OPTIMIZATION OF CITRIC ACID PRODUCTION FROM SUGARCANE MOLASSES USING *ASPERGILLUS NIGER* BY SUBMERGED FERMENTATION

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ABSTRACT

The potentiality of citric acid on economy is high because of its multi-purpose uses, particularly in the food and pharmaceutical industries. Bangladesh spent more than one million US dollars to import citric acid mostly from India and China. Its consumption is increasing 3.5–4%, annually, indicating the need for better manufacturing alternatives. Globally, citric acid is primarily produced through microbial fermentation with *Aspergillus niger.* To support the massive scale of production of citrate, the manufacturing process must be eco-friendly which should be inexpensive and available raw materials for maintaining high yielding in a cost-effective manner. In Bangladesh prospective, the current study has undertaken to optimize citric acid production using one of the most abundant raw materials sugarcane molasses. Moreover, the aim of this study was to determine the optimum conditions to produce citric acid from sugarcane molasses using *Aspergillus niger (F-81)* by submerged fermentation. The amount of citric acid production was determined by the Marier-Boulet colorimetric method. The optimization data suggested that 10% substrate (from processed cane molasses), 4% inoculum size of *A. niger*, and initial pH 6.0 allowed to produce around 25.8 g/L citric acid. Further study is warranted to assess the feasibility of citrate production in an industrial level as well as improvement of microbial strains is needed to further enhance citric acid production.

KEYWORDS: Citric acid, *Aspergillus niger*, Cane molasses, Submerged Fermentation, Marier-Boulet method.

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Introduction

Citric acid (CA) is one of the most important tricarboxylic organic and water-soluble acids that has both food-additive and pharmaceutical values. In 2016, the commercial value of CA was $2.5 billion, and its projected annual growth rate was approximately 5.0% from 2017 to 2025 (Research, 2018). In the early twentieth century, CA was mostly produced by citrus rich foods such as lemon and lime juices (King & Cheetham, 1987). Later on, *Aspergillus niger* was introduced to produce CA commercially in 1923 (Rohr, 1983). Since then, microbial fermentation has been recognized as a cost-effective, eco-friendly industrial process for CA production using *A. niger* and different raw materials (e.g. sugarcane, corns) as a source of hexose sugars in a commercial setting (Dhillon et al., 2011; Show et al., 2015). In Bangladesh, sugarcane is the second largest cash crops (Rahman et al., 2016) and approximately four million metric tons of sugarcane was produced in 2016-2017 (BBS, 2018). Besides, sugarcane molasses is a rich source of carbohydrates for CA production (GUPTA & SHARMA, 1994). In addition, sugarcane contributes to produce around 0.20 million tons of sugar and 0.60 million tons molasses in Bangladesh (BSRI, 2019). Although Bangladesh has sufficient raw materials (e.g. sugarcane molasses), the infra-structural setup to produce CA in the industrial level to meet their own demand is not substantial. In 2024, Bangladesh spent more than one million US dollar to import 448 shipments of citric acid from India and China (Volza, 2024). In China, submerged fermentation is the most common industrial plant for commercial CA production, where corn is the most common feedstock type and *A. niger* spores are inoculated with optimum settings (Wang et al., 2020). To address the market demand of citric acid in Bangladesh, the current study has undertaken to optimize the maximum production of citric acid by *A. niger* using one of the most abundant raw materials sugarcane molasses. The objectives of this study are the determination of the optimum inoculum size of *A. niger*, the nutritional composition of sugarcane molasses, and abiotic factors like metals, pH and temperature, which influenced submerged fermentation-based citric acid production.

Materials and Methods

A. niger growth and sugarcane molasses preparation for submerged fermentation

The fungal strain *Aspergillus niger* F-81 (91) was obtained from the "Molecular Biology Lab" of the Department of Biochemistry and Molecular Biology, University of Dhaka. The spores of *A. niger* were grown on Potato Dextrose Agar (PDA) plate and incubated at 28^0C for 7 days. Besides, *A. niger* also grows in PDB, a liquid media and incubated at 28^0C with 160 rpm and growth rate was recorded at 24 hours intervals at 240 nm.

Determination of moisture, ash, and sugar content in cane molasses

Raw cane molasses wet weight was measured and crucified, heated at 106^0C for 30 minutes. Subsequently, the dry weight was measured, and the moisture content was measured by the following equation (% moisture content = (Crucified weight – Dry weight)/Initial weight X 100). Besides, molasses was placed in a furnace and heated at 800^0C for 2 hours. Next, dry weight was measured, and the ash content was measured as % Ash = [(weight of ash) – (crucible weight)] x 100/ [(Total crucible and sample weight) – (Crucible weight). Moreover, the sugar content was determined by comparing with the standard maltose solution titration curve. Briefly, the standard titration curve was determined using 3,5-dinitrosalicylic acid (DNS), which changes color from yellow to red/orange in the presence of maltose. To determine the sugar content in molasses, 20 µL of 1M HCl was added in 1 mL of molasses solution and incubated at 90ºC for 5 minutes. Subsequently, 50 µL KOH (5N) was added to neutralize the acidic condition. The intensity of dark orange-red color was recorded at 540 nm using a UV-VIS Spectrophotometer as described (Krukowski et al., 2017). Finally, the amount of sugar was measured by comparing it with the maltose standard curve. The pH of the sample solution was recorded using a pH meter (HANNA, Germany).

Preparation of cane molasses and fungal inoculum

Raw cane molasses was either directly prepared in distilled water without pretreatment or pretreated with potassium ferrocyanide ($K_4Fe(CN)_6$) at 90^0C for 15 minutes. Then, ammonium salt (NH_4NO_3) and potassium dihydrogen phosphate (KH_2PO_4) were added into the molasses solution, which act as nitrogen and phosphate sources, respectively. The pH was measured and adjusted to 5.0-5.6. Finally, molasses solution was sterile at 121^0C under 15 psi for 20 minutes. Besides, suspension of spores was prepared from an 8-day-old *A. niger* culture plate and the spores were counted by Haemocytometer (Neubauer chamber) under a light microscope and the inoculum was adjusted to 1-5 million spores/mL. Finally, the spore suspension was quantitatively inoculated into molasses solution.

Different Fermentation conditions for citric acid production

A. niger spores were suspended in molasses media was placed into a shaking incubator for fermentation processes. Variations in fermentation conditions were maintained to observe the effect of different physical parameters on citric acid production. 10 mL and 15 mL of untreated/pretreated cane molasses were taken into three 500 mL Erlenmeyer conical flasks and diluted by adding 190 mL/185mL distilled water to make a substrate concentration of 5% and 7.5%, respectively. Similarly, to determine optimum concentration of molasses, 10% and 12.5% substrates were also prepared. The initial pH of the media was adjusted to 5.5. The media was then autoclaved at 121^0C under 15 psi for 20 minutes. Subsequently, 2% spore suspension was inoculated in 5-12.5% cane molasses containing solution, and incubated at 28^0C, 160 rpm for 13-14 days. Similarly, to determine optimum inoculum, 1%, 2%, 4% and 8% spore suspensions were also inoculated into a 10% substrate solution and incubated for 12 days at 28^0C, 160 rpm to observe the citric acid production. In addition, the optimum pH was also determined using 2.0, 4.0. 6.0 and 8.0 pH adjusted 10% molasses containing substrate media and 4% spore suspension as a source inoculum and incubated for 12 days at 28^0C with 160 rpm.

Precipitation and estimation of citrate

Fermentation broth was filtered through Whattman filter paper to remove fungal micelles and suspended materials. Then, an equal amount of 10% $Ca(OH)_2$ was added to the medium and heated for 1.30-2.0 hours at 60^0C -70^0C. Lime was then precipitated into tricalcium citrate tetrahydrate and the precipitate was filtered & washed with water. Thereafter, an equal amount of 20% H_2SO_4 was added and heated for 1.50-2.0 hours at 60^0C. Next, it was filtered, and the mother liquor was collected. The mother liquor was further filtered through a 0.2µm PTFE-Syringe filter (Sartorius company) and the estimation of citrate was done using the Marier-Boulet method (Pyridine-Acetic Anhydride method) (Alhadithy, 2020). The diluted (100 times) and filtrated mother liquor was treated with pyridine (MERCK), and then acetic anhydride (MERCK) was added and incubated at 32^0C for 30 minutes. Finally, the intensity of yellow color was measured at 405 nm. The amount of citrate was measured through by comparing with a standard calibration curve of citrate. Briefly, a stock citrate solution of 5 mg/mL was prepared by using tri-sodium-citrate dihydrate (MERCK). Then, 50-300 µL of citrate solutions were taken in separate test tubes and 4.95-4.70 mL distilled water was added to each test tube, respectively. After that, 1 mL of each diluent was taken into separate test tubes including a tube containing 1 mL distilled water as blank. Next, 1.30 mL of pyridine was added to each tube and mixed thoroughly. Next, 5.70 mL of acetic anhydride was added to each tube and placed the sample in a water bath at a constant temperature of 32^0C for 30 minutes. The optical density was recorded at 405 nm. Finally, a standard calibration curve was drawn taking the citric acid concentration at the X-axis and optical density at the Y-axis. The amount of Citrate was measured in mg/mL using the following equation. Concentration of Citrate (µg/mL) x Dilution Factor/1000.

Statistical Analysis

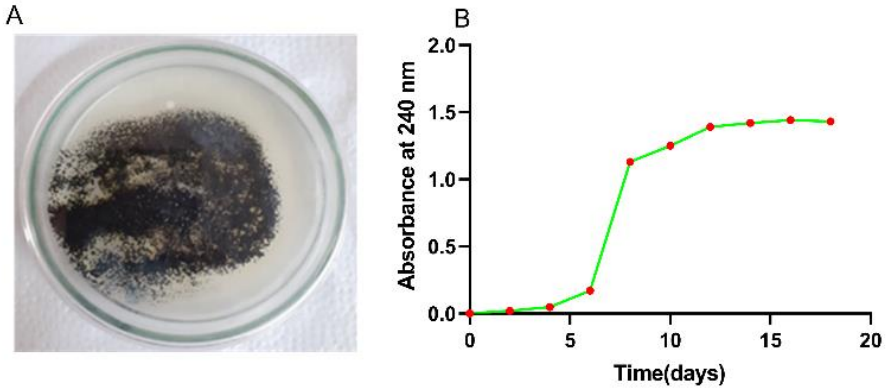
All statistical analyses were done using a software called GraphPad Prism version 8.0.2. Data were expressed as mean ± SD (Standard deviation). All experiments were performed at least three replicates.

Results

Preparation of *A. niger* inoculum and cane molasses suspension for submerged culture

It is well-established that the branches mycelium of acidogenic *A. niger* contains short hyphae with swollen tips, which are suitable for citric acid production irrespective of their pellets or

filamentous forms (Babitha et al., 2007; Snell, 1951). Moreover, the main substrates for citric acid production are solutions of sucrose, molasses or glucose (Domínguez, 2010). Previous studies suggested that raw materials which contains 14-22% sugar is sufficient for fermentation-based citric acid production (M. Hossain, 1984). Visible and filamentous forms of *Aspergillus niger* F-81 spores were grown in 10 days in potato dextrose agar. Similarly, the suspension culture of *A. niger* was grown to the highest level by 10 days in a potato dextrose broth media. As a source of sugar, cane-molasses was processed and crushed into a solution. The physico-chemical properties of cane-molasses suggested that it has 53.5% sugar residue, 24.7% moisture, 16.3% ash and pH was acidic (5.6).



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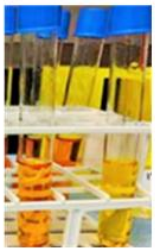

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Content	Amount/value
Total Sugar	53.5%
Moisture	24.7%
Ash	16.3%
pH	5.6

Figure 1. Optimization of *Aspergillus niger* growth in potato dextrose agar/broth. Figure A shows formation of fungal (*A. niger*) spores after 10 days of culture on potato dextrose agar at 28°C. (B) shows the growth curve of *A. niger* in potato dextrose broth. (C) shows the raw and processed cane molasses (D) shows the maltose color reagent which was used to measure sugar content in cane molasses. Table (E) shows the physico-chemical properties of cane-molasses which were assessed by appropriate methods.

Determination of optimum concentration of substrate, microbial inoculum, and pH for fermentation-based citrate production

The effect of substrate concentration on citric acid production was determined at 5%, 7.5%, 10% and 12.5% substrate concentration (V/V) with certain inoculum size (2%) of *A. niger*, initial pH (5.6), and 10 days cultivation at ambient temperature (circa 28°C) with 160 rotation per minute (rpm). The maximum yield of citric acid has been recorded 12.09 mg/mL with 10% substrate concentration (V/V). Moreover, citric acid production has also recorded 5.18 mg/mL, 6.36 mg/mL, and 7.48 mg/mL with a 5%, 7.5% and 12.5% substrate concentration, respectively (Figure 2B). Whereas the effect of fungal inoculum size was determined using 1%, 2%, 4% and 8% inoculum sizes with a certain amount of substrate concentration 5%, initial pH 5.6- and 12-days cultivation setup at an ambient temperature and 160 rpm. The maximum yield of citric acid was 17.67 mg/mL in 4% inoculum size of *A. niger*. Besides, 1%, 2% and 8% inoculum of *A. niger* spores allow to yield 3.76 mg/mL, 10.80 mg/mL, and 13.98 mg/mL of citrate, respectively (Figure 2C). In addition, pH optimization data shows that the maximum yield of citric acid was 9.70 mg/mL at initial pH 6, whereas the citrate yielding were 3.24 mg/mL, 6.62 mg/mL, and 5.85 mg/mL at pH 2, 4, and 8, respectively (Figure 2D).

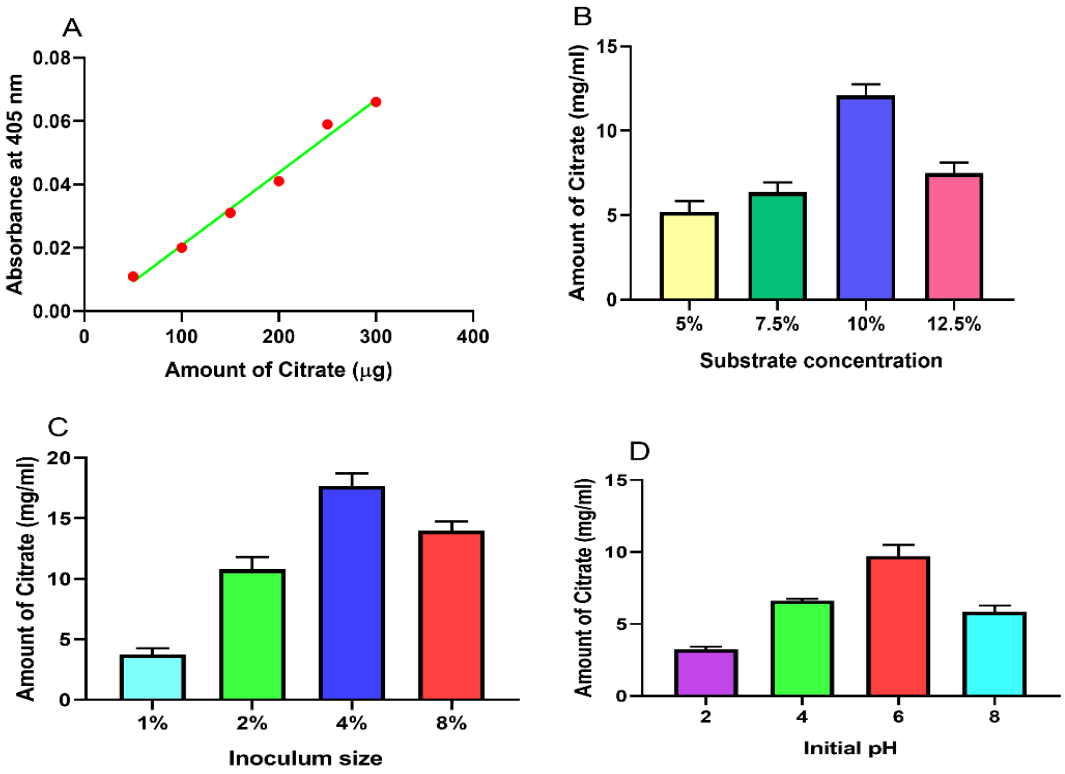

Figure 2. Optimization of molasses concentration, fungal inoculum, and physico-chemical features of submerged fermentation. (A) shows the titration data of citrate, which were generated by the Marier-Boulet method using a standard citrate solution. (B) shows the amount of citrate production using different concentrations of molasses as a source of substrate. (C) shows the amount of citrate production using different sizes of inoculum of *A. niger*. And (D) shows the amount of citrate production at different pH of broth. Data shows as mean ± standard deviation. N=3.

Citric acid production from different sugar concentrations by *A. niger*

Aspergillus niger was first introduced to produce fermentation-based citric acid in 1919 (Papagianni, 2007). To produce citric acid (CA) by a submerged fermentation process, *A. niger* inoculum spores (2% or $1.47x10^6$ spore/mL) were prepared from potato dextrose agar and sugarcane molasses were utilized as a source of cheap raw material. To determine citric acid production capacity from sugarcane molasses, a titration curve was generated by the Marier-Boulet method (colorimetric) using a standard citrate solution (Figure 3A). The amount of citrate production in different batches of fermenter were 5.2 mg/mL to 6.2 mg/mL in 5% substrate and 6.4 mg/mL to 7.8 mg/mL in 7.5% substrate, and 13.5 mg/mL to 17.7 mg/mL in 10% substrate containing with 8 days of fermentation (Figure 3B and 3C). As expected, there was no significant difference of citrate among the batches of fermenter.

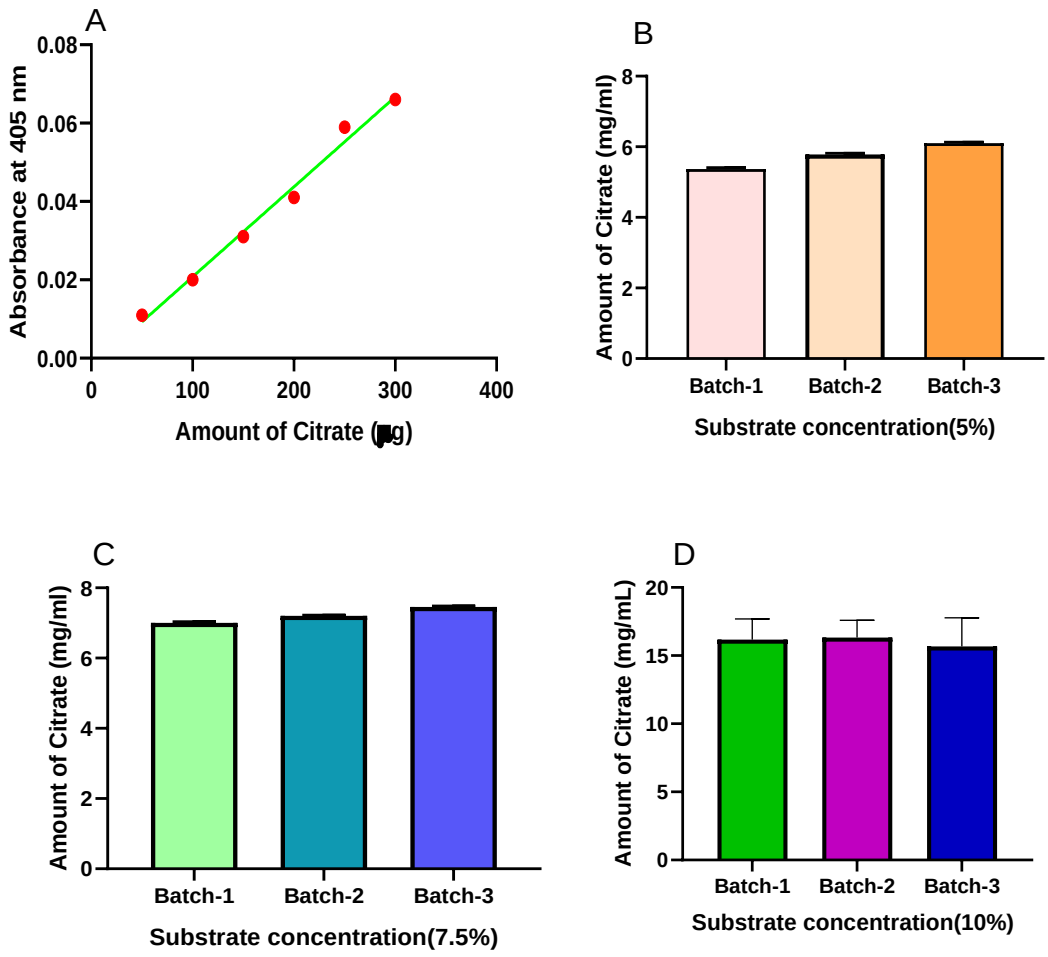

Figure 3. Standardization of Citric acid production using molasses as a raw material**. (A) shows the** titration data of citrate, which were generated by the Marier-Boulet method using a standard citrate solution. (B) shows the amount of citrate production in different batches of submerged fermenter using either 5% or (C) 7.5% of molasses as a source of initial substrate. The amount of citrate was determined by non-linear regression method. And (D) shows 10% of molasses as a source of initial substrate. Data shows as mean ± standard deviation. N=3.

Maximum production limit of citrate in submerged fermentation

To observe the effect of pretreatment with potassium ferrocyanide, 5% molasses media was inoculated with inoculum size 2%, initial pH 5.6 for 10 days of fermentation. The result showed that the maximum yield of citric acid was observed in a pretreated batch (12.49 mg/ml) compared with an untreated batch of fermentation (Figure 4A). The continuous-batch fermentation with 5% substrate of molasses, 2% inoculum of *A. niger* and initial pH 5.6 has shown 16.0 mg/mL (circa) citrate has yielded by 16 days of cultivation (Figure 4B). In addition, 4% inoculum size of *A. niger* F81 and 10% pretreated sugarcane molasses as a substrate in the above-mentioned optimum pH and temperature yielded maximum citric acid (28.25 mg/mL) after 16 days of fermentation (Figure 4C).

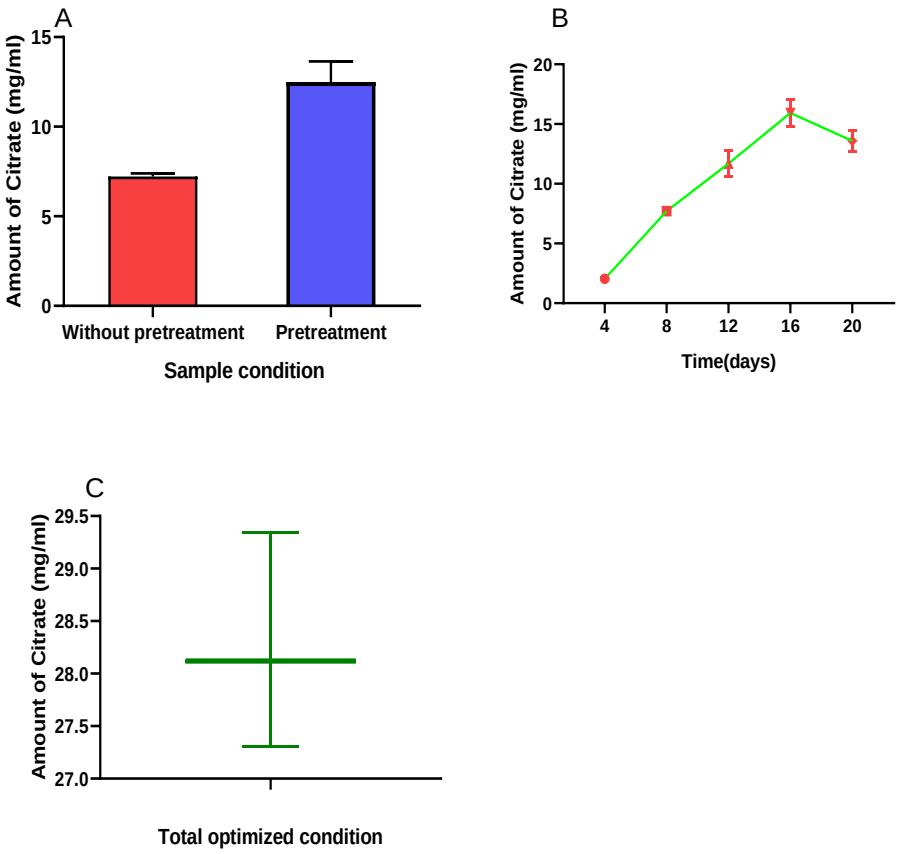

Figure 4. Citrate production limits in sugarcane molasses and *A. niger* containing submerged fermentation. (A) shows the amount of citrate production with/without pretreated sugarcane molasses. (B) shows the optimum duration to produce maximum amount of citrate by the submerged fermentation using sugarcane molasses as a raw material. (C) shows the highest amount of citrate production using an optimized amount of sugarcane molasses, temperature, pH, and inoculum of *A. niger*. Data shows as mean ± standard deviation. N=3.

Discussion

Citric acid, one of the most demanding organic ingredients, is used in 70% food-additives, 12% pharmaceuticals and 18% other industrial products (Dhillon et al., 2011). It can be derived from lemon, lime and oranges as a natural source (Penniston et al., 2008). In addition, citrate can be produced synthetically by chemical reaction, but microbial fermentation met 99% of citric acid requirements globally (Kuforiji et al., 2010). Hence this study has adopted the submerged fermentation technique, which is less sensitive to medium composition, allowing a broad range of substrates with flexible substrate control. In addition, this technique is cost-effective with minimum risk of batch contamination and high yielding capacity (Max et al. 2010). Besides, this also allows to use different microbes, including bacteria and fungi to produce citric acid. Current study has utilized *A. niger*, which is superior to other microbes particularly for citric acid production because its high yielding capacity using cheap raw materials like cane molasses as a source of carbohydrate (Alhadithy, 2020; Show et al., 2015).

Apart from carbohydrates, cultural conditions of *A. niger* are crucial factors for submerged fermentation-based citrate production (Çevrimli et al., 2009). In this study, submerged fermentation has been adopted to produce citrate because of its cost-effective implementation and reasonable reproducibility. However, other parameters for this process have been optimized to obtain maximum amount of citric acid. In addition, we also demonstrated that the yielding of citrate increased by a decrease of the pH value, and the sugar content in the solution. In this study, fermentation conditions and *A. niger* F81 isolates with pH 6.0 appears to be the best initial pH for maximum production of citrate (28.25 mg/mL). Overall, these findings were corroborated with previous findings, where an initial pH 5.5 contributes to the maximum level of citric acid biosynthesis (Ikram-ul et al., 2004; Sikander et al., 2002). However, Khurshid et al., demonstrated that a higher pH causes accumulation of oxalic acid and a low, or high pH causes a negative impact on citric acid production due to the release of toxic ions (Khurshid et al., 2024). Furthermore, Dashen and colleagues demonstrated that pH of the medium changes constantly due to microbial metabolic activities, primarily due to the secretion of organic acids such as citric acid, as well as the unwanted gluconic and oxalic acid (Dashen et al., 2014). We also observed that the pH of the fermenter is critical during sporulation of *A. niger* and citrate production. During the germination stage, the germinating spores absorb ammonia and release protons, which increasing the acidity of the medium and favoring citric acid production.

In addition, it is reported that 25-30^{0}C is optimum for citrate production by submerged fermentation. To enhance citrate production wild type strain of *A. niger* needs to acclimatize as an industrial strain, which is yet to establish in our study. This

is mostly done through strain improvement process. Moreover, mutagenesis can be applied to improve citric acid producing *A. niger* through inducing the parental strains. For example, mutagens including gamma radiation, ultraviolet radiation and chemical mutagens are applied to induce mutation. Besides, the ultraviolet and chemical mutagens-based combined method produces citrate hyperproducer strain, reviewed in (Chandra et al., 2021). The passage and single spore techniques are used for selection. The passage method is preferred because organic acids (oxalic and gluconic acids) and mineral acids simulate the presence of citric acid in the single-spore method (Soccol et al., 2006). Moreover, this study also optimized the spectrophotometry method to measure the level of citrate in each batch of fermenter, which is reproducible, less time consuming and cost-effective.

Conclusion

Sustainability is the main concern to protect the environment from negative ecological impact. Microbes can, as a part of the sustained that utilize in an agro-based biomass to produce industrial byproduct through fermentation. In addition, it's produced a value-added product and helped to make a green environment. *A. niger* is a potential source for citric acid production, which is used particularly in beverages, food, detergents, cosmetics, and pharmaceutical industries that generate through submerged fermentation. Indeed, in South Asia, particularly in Bangladesh, the demand of citric acid met through imports. The rate of utilization for citric acid substantially increased, leading to industrialization. The object of this study is to optimize citric acid production from sugarcane molasses using *A. niger* containing fermentation-based platform. This study demonstrated that yielding a decent amount of citrate is possible using optimum pH, inoculum of *A. niger* and optimized concentration of sugarcane molasses. However, identification of regulatory genes of citric acid metabolism could be extemporaneous in *A. niger* for further enhancement of citric acid production.

Acknowledgements

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Author Contribution

Alamin and RA have equally conducted and analyzed experiments. TNI reviewed the manuscript. MM and MI designed experiments and reviewed analysis with Alamin and RA. MM wrote the manuscript with input of all authors.

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