-methylenesuccinic acid or propylenedicarboxylic acid is a whitish crystalline unsaturated five (C5) carbon dicarboxylic acid with a chemical formular $C_5H_6O_4$, melting point range of 167-168 ℃, molecular weight 130.1, density of 1.633g/L at 20 ℃; and at

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moderate temperature remains stable at neutral, mid-basic and acidic conditions (An *et al*., 2012; Pathak and Rajwinder, 2015; Omojasola and Adesina, 2017). IA is among the 12 vital organic acids with high industrial potential (Omojasola and Adeniran, 2014); and its unique nature is the methylene and two carboxylic groups where the former is responsible for its polymerization with carboxylic groups (Paranthaman *et al.,* 2014).

Considering the menaces caused by the use of chemical and pure synthetic products in (IA) production, which are not in support of the bio-refinery concept, the process of production of sustainable products should be highly sustainable both environmentally and economically. To obtain this goal, there is need to use lignocellulosic agro-waste materials which are by no means in competition with human nutrition and are diminutively valued economically (Jiménez-Quero *et al.,* 2016). The application of wastes in the biotechnological industry has been making a big wave and this has been greatly involved in waste management (Agwa *et al.,* 2013). Lignocellulosic materials are the most abundant biomass on earth, and are essential in bio-production of some vital renewable with no emission of CO. Starch, which is the major component of lignocelluloses, is the cheapest and most reserved carbohydrate with the principal sources being root-tubers, cereals (maize and millet), fruits (Daramola and Falade, 2006) and lower plants such as algae. These materials over time have proven to be better option in the biotechnological industries because of their little or no significant value.

1.1. IA Biosyntheis in Aspergillus sp.

Biosynthesis and production of IA Itaconic acid can be produced by several microorganisms preferably fungi such as *A. terreus, U. maydis*, *Candida* sp., and *Pseudozyma antarctica* but *A. terreus* and *U. maydis* have served as model systems for investigating itaconic acid biosynthetic pathways (Meilin *et al.,* 2018). The biosynthesis of IA observes two pathways, the pentose phosphate (glycolytic) pathway and the tricarboxylic acid pathway. Glucose or other carbon sources are taken up from the extracellular environment and converted to pyruvic acid via glycolysis. The pyruvic acid is metabolized to acetyl-CoA releasing carbon dioxide (CO_2) molecule. The acetyl-CoA is partially transformed to oxaloacetate with the inclusion of the liberated CO_2 molecule before the metabolisation of acetyl-CoA in the mitochondrion. From oxaloacetate and acetyl-CoA, citrate and cis-aconitate are produced in the TCA cycle. The cis-aconitate is transferred into the cytosol by the mitochondrial tricarboxylate transporter (*mttA* gene) where the cis-aconitate decarboxylase (encoded by the *cadA* gene) facilitates the production of IA by discharging CO_2 and at the same time, the IA is transferred outside the cell by the facilitator super-family protein (*mfsA* gene) (Li *et al.,* 2011; Meilin *et al.,* 2018; Teleky and Dan, 2019). The key enzyme for itaconic acid formation is CAD which is encoded by the *cadA* gene (Kanamasa *et al*., 2008).

1.2. Applications of IA

The rising challenges from the use of non-degradable materials have been a major drive in the production of bio-degradable polymers and elastomers from bio renewable resources. The applications of Itaconic acid and its polymers are numerous and cut across almost all human strata and various fields either as a drop-in or novel substitute monomers providing better characteristics on the end products which make it superior when equated to its conventional substitutes (El-Imam and Du, 201). Its applications have been evident in medicals, agriculture, textile, and production industries; and in some hygiene industries. The polymerization and cross-linking of one or more poly-functional monomers such as IA, its esters and acrylamide are very vital in the generation of hydrogels and other biomaterials. The addition of IA to a polymer favors the overall hydrophilicity, strengthens the polymer-water interaction and finally improving its solubility. In the preparation of hydrogels, micro-gels or nano-gels are majorly used in the control of rheology, encapsulation, and targeted delivery in various industrial applications such as food, drugs delivery, cosmetics, personal care and water decontamination.

IA hydrogels have been vital in deflocculating and sequestering metallic ions such as Fe^{3+}, Fe^{2+}, Pb^{2+}, As^{5+}, Cu^{2+}, Cd^{2+}, Cr^{3+}, Cr^{6+}, Al^{3+}, Zn^{2+}, and Ni^{2+} and by indication decontaminate the water bodies in question (Teleky and Dan,2019).

1.3. IA in food industry

The U.S. Food and Drug Administration (FDA) classify IA as an important additive used in food packaging and contact substances. When it is used as a component of active packaging, interacts with food and prevents the growth of microbial food spoilage and pathogens, thereby increasing shelf-life of the food. On a separate note, the Food Safety Commission of Japan (FSCJ) included IA in the list of food additives and its importance was confirmed by the study that IA is a major fructose-6-phosphate-2-kinase inhibitor which suppresses the activity of phosphofructokinase, a regulatory enzyme for glycolytic pathway metabolism (Juliana *et al.,* 2018). In the animal nutrition, IA has shown to suppress weight gain, lowered plasma triglycerides level and blood glucose following ingestion. Also, the evidence of the glucose suppression in streptozotocin-induced diabetic rat has shown its potential as anti-obesity, antidiabetic, and anti-lipemic effects because of its function in suppressing the glycolytic pathway and the activity of phosphofructokinase.

1.4. Medical applications

Itaconic acid is majorly used in the preparation of nano-hydrogel improving its solubility, absorption rate and pH sensitivity. These make nano-hydrogels to have a better drug delivery which invariably betters the therapeutic effects of the drugs such as application of ampicillin, paracetermol, and anti-cancer/tumor drugs (doxorubicin) (Teleky and Dan, 2019). Also, Okabe *et al*. (2009) gave an elucidatory application of IA in preparation of Glass Ionomer Cement (GIC) with its inaugural introduction about 40 years ago playing a vital role as adjunct in restorative dentistry. The polycarboxylic carriers such as the polyacrylic acid and polyitaconic acid in a sub-colloidal nano-particulated hydrogel-form have high potential use in sustained drug release during ocular delivery (Okabe *et al*., 2009; Dowlathabad *et al*., 2018). Furthermore, IA can as well be applied as hardening agent in organosiloxanes especially majorly in contact lenses uses (Willke and Vorlop, 2001; El-Imam and Du, 2014; Dowlathabad *et al.,* 2018). Both the diesters and monomers of partially substituted IA have substantial analgesic or anti-inflammatory activities to suppress swell-ups and pains respectively (Willke and Vorlop, 2001; Dowlathabad *et al.,* 2018).

1.5. Industrial application of IA

IA polymerization and its incorporation in the production of biomaterials have been very vital in the industries across the globe such as in the improvement of polymer adhesion in the production of emulsion paint, the use of polymerized esters of IA such as the vinyl, methyl or ethyl in the development and improvement of coatings, adhesives, plastics, and elastomers (Willke and Vorlop, 2001; Okabe *et al*., 2009; El-Imam and Du, 2014; Dowlathabad *et al.,* 2018). The co-polymers of itaconic acid are known to produce resins of rubber-like texture exhibiting splendid flexibility and strength with waterproofing coating giving it an appreciable electrical insulation, and the use of non-woven binders in fabric industries have been documented very successful and reliable with acrylic lattices when supplemented with itaconic (Willke and Vorlop, 2001). Also, the alkali salts of itaconic acid homopolymer are strongly recommended detergents and sequestrants production, while the reactions with amine yields N-substituted pyrolidones can be applied as thickeners in detergents, pharmaceuticals, grease; shampoos, and herbicides productions (Willke and Vorlop, 2001; Okabe *et al*., 2009). Finally, IA can also be used for the synthesis of a potential biofuel 3-methyltetrahydofuran (Badal *et al.,* 2018), and in the production of adsorbent fibers for manufacturing dippers and feminine hygienic products (Juliana *et al*., 2018).

1.6. Economic value of IA

Irrespective of the technological advantages, there still loom the high cost of production stemming from the process and constituents of production, and an appreciable reduction of its production cost will directly increase the economic possibilities and expansion (Juliana *et al*., 2018). The annual global production of IA was over 141, 000 tons worth $74.5 million in 2011 and projected to surpass 50, 000 tons annually to over $567 million by the year 2020 (El-Imam and Du, 2014). IA was first produced commercially in 1945 by the Pfzer Company and there have been subsequent companies involved in its production such as the Iwata Chemical (Japan in 1970), Rhodia (France 1995), Cargil (USA 1996) (Juliana et al., 2018). These companies in one way or the other encountered a stumbling block which then led to the disruption of its large scale production. This however created a vacuum which has till date made China to be the front spot in the bio-based production of itaconic acid (El-Imam and Du, 2014). In the early 1990's, the output of IA in China was very low and they relied on importations of IA in other to meet their domestic demands. After 1993, they started setting up some IA production units and seven years later, they had up to 10 enterprises with combined output of 20, 000 tons which was only second to USA (Okabe *et al*., 15 2009). Among the Chinese companies, the Qingdao Kehai Biochemistry Company produces 50% in China, while their global strength amasses 18% of IA production. They also reported that the major contributing countries with regards to IA are USA, China and India while the individual players include: Jinan Huaming Biochemistry Company Limited (China), Alpha Chemika (India), Zhejiang Guoguang Biochemistry Company Limited (China), Chenggdu Jindai Biology Engineering Company Limited (China), Shandong Kaison Biochemistry Company Limited (China); Qingdao Kehai Biochemistry Company Limited (China), and Itaconix Company (USA) (Juliana *et al*., 2018).

1.7. Fermentation of IA

The fermentation of IA can be by submerged or solid state fermentation (Willke and Vorlop, 2001). This biosynthetic (fermentative) method was first carried out by Kinoshita in 1932 using Aspergillus itaconicus isolated from the juice of salted plum (Willke and vorlop, 2001; Okabe *et al*., 2009; Juliana *et al*., 2018; Dowlathabad *et al.,* 2018). Succeeding Kinoshita's innovation in 1932, Calam in 1939 produced a higher concentrated IA using Aspergillus terreus as a surface culture, while in 1948 Charles Pfizer Company applied the first patency for an industrial production process by submerged cultivation of A. terreus and built the first production plant in Brooklyn, New York, USA in 1955 (Anja and Susan, 2018). Itaconic acid is majorly produced by fungi organisms such as *Aspergillus terreus* (Willke and Vorlop, 2001),

A. flavus, A. terreus, *A. niger* and *A. nidulans* (Meena *et al*., 2010), *Ustilago maydis* (Badal, 2017), *U. vetiveria* TZI (Thiemo *et al.,* 2017), Pseudozyma antaritica (Levinson *et al*., 2006) and *A. oryzae* (Jimenez, 2016).

Itaconic acid is best produced using refined sugar such as glucose or sucrose as carbon source (Willke and Vorlop, 2001) but their prices $0.35-0.6/kg and $0.45-0.72/kg drove the production cost high and directly affected the cost prices (El-Imam and Du, 2014). This has propelled the need to subsidize the price by going for cheaper raw material in the nature of biomass which can be readily sourced from the water bodies (algae and seaweeds), Domestic (municipal solid waste and wastewaters) and industrial organic residues (process residues and leftovers) (Cherubini, 2010) and some non-biomass e.g. glycerol and citric acid though they can pose difficulties such as the introduction of impurities into the fermentation medium (El-imam and Du, 2014). The aquatic biomass comprises all biomasses sourced from the water bodies ranging from the macro-algae (multicellular) to the microalgae (unicellular) (Bharathiraja *et al.*, 2015) and they have today received an increasing interest as top sources of high-valued products applied across all human strata (Alvarado-Morales *et al.,* 2015; Chew *et al*., 2017). This invariably has led to the relentless and ongoing research in their cultivation, harvesting and applications (EL-Moslamy *et al*., 2016) of the production of biofuels, foods, feeds, bio-fertilizers, pharmaceuticals, organic acids in a bio-refinery system (Das, 2015).

2. Material and methods

2.1. Preparation of *C. vulgaris* biomass

The carbon substrate used in this study was *C. vulgaris* and it was bloomed from the stuck culture in Microbiology laboratory, University of Port Harcourt via heterotrophic method using hot poultry dropping extract as the sole source of nutrient (Agwa and Abu, 2014). It was harvested using centrifugation at 10,000 rpm for 10 minutes and the residue stored. Corn steep liquor was sourced from market pap (akamu) seller at Choba market, Rivers State, while the fish bone was sorted from the campus' restaurant bins, washed, dried, grinded, and nutrients extracted using hot water extract method prior to sterilization at 120 ℃, 15psi for 15 minutes (Amitha *et al*., 2019).

2.2. Pretreatment of biomass

Following the harvesting of the biomass, the paste was treated with 20% v/v of 2M of H_2SO_4 and sterilized with autoclave at 120 ℃ for 38 minutes (Khan *et al*., 2017).

Nutrient estimation: Proximate analysis was carried out on *C. vulgaris* biomass for the presence of protein, carbohydrate, crude fibre, ash, moisture content, and lipid (Omojasola and Adesina, 2017), while corn steep liquor (CSL) and fish bone extract (FBE) were examined for their nitrogen and phosphorous content respectively.

2.3. Test organism

The test organism used for this study was *Aspergillus alabamensis* MN907795 sourced from stuck culture in Microbiology department laboratory, University of Port Harcourt and sub-cultured into czapek dox broth medium and incubated at room temperature for five days, and then maintained at 4 ℃ until when needed.

2.4. Screening of organism

Aspergillus alabamensis MN907795 was subjected to both quality and quantity ability to produce itaconic acid (Nkwocha and Agwa, 2021).

2.5. Production media

The medium for production was composed of 0.8 g $MgSO_4$, 0.01g $FeSO_4$, 0.5g KCl, 2g Na in 1liter of distilled water. 10ml of *C. vulgaris* biomass, 5 ml of CSL, and 0.5 ml of FBE were measured into 250 ml Erlenmayer flask and the volume was made up to 100 ml with the prepared medium. The setups were sterilized with autoclave at 120 ℃ for 15minutes at 15psi and allowed to cool to room temperature. It was inoculated with 10 ml of *A. Alabamensis* MN907795 from the growth medium, and were incubated in a rotary shaker at 150rpm at 37 ℃ (Wilke and Vorlop, 2001; El-Imam and Du, 2014). The fermented crude liquors were analyzed for the quantity of IA using bromination method (Friedkin, 1945; Omojasola and Adesina, 2017) every 24 hours for 10 days.

2.6. Estimation of microbial dry weight

The dry mass of the microorganism was determined using a direct method. From the setups, 5 ml of the fermented liquor from every flask was transferred to centrifuge tubes. The tubes were centrifuged at 10000rpm for 20minutes.

Centrifugation was repeated thrice under similar conditions to achieve complete separation of fungal mass from the substrate. At the end of centrifugation, the fungal mass with lower density than the substrate floated while the substrate settled to the bottom. The biomass alone was transferred to a pre-weighed filter paper and dried in hot air oven for 2hrs at 105 °C to obtain a constant weight (Asha *et al*., 2006) and the initial weight is subtracted from the eventual.

2.7. One-factor-at-a-time studies

Some of the fermentation parameters were varied to observe their respective impact(s) on IA yield and growth of the test organism (*A. Alabamensis*) and they include: carbon concentration (8.0, 10.0, 12.0, 14.0 % v/v), nitrogen concentration (3.0, 5.0, 7.0, 9.0 % v/v), phosporus concentration (0.5, 1.0, 1.5 % v/v), inoculum size (5.0, 8.0, 10.0, 12.0, 15.0 % v/v), pH (2.5, 3.0, 3.5, 4.0, 5.0) for a period of 10 days while temperature was kept constant at 37 °C. The setups were analyzed for IA yield and organism's growth.

2.8. Data analyses

The data from this study were statistically (ANOVA) analyzed using F-test where $P \leq 0.05$ is considered significant.

3. Results

3.1. Nutrient estimation of substrates

The proximate analyses of C. vulgaris biomass in this study elucidated that it comprises nutrients in certain degrees: ash 13.64, lipid 4.6, carbohydrate 43.66, moisture content, 9.53, crude protein 15.90, crude fiber 12.57 as seen in table 1 below; while SCL and FBE had 8.05% and 16.53% of nitrogen and phosphorous respectively.

3.2. IA fermentation

The fermentation of IA prior to OFAT studies yielded varying concentrations of IA in fermented liquor from day 1 to day 10 as seen in figure 2. There was no result on the first day while 2.89g/L, 7.72g/L, 11.75g/L, 15.02g/L, 17.09g/L, 16.91g/L, 16.02g/L, 12.73g/L, and 10.0g/L were recorded on subsequent day till the 10th day with the maximum yield recorded on day 6 as seen shown in table 2.

3.3. OFAT studies

The effects of some fermentation parameters were varied and yielded various concentration of itaconic acid as seen in table 3 and figures 3 to 7 below. The concentration of itaconic acid in the fermented liquors varied and yielded a maximum of 17.8g/L at 10% v/v of carbon when it was varied. This was followed by 17.10g/L and 16.70g/L from 12% v/v and 14% v/v respectively on the 5th day of fermentation while 16.30g/L of IA was recorded on day 6 from 8% v/v. The variation of inoculum size (*A. Alabamensis*) resulted to maximum production on day 18.0g/L at 12% v/v on the 4th day followed by 17.9g/L, 17.5g/L, 16.7g/L, and 16.5g/L from inoculum size of 10% v/v, 8% v/v, 5% v/v, and 15% v/v respectively, recorded on the 6th, 5th, 6th, and 4th day in the same order. IA's yield was at best on day six with 18.65g/L at 3% v/v when the nitrogen was varied while the concentration of 5% v/v, 7% v/v, and 9% v/v yielded their respective best on day 6, 5 and 6, and 5 with 18g/L, 17.68g/L, and 17.5g/L respectively. Furthermore, pH factor was analyzed and all best results were recorded on the 5th day with pH 3.0 producing the overall best of 18.9g/L, while pH 2.5, 3.5. 4.0, and 5.0 yielded their personal best with 18.6g/L, 17.0g/L, 15.0g/L, and 12.8g/L of IA respectively. The last factor to analyze was the effect of increment of phosphorous where 0.5, 1.0 and 1.5% v/v were used. All the best results were recorded on the 5th day of fermentation as the least concentration (0.5% v/v) yielded the maximum result with 18.55g/L followed by 1.0% v/v and 1.5% v/v with personal bests of 17.68g/L and 8.6g/L IA respectively.

The different concentrations of the fermentation parameters also had a varying degree of effect on the growth of the production organism *A. Alabamensis* as seen in table 4 and figures 8 to 12. The highest dry biomass (1.73g) of the organism was recorded from 12% v/v on the 5th day of fermentation when carbon concentration was varied. This was followed by 1.7g, 1.68g and 1.38g from 10% v/v, 8% v/v, and 14% v/v on the 5th, 6th, and 4th day respectively. The variation of the test organism's size yielded an overall best of its dry biomass as 2.34g from 15% v/v on day 5, while individual bests dry biomass of 2.24g, 2.1g, 1.12g, and 0.98g were recorded from 10% v/v, 12% v/v, 8% v/v, and 5% v/v respectively and on the 6th, 5th, 5th, 7th day of fermentation in that same order. The concentration of nitrogen elucidated its influences on the inoculum's dry biomass when varied at 3, 5, 7, and 9% v/v with best results of 1.6g, 1.38g, 1.01g, and 0.95g respectively, and all on the 5th day. On the other hand, the different initial acidity levels of the analyses setups demonstrated their effects on the organisms (dry) biomass. The overall best dry mass of 1.47g was recorded from pH 3.0 on day 5, followed by 1.4g, 1.23g, 1.16g, and 1.08g from pH 2.5, 3.5, 4.0, and 5.0 respectively; but on days: 5 and 6, 5, 6, and 6 in same order. Also, phosphorous concentration of 0.5, 1.0, and 1.5% v/v yielded varying

resulted with respect to the organism's dry mass. The best result of 1.88g was gotten from 1.0% v/v while 0.5 and 1.5% v/v produced personal best of 1.2 g and 1.06 g, but on days 5 and 6 respectively.

3.4. Statistical analyses

The statistical analyses (ANOVA) of impacts of the fermentation parameters on IA yield (tables 5 to 9) and cell growth (tables 10 to 14) were carried out using F-test. The P-values of 0.9, 0.9, 0.9, 0.4, and 0.05 from carbon, inoculum size, nitrogen, pH, and phosphorus respectively for IA yield, while for the inoculum's growth, P-values of 0.9, 0.05, 0.4, 0.2, and 0.7 were calculated from carbon, inoculum size, nitrogen, pH, and phosphorus respectively.

4. Discussion

The proximate analysis of *C. vulgaris* biomass elucidated the presence of ash, lipid, carbohydrate, moisture, protein and fiber in varying degrees. The estimate of 13.64% ash is comparatively higher than those recorded by Yasmin *et al*. (2011) (5.16-12.98%), Agwa *et al*. (2013) (2.65%), and Megan *et al*. (2015) (5.71%) from *C. vulgaris*. The presence of ash indicates that the biomass has high content of minerals and therefore is suitable as feedstock and production of industrial products such as organic acids. The estimation of 4.6% of lipid depicts that the substrates is a suitable source of some fat soluble vitamins which plays a vital role in the contribution of energy in the fermentation medium. This is in range with the documentation of Yasmin *et al*. (2011) who recorded lipid content of 1.0-5.6%, but the value from the present study fall below the estimation of Agwa *et al*. (2013) who documented 38.30% of lipid from *Chlorella* cells. The presence of carbohydrate in this study was calculated to be 43.66% and it is the most abundant of other nutrients and when compared with the studies of Agwa *et al*. (2013), Uchechukwu, (2013), and Megan *et al.,* (2015) who recorded 20.46%, 12.09%, and 24.93% is said to be high, but in alignment with the findings of Yasmin *et al*. (2011) who had a range of 25.5-48.19%. Carbohydrate is a crucial source of energy with high suitability in bio-production of organic acids, and this high content deems the biomass very suitable for the fermentation of itaconic acid and other organic acids. The moisture content of the biomass indicates the amount of water that could be lost during drying process and it is a key factor in the degradative susceptibility of the biomass (Adeolu and Adewoye, 2018). The moisture content of 9.53% when compared to 3.37% and 1.3% from Agwa *et al*. (2013) and Megan *et al.* (2015) is high, but in range with those of Yasmin *et al*. (2011) who recorded a range of 2.67-9.95% from *Chlorella* cells. Protein is the major source of nitrogen in the fermentation medium and the higher its contents, the more adequate amino acids. An estimate of 15.9% was recorded from the present study and it is relatively low when compared with 48.19% and 54.65% from the findings of Yasmin *et al*. (2011) and Megan *et al.* (2015). The calculated amount of fiber in the biomass is 12.57% as seen in table 1. This relatively lower than 16.37% from Yasmin *et al*. (2011) and higher than 10.79% from Agwa *et al*. (2013). Its contents in the fermentation medium are very vital because of its ability to increase the removal of potential mutagens and xenobiotics. Finally, 16.53% of phosphorus estimated from fish bone extract used as the phosphorus source is marginally lower than 17.2% from Logesh *et al.* (2012). While the 18.05% of protein was estimated from CSL which served as nitrogenous source which is relatively lower than 20% documented by Corn Refiners Association, (2006).

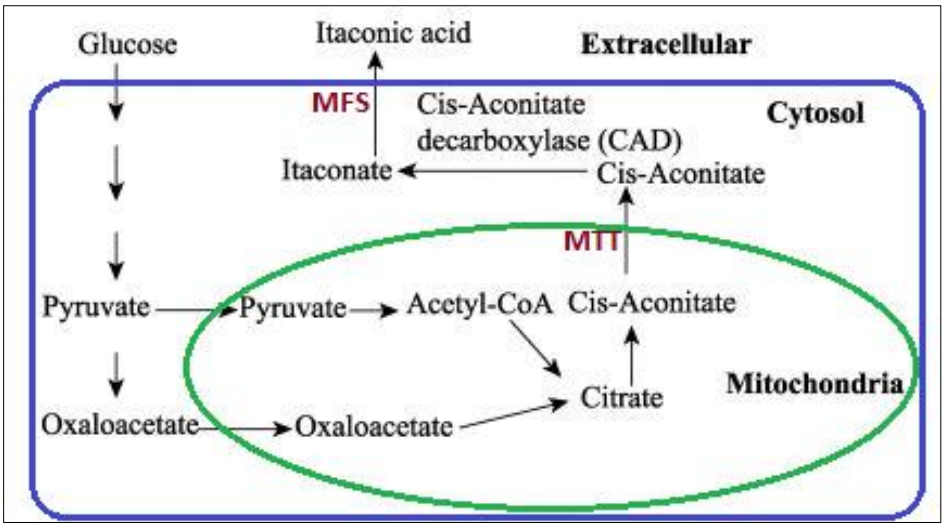
The pre parameters analysis of production of IA spanned for a period of 10 days and an increase in concentration was observed from day 1 till the 6th with maximum yielded of 17.09g/L and a gradual decrease till the 10th day of fermentation as seen in figure 2 and table 2 With respect to incubation time, this aligns with the trends of IA fermentation from the several researches such as Omojasola and Adeniran, (2014) who achieved a maximum concentration of 67.67g/L and 29.0g/L after the fifth day from *A. niger* and *A. terreus* respectively using sweet plantain peel as their production substrate but the value from this present study is comparably low. Also, there is a complete agreement with the findings of Paranthaman *et al*. (2014) but in their value of 8.6g/L which is relatively lower than the present findings. In their study, the maximum yield was recorded on the 6th day using banana peel as substrate and *A. niger* as the production organism. Omojasola and Adesina, (2017) found that *A. niger* and *A. terreus* during IA fermentation could yield high concentration: 65g/L and 72.5g/L of IA respectively on the 6th day using banana peel as their carbon substrate. Other researchers such as Meena et al. (2010) who used several species of Aspergillus had their best result of 18-27g/L on the 6th day of fermentation likewise Gregor et al. 2010 who documented a maximum production of 13.5g/L IA. The production of IA through fermentation is extremely medium dependent and these variations in value could be as a result of differences in fermentation medium, test organism, and other abiotic factors.

The varied fermentation parameters demonstrated their respective impacts on the yield of itaconic acid. Carbon is a credible source of energy in fermentation (Meena *et al.,* 2010) and when varied, its concentration of 10% produced the overall best result of 17.80g/L of itaconic acid on the 5th day. This trend is in accordance with the findings of Omojasola and Adeniran, (2014) who recorded a maximum of 96g/L and 104.67g/L from *A. niger* and *A. terreus* respectively using sweet potato peel as a source of carbon. In contrast, Omojasola and Adesina, (2017) used banana peel as a source of carbon but recorded the best results of 163.2g/L and 165.7g/L from *A. niger* and *A. terreus* respectively with a relatively

low concentration (4%) of the carbon source. The inoculum size is very essential in the fermentation medium and must be in the right concentration because a low concentration results to slow growth and low yield of biomass while when in excess leads to increased nutrient completion (Chandragiri and Sastry, 2011). The inoculum size of 10% demonstrated the most suitable concentration in this study with a maximum yield of 17.9g/L, and this is agrees to the studies of Dowlathabad *et al.* (2007), Juy *et al*. (2010) Omojasola and Adeniran, (2014), and Omojasola and Adesina, (2017) who recorded their respective best results of 24.46g/L from jatropha seed cake, 13 and 17.83g/L from glucose and glycerol, 77.33 and 88.33g/L from sweet potato peel, and 87.3 and 92g/L using banana peel. The presence of nitrogenous source in fermentation is very crucial for the supply of amino acids needed during metabolic processes. The least concentration of nitrogen (3%) produced the best concentration (18.65g/L) of IA in the fermentation liquor. Though, the concentration when compared to 0.35% and 0.3% from the works of Meena et al. (2010) and Aml *et al*. (2018) is high, but with a lower concentration of IA as the both recorded a higher yield of 25g/L and 35.77g/L respectively. However, the data is in total agreement with that of Anja *et al.* (2014) who produce a higher concentration of IA (36g/L) with 3% nitrogen source. The initial acidity of the fermentation medium is very vital. In as much as microorganisms have the potential to stabilize their internal pH levels, the external acidic level plays a part in the sustenance and physiological activities (Andro, 2015; Omojasola and Adesina, 2017). The assimilation of nutrients from the environment is therefore a function of the pH level, and from the study, 18.9g/L of IA was produced from initial pH 3.0. This is in accordance with the data from Lingyun *et al.* (2013), Antje *et al*. (2014), Omojasola and Adeniran, (2014), Jiménez-Quero *et al*. (2016), Omojasola and Adesina, (2017) who produced maximum IA yields of 35g/L, 146g/L, 96.67 and 106.67g/L, 32.2g/L, and 64 and 73.1g/L respectively. While the works of Paranthaman *et al.* (2014) and Badal, (2017) elucidated a contradicting respective bests of 7.5mg/Kg and 51.9g/L at pH 4 and 5 respectively. Phosphorous is important for microbial growth but hinders the metabolic processes of the organisms for IA production (Anja *et al*. 2014). Quite a few reports have been documented on the influence of phosphorus on IA production, but Anja *et al.* (2014) recorded a personal best of 36g/L of IA using 0.1g/L. This is slightly different with the current study of 18.55g/L with 0.5%. These differences could be as a result of many factors from the setup down to other environmental factors.

The performance of the inoculum influences the yield of itaconic acid and it is a function of the fermentation medium. The fermentation parameters were varied over 10 days and they elucidated their respective impacts on the growth of the *A. alabamensis* as seen in table 4 and figures 9 to 12. The variation of carbon concentration in the medium yielded 1.73g dry weight on day 5 of the fermentation and from 12% carbon concentration, while the best growth from inoculum size analysis was recorded from 15% concentration on the fifth day. When nitrogen was varied from 3% to 9%, the best cell growth was achieved from 7% on day 5. On the same note, both pH and phosphorus were varied and their respective maximum growths were recorded on the fifth day of fermentation where pH had the highest from 1.47g and the best (1.88g) for phosphorus examination was recorded from 1.88g. There are very few reports on the effects of fermentation parameters on IA yield, but Aml *et al*. (2018) recorded their best result of 8.88g on the 7^{th} day, while 0.6% concentration of urea (nitrogenous source) yielded the maximum growth of 7.42g.

One way analysis of variance was implemented to confirm the significance of the independent variables on the dependent variable such as IA concentration and inoculum growth using F-test at 95% confidence interval (CI) where P-value of or less than 0.05 ($P \leq 0.05$) is considered to be significant. From the statistical analysis, only phosphorous can be considered to have a significant effect on the production of itaconic acid with $P = 0.05$, while the rest had no significant effect. From the statistical data extrapolated from the effects of fermentation parameters on the growth of the inoculum, only inoculum size was considered to have a significant effect with P-value of 0.05 ($P = 0.05$) while the rest though influenced the cell growth but does not have a significant impact.



Key: MFS: Major facilitator super-family; MTT: Mitochondrial tricarboxylic transporter

Figure 1 Itaconic acid biosynthetic pathway in *Aspergillus sp*

Table 1 Proximate analyses of C. vulgaris biomasses used for fermentation (%)

Ash	Lipid	Carbohydrate	Moisture content	Crude protein	Crude fiber
13.64	4.6	43.66	9.53	15.90	12.57

Table 2 IA production

	Days									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
IA Conc. (g/L)	0.00	2.89	7.72	11.75	15.02	17.09	16.91	16.02	12.73	10.00

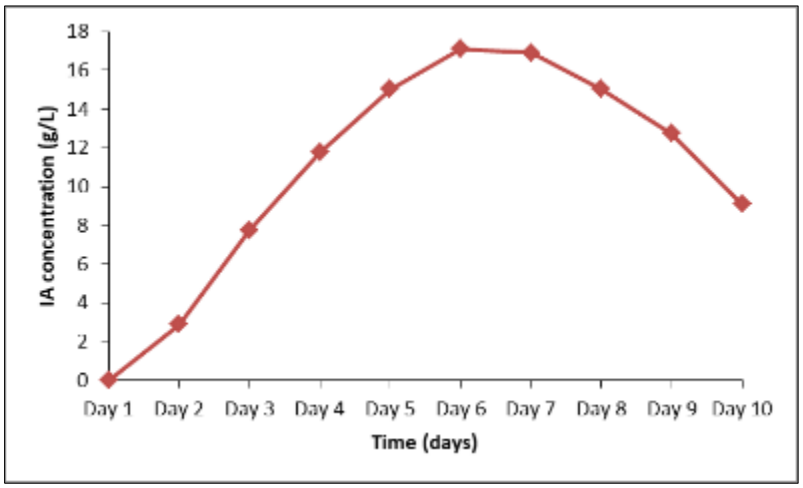


Figure 2 Itaconic acid production using *A. alabamensis*

Table 3 OFAT studies of IA production using *C. vulgaris* biomass as carbon source

Parameter	IA conc									
	Day 1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
N 3ml	0.00	4.41	6.82	10.8	13.81	18.65	17.32	15.56	11.82	8.41
N 5ml	0.00	3.05	8.11	11.61	14	18.00	17.01	14.62	10.01	9.82
N 7ml	0.04	3.42	7.5	12.01	17.68	17.68	15	12.93	9.55	7.22
N 9ml	0.00	4.00	10.65	15.39	17.5	16.57	12.33	10.61	7.98	6.11
pH 2.5	0.00	4.40	6.00	13.80	18.60	17.20	14.00	13.10	12.30	11.50
pH 3.0	0.00	4.80	10.00	15.00	18.90	16.20	15.60	15.10	13.00	11.10
pH 3.5	0.00	4.20	6.80	11.40	17.00	14.80	12.00	11.30	10.80	10.10
pH 4.0	0.00	3.90	8.10	13.30	15.10	14.20	13.80	12.60	12.00	10.20
pH 5.0	0.00	3.10	7.60	10.20	12.80	10.00	8.60	9.10	8.10	7.30
In. size 5ml	0.00	2.40	5.80	9.70	11.60	16.70	16.00	14.30	11.80	9.20
In. size 8ml	0.00	2.70	6.80	12.50	17.50	16.80	13.60	12.10	11.70	8.80
In. size 10ml	0.00	3.20	9.30	12.90	16.20	17.90	14.60	10.40	9.00	8.10
In. size 12ml	0.10	5.80	10.30	18.00	16.60	14.60	11.50	9.10	8.90	6.30
In. size 15ml	0.10	5.60	11.90	16.50	16.00	13.60	12.80	12.95	8.30	5.10
Ph 0.5ml	0.04	3.4	8.52	13.07	18.55	18.03	16.7	12.8	11.65	9.91
Ph 1.0ml	0.00	2.89	7.16	11.5	17.68	16.95	13.83	10.66	9.18	5.01
Ph 1.5ml	0.00	3	6.31	7.95	8.6	6.5	8	6.5	5.72	4.01
C. 8ml	0.00	4.00	7.61	12.33	14.03	16.30	12.80	10.02	8.55	4.91
C 10ml	0.00	4.01	6.25	11.18	17.80	15.50	12.80	9.60	9.00	4.20
C 12ml	0.00	4.28	7.99	12.04	17.10	16.50	14.15	11.60	10.00	8.60
C 14ml	0.04	4.00	9.60	15.50	16.70	15.80	12.10	9.08	9.35	8.50

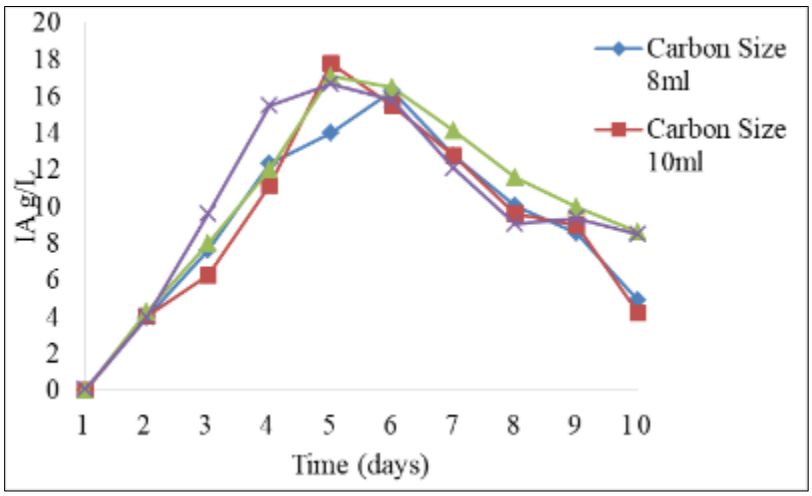


Figure 3 Influence of carbon concentration on IA production using *C. vulgaris* biomass

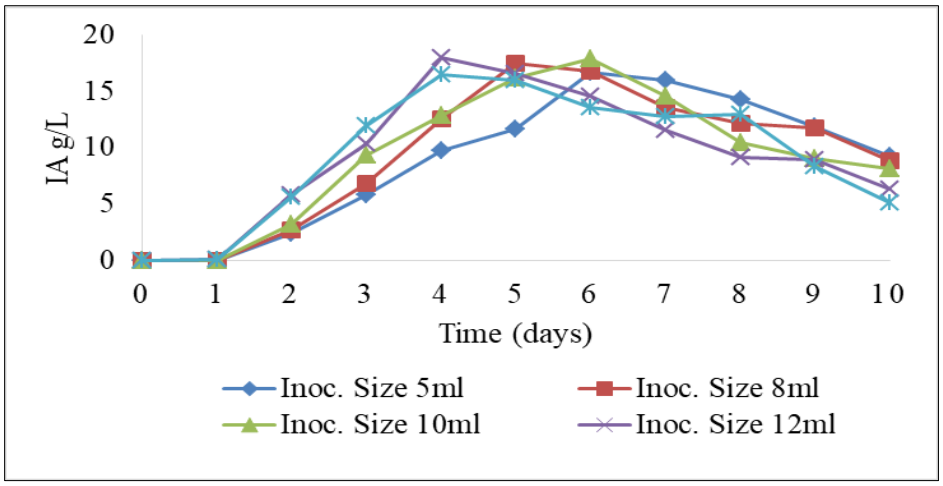


Figure 4 Influence of inoculum size on IA production using *C. vulgaris* biomass

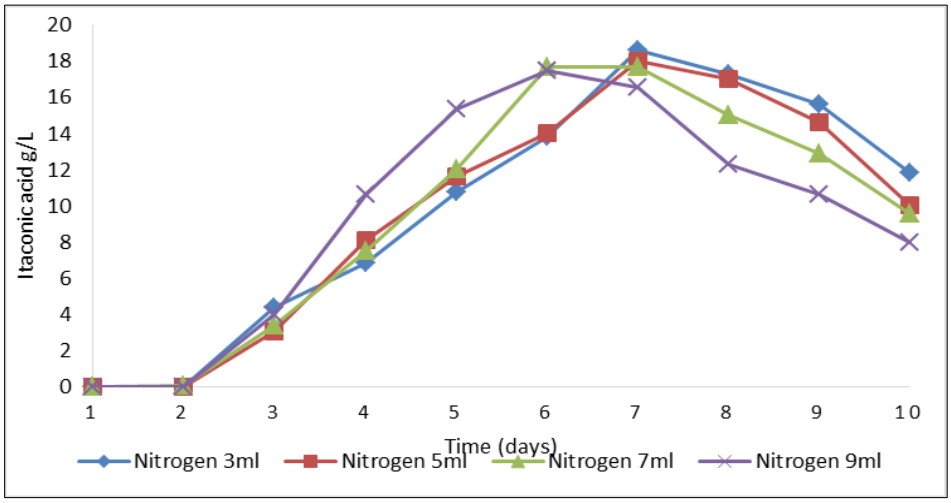


Figure 5 Influence of nitrogen on IA production using *C. vulgaris*

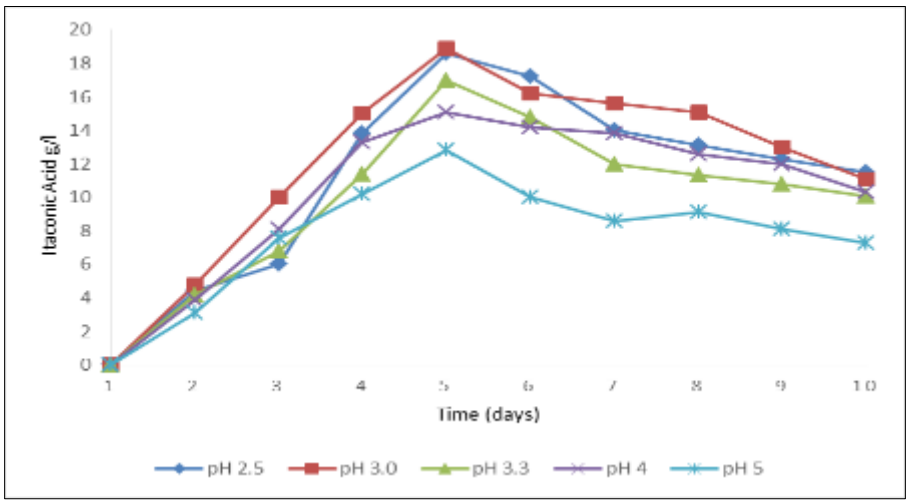


Figure 6 Effects of pH on Itaconic acid production using C. vulgaris biomass

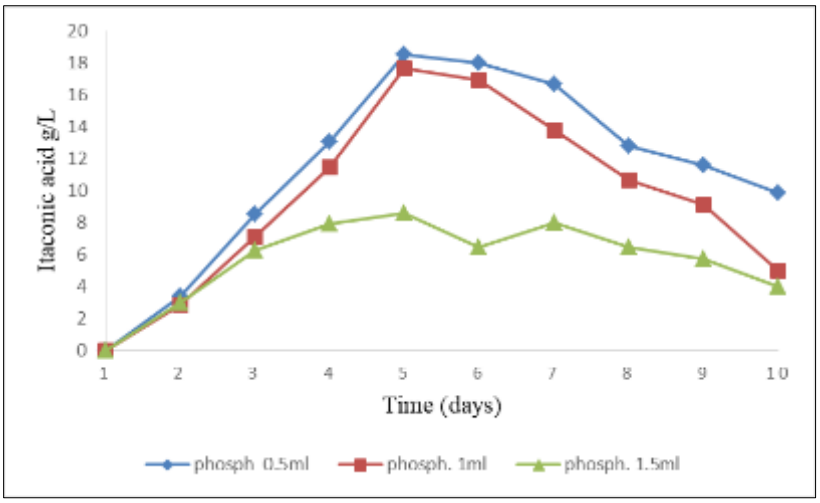


Figure 7 Effect of phosphorus on IA production

Table 4 OFAT studies of inoculum growth using *C. vulgaris* biomass as carbon source

Parameter	**Dry biomass**									
	Day 1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
N 3ml	0.09	0.50	0.63	0.98	1.01	0.89	0.62	0.55	0.51	0.42
N 5ml	0.11	0.52	0.77	0.78	0.95	0.86	0.77	0.63	0.47	0.33
N 7ml	0.25	0.34	0.59	1.00	1.38	0.98	0.73	0.59	0.44	0.32
N 9ml	0.53	0.65	0.96	1.03	1.6	1.00	0.80	0.61	0.69	0.31
pH 2.5	0.11	0.40	0.80	0.96	1.40	1.40	1.35	1.08	0.73	0.55
pH 3.0	0.16	0.41	1.00	1.09	1.47	1.39	1.30	1.12	0.93	0.78
pH 3.5	0.11	0.36	0.76	0.99	1.23	1.20	0.96	0.73	0.67	0.49
pH 4.0	0.10	0.23	0.58	0.90	1.14	1.16	1.00	0.86	0.59	0.33
pH 5.0	0.09	0.12	0.47	0.78	0.97	1.08	0.89	0.65	0.50	0.41
In. size 5ml	0.09	0.16	0.30	0.42	0.82	0.12	0.98	0.72	0.47	0.46
In. size 8ml	0.14	0.14	0.48	0.72	1.12	1.04	0.85	0.67	0.42	0.40
In. size 10ml	0.12	0.21	0.61	0.79	0.95	2.24	2.00	1.65	0.91	0.84
In. size 12ml	0.22	0.81	1.35	1.50	2.10	1.75	1.35	0.85	0.78	0.52
In. size 15ml	0.47	0.78	1.73	1.90	2.34	2.01	1.53	1.00	0.81	0.44
Ph 0.5ml	0.07	0.35	0.53	0.74	1.06	1.02	0.87	0.67	0.32	0.19
Ph 1.0ml	0.04	0.29	0.45	0.89	1.88	1.23	0.92	0.73	0.45	0.40
Ph 1.5ml	0.05	0.32	0.42	0.61	1.03	1.20	0.89	0.71	0.42	0.38
C. 8ml	0.11	0.57	0.85	1.16	1.24	1.68	1.25	0.88	0.72	0.46
C 10ml	0.22	0.70	0.82	1.29	1.70	1.41	0.99	0.75	0.56	0.49
C 12ml	0.20	0.73	0.96	1.45	1.73	1.38	1.01	0.76	0.69	0.61
C 14ml	0.24	0.60	1.16	1.38	1.21	0.98	0.92	0.87	0.80	0.72

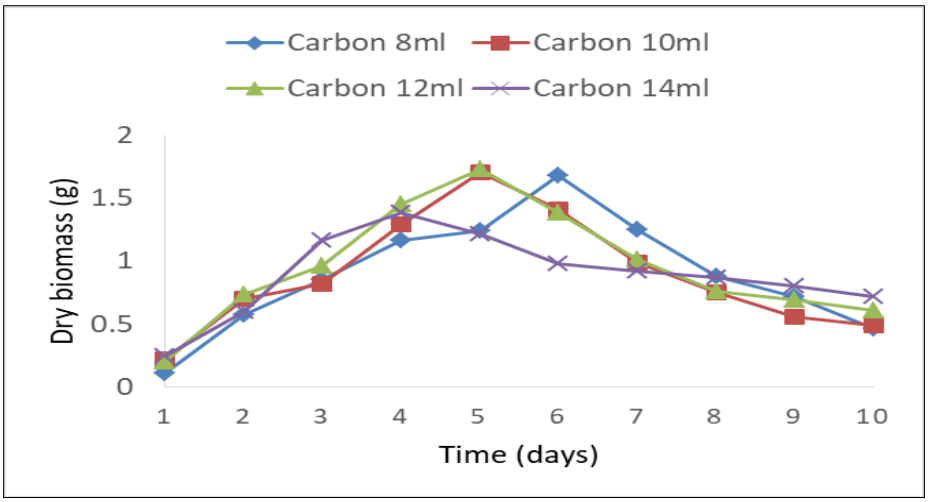


Figure 8 Effects of carbon on inoculum growth using *C. vulgaris* biomass

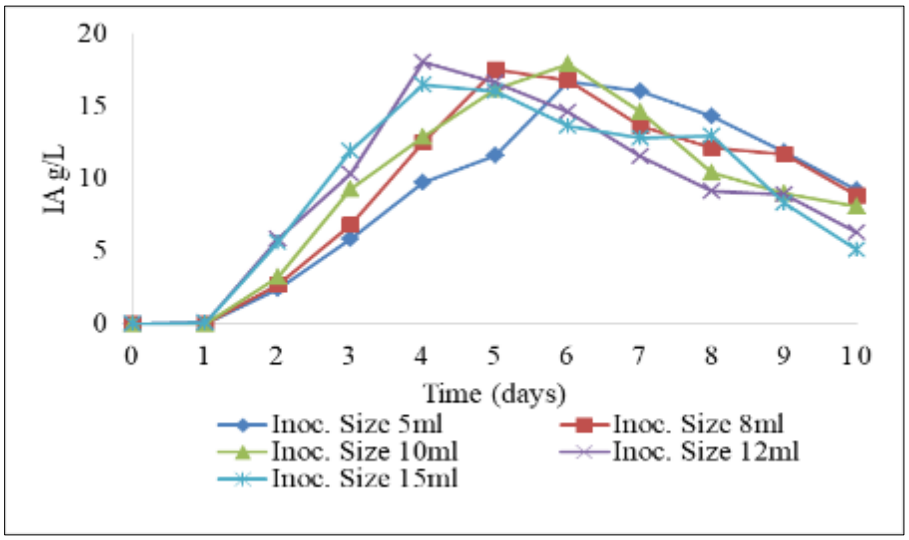


Figure 9 Influence of inoculum size on IA production using *C. vulgaris* biomass

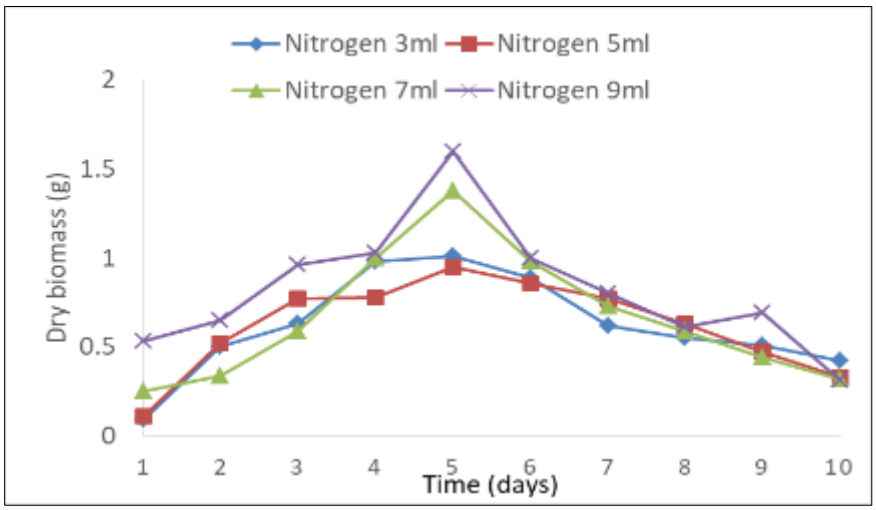


Figure 10 Effects of nitrogen on inoculum growth using *C. vulgaris* biomass

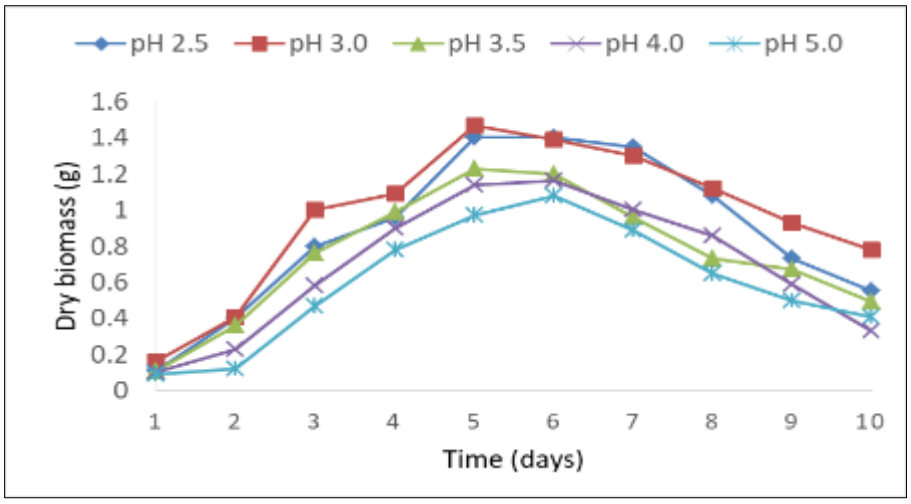


Figure 11 Effects of pH on inoculum growth using *C. vulgaris* biomass

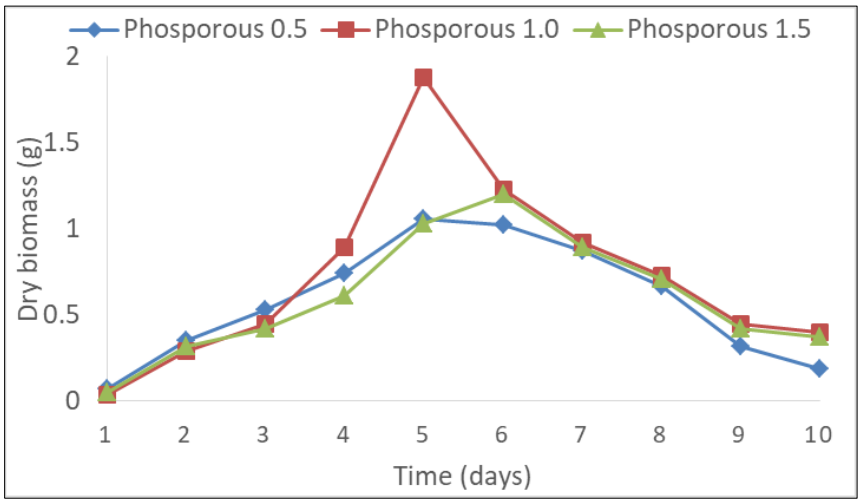


Figure 12 Effects of phosphorus on inoculum growth using *C. vulgaris* biomass

Table 5 Statistical analyses of carbon variations on IA yield

Source	Source of square (SS)	df	Mean square (MS)	F	P
Treatment	13.0	3.0	4.3	0.15	0.9
Error	1011.6	36.0	28.1		
Total	1024.6	39.0			

Table 6 Statistical analysis of impact of Carbon on cell growth

Source	Source of square (SS)	df	Mean square (MS)	F	P
Treatment	0.0	3.0	0.0	0.05	0.9
Error	6.6	36.0	0.2		
Total	6.6	39.0			

Table 7 Statistical analysis of impact of Inoculum size on cell growth

Source	Source of square (SS)	df	Mean square (MS)	F	P
Treatment	5.2	4.0	1.3	4.2	0.05
Error	13.7	45.0	0.3		
Total	18.9	49			

Table 8 Statistical analysis of impact of nitrogen on cell growth

Source	Source of square (SS)	df	Mean square (MS)	F	P
Treatment	0.3	3.0	0.1	0.9	0.4
Error	3.6	36.0	0.1		
Total	3.9	39			

Table 9 Statistical analysis of impact of pH on cell growth

Source	Source of square (SS)	df	Mean square (MS)	F	P
Treatment	0.9	4.0	0.2	1.4	0.2
Error	6.9	45.0	0.2		
Total	7.7	49.0			

Table 10 Statistical analysis of impact of Phosphorus on cell growth

Source	Source of square (SS)	df	Mean square (MS)	F	P
Treatment	0.1	2.0	0.1	0.3	0.7
Error	4.8	27.0	0.2		
Total	4.9	29.0			

5. Conclusion

This study has shown that IA can be produced using A. alabamensis MN907795 as production organism and C. vulgaris biomass, CSL and FBE as sources of carbon, nitrogen and phosphorus respectively. The best yield before OFAT studies was 17.09g/L and it was recorded on the 6th day of fermentation. The data from OFAT studies elucidated the impacts of the fermentation parameters on both IA and cell growth yield with the best results of 17.8g/L, 18.0g/L, 18.65g/L, 18.9g/L, and 18.55g/L observed from carbon 10%, inoculum size 12%, nitrogen 3%, pH 3.0, and phosphorus 0.5% respectively for IA yield, while the best cell dry masses of 1.73g, 2.24g, 1.38g, 1.47g and 1.88g were recorded from 12% carbon, 10% inoculum size, 7% nitrogen, pH 3.0 and1.0% phosphorus respectively. The F-test statistical analysis confirmed the significance of phosphorus and inoculum size at $P \leq 0.05$ for both IA and cell growth respectively. The industrial potential of both A. alabamensis and C. vulgaris should be explicitly explored in the industries for both itaconic acid and other bio-products productions.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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