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Citric acid production by the solid-state fermentation potential of *Aspergillus Niger* using different substrates from northern Gondar zone of Ethiopia

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Abstract: Citrus fruits, of course, contain citric acid. Synthetic forms are frequently used as an extra ingredient and are made from a particular kind of fungi. There is a higher requirement for citric acid manufacturing because it is utilized in many advanced medical fields as well as the food and beverage industries. There is a dearth of prior research on the extraction of organic acids, such as citric acid, from filamentous fungi in Ethiopia. Therefore, the purpose of this work was to use wheat straw, sugarcane waste, and a combination as a substrate to manufacture citric acid from *Aspergillus niger* that was isolated from agricultural soils in Ethiopia's northern Gondar zone. Aspergillus isolates were found in agricultural soil samples used in this investigation. The ideal pH and temperature for the chosen isolate to produce the most citric acid were also investigated. Morphological techniques were used for the initial identification of the chosen isolates. An isolate from a mixed substrate showed the highest citric acid output (1.6 g/100 ml) on basal screening media when the isolates were screened for citric acid synthesis. For this isolation to produce the most citric acid, the ideal conditions were 30°C, 129 hours, pH 6.5, and 70% moisture. This study suggests that *A. niger*, which was isolated from agricultural soil in Ethiopia's north Gondar zone, could generate more citric acid with a different substrate.

Keywords: Solid-state fermentation, Citric acid, Agro-industry waste, *Aspergillus niger*

1. Introduction

Citric acid is a white, crystalline powder that dissolves in water and has a strong buffering effect on water. It is specialized organic acid. It is anticipated that the global citric acid market will reach $3.2 billion by 2023. Over a million tons of citric acid are thought to be generated annually worldwide. The World Health Organization has classified citric acid as safe. Citric acid has several uses, including those as an antioxidant, ch-elating agent, preservative, pH regulator, and flavor enhancer (Behera et al., 2021). There are several industrial uses for citric acid in meals and drinks. It has little aftertaste and is used as an acidifies in meals. Since citric acid adds a sour flavor to drinks, it is utilized to temper sweetness. Citric acid is known to improve renal and digestive function. Pharmaceuticals employ citric acid as an antioxidant to preserve vitamins and to adjust pH. Citric acid is used as a foaming agent in the textile industry to increase the softness of textiles. To make detergents more ecologically friendly, citrate is also utilized in the detergent business to replace phosphate. Furthermore, to be mentioned is the possibility of using an organic acid, like citric acid, as a component in the synthesis of commercial chemicals (Hu et al., 2019). In most plants and animals, citric acid (2-hydroxy2, -propane tricarboxylic acid) is one of the most significant and adaptable carboxylic acid intermediates of metabolism. Many acidic fruit juices, especially those that are citric, include citric acid (CA). However, the natural methods of extracting it from lemons and other natural sources have gradually been superseded by biological processes, which are principally based on the employment of fungi and are today the most extensively used method (Shah et al., 2020). Solid-state cultivation (SSC) can be characterized as microbial development on solid substrates

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without the presence of free water; this is analogous to the natural occurrence of fungi (Pandey, 2003). The chemical, food, pharmaceutical, and agricultural industries have all emphasized this process because it is low-power, low-waste, and it reuses industrial residues as substrates. Because of their physiological and biochemical traits, filamentous fungi—a subclass of microorganisms that may be cultivated on solid supports-have the best capacity to develop under these circumstances (Pandey et al.,2000).
Recently, microbial fermentation has been used extensively in the synthesis of citric acid due to its ease of handling and low cost. In the food and beverage sectors, citric acid is used extensively as an antioxidant and flavor enhancer. It is also used in the chemical, cosmetic, and pharmaceutical sectors, among other industries. Therefore, the goal of the current study was to ascertain if it would be feasible to produce and optimize citric acid under fermentation conditions from various substrates using inexpensive, raw materials like wheat straw, sugarcane waste, and their mixture.

2. Materials and methods

2.1. Isolation and identification of the fungus

The soil samples were taken from fields in Ethiopia's Amhara regional state's North Gondar Zone. A total of 50 soil samples were collected in clean nylon bags and taken to the Laboratory of the Department of Biology, College of Natural and Computational Sciences, University of Gondar. Wheat straw, sugarcane waste, and mixture of these substrates was gathered from farms for the substrate preference. In 90 milliliters of sterile distilled water, five milligrams of every soil sample were suspended. Next, 0.1 ml of the resultant mixture was applied using a glass rod to the surface of solid potato dextrose agar (PDA) plates. The composition of PDA was Potato, 100g; Dextrose, 10g; Agar, 10g and distilled water, 500ml. pH: 5.6 ± 0.2 at 25°C. These plates were then incubated at room temperature (25°C) for five days, during which time the growth was monitored every day for fungal development. The isolated fungus was identified using morphological characteristics, and the growth was confirmed microscopically using lactophenol cotton blue staining to confirm its purity (Salano, 2014).

2.2. Preparation of basal medium and Fungal strain inoculum

To prepare the basal medium for solid-state fermentation, the following ingredients were dissolved in 1 liter of 0.1M sodium acetate buffer (pH 5.5), autoclave for 20 minutes at 121°C and 1.5 atim, and then added to KCl 0.5, MgSO4.7H2O 0.2, CaCl2 0.1, yeast extract 0.5, and FeSO4.7H2O 0.01. (Shyamala.,2009) We employed a strain of *Aspergillus niger* that was stored at 80 °C in spore form as a 70% (v/v) glycerol stock. Conidiospores were obtained by shaving and extracting the spores from the 7-day culture on potato dextrose agar plates that had been incubated at 30 °C using sterile water containing 0.04% (v/v) Tween 80. To achieve the required concentration of 106 spores per milliliter of the medium, the spore suspension was diluted with sterile deionized water (Kennedy and Saha 2020).

2.3. Screening of isolates

Five days old strain of *A. niger* 1 milliliter of spore suspension was inoculated in a sterile liquid basal medium to screen the isolates of *A. niger* quantitatively for the generation of citric acid. After adjusting the pH to 6.0. At room temperature, the fermentation process was conducted in 250 ml Erlenmeyer flasks with 100 ml fermentation medium. Titrimetric analysis was used to quantify the content of citric acid in the fermentation medium (Ali, 2002).

2.4. Inoculum preparation

The five-day-old culture was inoculated with 10 milliliters of distilled water containing two drops of 0.1% Tween 80. The spore clusters were removed from the sterilized conditions using a sterile wire loop, and the spore suspension was homogenized by vigorous shaking after mixing (Pandey, 1992).

2.5. Samples Collection

Samples such as wheat straw and sugarcane waste were collected from the local market of North Gondar Zone, Amhara region, Ethiopia (Figure 1).

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Sugarcane waste Wheat straw

Figure 1. Wheat strow and sugarcane samples

2.6. Fermentation

Wheat straw, sugarcane wastes, and mixed substrate were fermented using *A. Niger*. The substrate was cleaned, let the air dry, and then dried in a hot air oven at 70°C for two to three hours. After that, the substrate was grounded to a size of 1-2 mm. The substrate preference experiment was conducted using the (Bapat et al., 2003) method. Seven (7) grams of natural substrate, seven grams of sugarcane waste, seven grams of wheat straw, and fourteen grams of mixed were placed in a sieve and left to soak in distilled water for three minutes. After that, the strainer was taken out, and the extra water was emptied for the same amount of time. To get rid of the dust and make sure there was enough soaking, this process was done three times. After being moved into 250 ml conical flasks, the substrate was sterilized for 20 minutes at 121°C. To modify the ultimate moisture content, 14 grams of mixed substrate and seven milliliters of sterile water were aseptically added. One milliliter (ml) was added to each flask to introduce *A. Niger* isolates. For seven days, inoculated flasks were stored at room temperature. To guarantee that the mycelia were evenly distributed throughout the solid particles, the flasks were gently shaken every day for seven days.

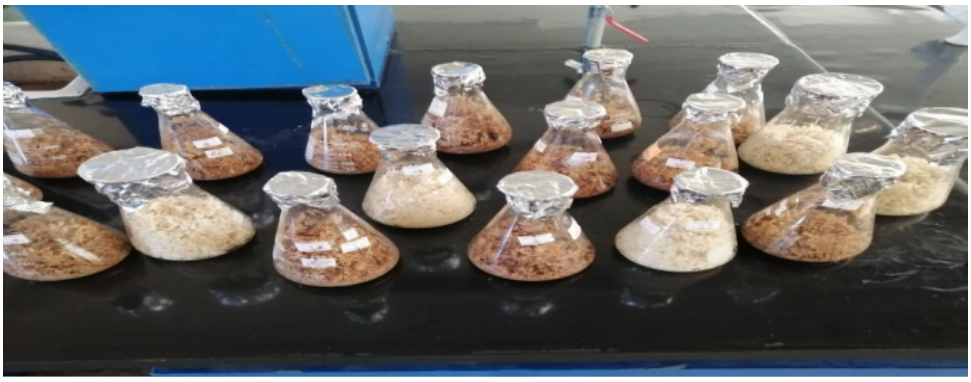

Figure 2. Solid-state fermentation

2.7. Spore Extraction and Quantification

The slants and flasks were filled with fifty (50) milliliters of sterile water, respectively. Following 15 minutes of vigorous shaking of the flasks to harvest the spores on the natural substrates, filter paper was used to filter the mixture. For a full day, the brown solution was stored in the refrigerator. Conidia were quantified at 560 nm using a spectrophotometer (Stinson.,1954)

2.8. Optimization of citric acid production

To optimize the synthesis of citric acid, several physical and chemical parameters were chosen, including temperature, pH, incubation duration, and the amount of substrate used (10g and 15g). Additionally, the size of the substrate inoculates (6.0X106) was examined. The optimization process involved adjusting a single

parameter at a time for each variable, such as the incubation time (48, 96, 144, 192, 240, and 288 hours), reaction temperature (20, 25, 30, 35, and 40 degrees Celsius), reaction pH (5.5, 6, 6.5, 7, and 7.5), moisture content starting at (60, 65, 70, 75, 80), and the impact of the agricultural substrate (Kessas et al., 2012; khattab et al., 2017).

3. Results and Discussion

3.1. Identification of Isolate

In the present study, Soil samples were selected at random from farms that practice agriculture. To demonstrate the isolate's purity, it was refined, and examined under a microscope, and its cultural traits were noted. By looking at physical traits and using lactophenol cotton blue staining, the isolated fungus was recognized as *A. niger* (Figure 3). Solid-state fermentation is a microbial fermentation process through which selected microorganisms (bacteria, fungi, and yeasts) are cultivated on a moist, solid, non-soluble organic material that acts as a support and nutrient source for the growth of microorganisms, in the absence or near absence of free-flowing water. It is considered an important, viable food processing approach for bioconversion of organic agro-industrial wastes (Mansor et al., 2023). Globally, the food, pharmaceutical, energy, and chemical industries are the main beneficiaries of the application of solid-state fermentation, because, through microbial biotechnology, it is conveniently used in the production of fermented foods and other useful industrial products (Ghosh, 2016). The purpose of this study was to find fungi that produces citric acid in a variety of naturally existing agricultural soil sources, and it is the high yield of citric acid production. To find possible fungi that produces citric acid, the researchers concentrated on several decomposing soil samples that were collected on PDA agar plates. acid, the researchers concentrated on several decomposing soil samples that were collected on PDA agar plates. Identification of Aspergillus niger morphologically: In this study the isolates grew initially on PDA with white mycelia, which matured into velvety black spores. The conidial head also displayed black coloring.

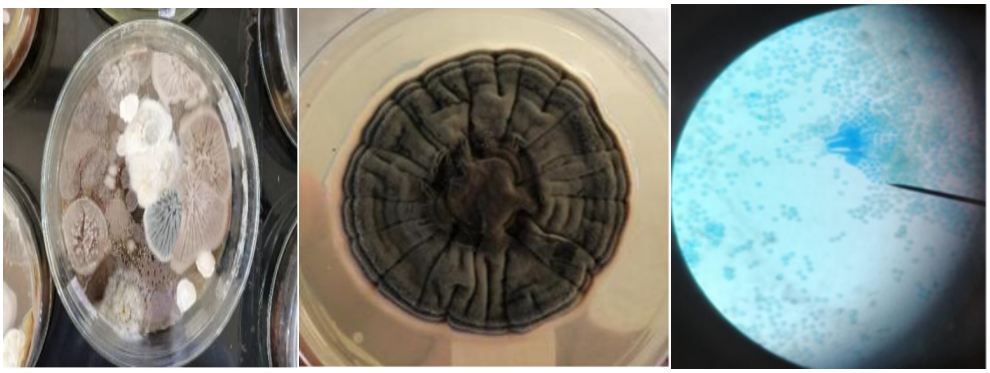

Figure 3. Matured *A. niger* as viewed under macroscopic and the microscope

3.2. Production of citric acid

This study used an alternative raw material to evaluate the filamentous fungal isolate's capacity to produce acetic acid. Isolates from several places are screened for the synthesis of citric acid: The amount of citric acid produced from agricultural wastes. Over different hours, the amount of citric acid generated by the *A. niger* isolates was measured at recorded regular intervals of hours. A maximum mean value of 1.6 g/100ml citric acid was produced from an isolate using a mixed substrate in the north Gondar zone after 129 Hrs of incubation. A mean value of 1.4 g/100ml citric acid was produced from an isolate using a sugarcane wastes substrate and 1.2 g/100ml citric acid was recorded from *A. niger* isolates using wheat straw substrate respectively. *A. niger* strains that were isolated from agro-ecologic zones in north Gondar, Amhara regional state, Ethiopia, generated citric acid. Numerous studies have sought to achieve high yields using low-cost optimization techniques. improvement efforts of *A. niger* for citric acid production were often focused on traditional optimization techniques, and numerous publications exist regarding these techniques, which vary one element at a time to produce citric acid (Ambati and Ayyanna, 2001; Maharani et al., 2014; Adeoy et al., 2022).

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Table 1: Maximum mean value of citric acid production (g/L) by A. Niger isolates from agricultural soils collected from north Gondar zone

Substrate	Citric acid production g/100ml
Wheat straw	1.2
Sugarcane wastes	1.4
Mixed	1.6

3.3. Optimization of Citric acid production using *Aspergillus niger*

3.3.1. Effect of fermentation time

In this study, the generation of citric acid was assessed using a variety of physical and chemical criteria. The impact of various incubation times on the synthesis of citric acid was investigated. *A. niger* produced the highest amount of citric acid in a mixed substrate after 129 hours of incubation (1.60 gm/100 ml). It was observed that the generation of citric acid reduced with increasing incubation time (Figure 4). Maximum production of the citric acid peaking at 1.6 g/100 ml after 129 hours by mixed substrate and then declining to 0.3 g/100 ml after 48 hours. The *A. niger* has been incubating for a few hours. Our findings are consistent with those of some earlier research that indicated a seven-day fermentation period was ideal. In This study at 129 Hrs, where maximum production was observed, the best results were obtained (Iqbal et al., 2015). The other research report was by (Cevrimli et al., 2009). The greatest amount of citric acid was generated after 216 hours, and this was further enhanced by 144 hours of incubation. It was reported that 192 hours of incubation produced the highest quantity of citric output. As anticipated, it is evident that the fermentation period had an impact on citric acid production. More interaction between the fungi and the substrate results from longer fermentation times.

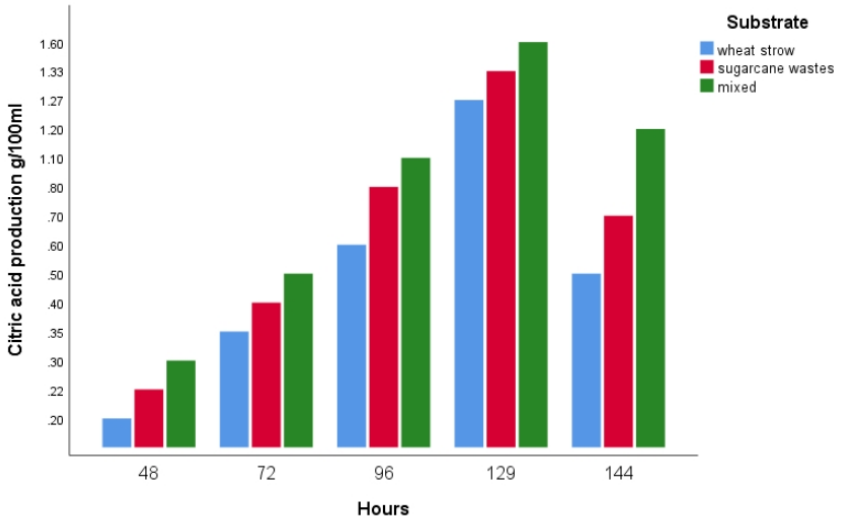

Figure 4. Isolates from different substrates and citric acid produced at different Hrs

3.3.2. Effect of fermentation pH

In this study, the greatest synthesis of citric acid by *A. niger* was observed at pH 6.5 (2.0 gm/100 ml) and pH 7.0. value was 1.5 grams per 100 milliliters, at pH 7.5 1.2 grams per 100 milliliters, and the generation of citric acid was decreased as the medium's pH increased to mixed substrate (Figure 5). When employing fungi or any other microbe to produce organic acid or acid, the impact of pH is a crucial factor. For Aspergillus niger, high citric acid was obtained in this work at pH 6.5. We have noted the following during the fermentation of the basal medium for the three substrates (wheat straw, sugarcane wastes, and mixed) kinds at 30 °C and 128 hours of incubation. Figure 5 indicates citric acid by *A. niger* was observed at pH 6.5 (2.0 gm/100 mL) and pH 7.5. value was 1.2 g per 100ml. The pH gradually decreased as the fermentation period lengthened; to successfully

produce citric acid in subsequent studies, a pH of 6.5 is required. This result agrees with the previously published works of (Almousa et al., 2018). The culture medium's initial pH value primarily regulates the growth of the fungus and the synthesis of citric acid. Numerous research has been reported on various pH optima. The obtained results are consistent with (Laltha et al., 2022) who investigated the effects of low and high starting pH values and concluded that the generation of citric acid is reduced when the initial pH value is less than 3.5. It could be because the ferrocyanide ions were more harmful to mycelium growth at low pH levels. Oxalic acid builds up when the starting pH is higher. But the greatest amount of citric acid (1.2 g/l00ml) was generated when the starting pH of the fermentation medium was set to 6.5, according to research on the effects of varying initial pH (0.5 - 7.5) of the fermentation media. Protein and citric acid output were both decreased when pH dropped which agrees with the study of (El-Aasar, 2006).

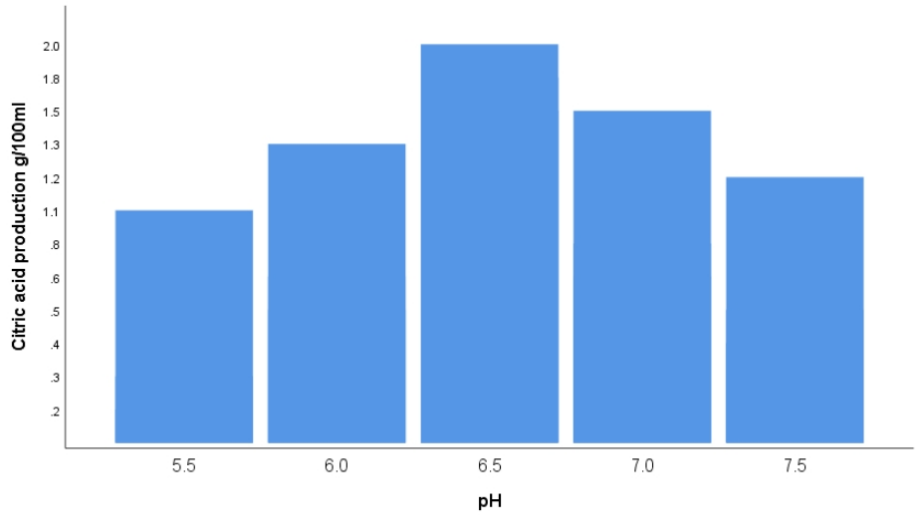

Figure 5. Citric acid production by *A. niger* mixed substrate at different pH

3.3.3. Effect of fermentation temperatures

As demonstrated in (Figure 6) out of all the temperatures that were tested, 30°C produced the most citric acid (2.26 gm/100 ml); at 35°C and 40°C, the values were 1.2 gm/100 ml and 0.6 gm/100 ml, respectively. Therefore, 30 OC was the ideal temperature for the manufacture of citric acid in this investigation, and any higher temperature reduced the production of citric acid. Effect of incubation temperature: Figure 4 illustrates how temperature affects the synthesis of citric acid. We observed that the average amount of citric acid produced at 30°C was 2.2 g/100 ml, at 35°C it was 1.2 g/100 ml, and at 40°C it was 0.6 g/100 ml in this investigation, which employed a pH of 6.5 at various temperatures. Thus, 30°C is the ideal temperature for the synthesis of citric acid in this investigation. Several studies found similar results (Alsudani and Al-shibli, 2015).

In addition, 20–40°C is thought to be the ideal temperature range for mushrooms. On the other hand, a temperature higher than 40°C promotes oxalic buildup and suppresses mycelial growth. *A. niger* cultures that are thermotolerant, however, may manufacture citric acid at temperatures as high as 25–40°C studies found similar results (Kareem et al.,2010). These cultures have demonstrated that one of the key variables that significantly affects citric acid synthesis is the temperature of the medium fermentation. Lower than 27°C temperatures significantly slowed down growth and productivity. reduced middle temperature also resulted in reduced enzyme activity, which did not affect the generation of citric acid. However, the biosynthesis of citric acid was reduced when the medium temperature rose over 30°C. The generation of citric acid at varying moisture levels was demonstrated by the differences in substrates, according to the results.

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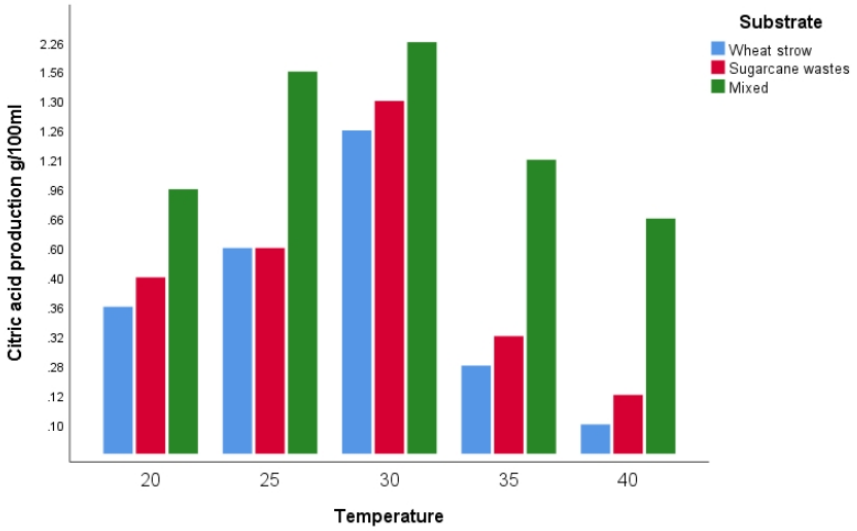
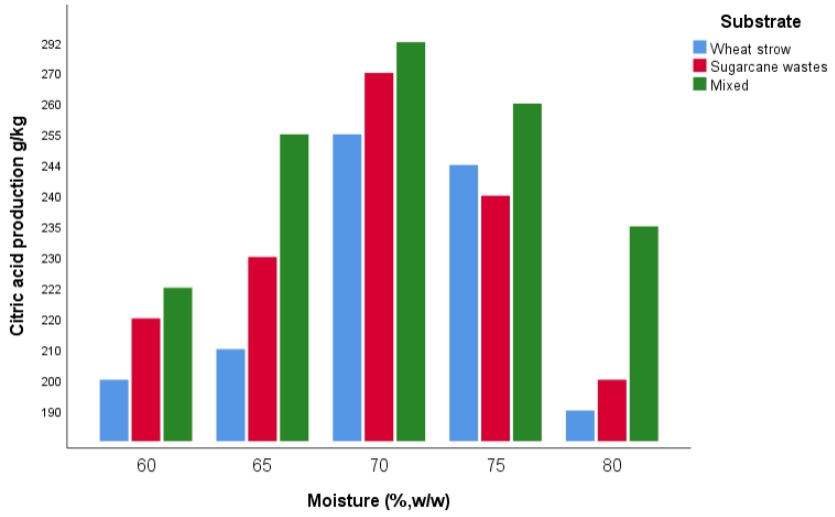


(Figure 6). Citric acid production by *A. niger* mixed substrate at different temperatures

3.3.4. Effect of fermentation moisture content

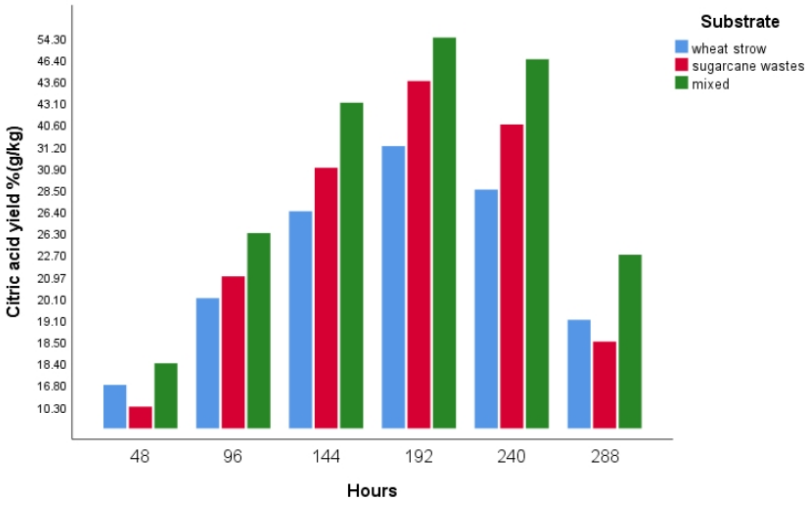
As seen in (Figure 7), citric acid rose as the moisture content grew from 70 to 75% and then sharply reduced as the moisture level increased even further. In these studies, at a moisture percentage of 70%, the greatest citric acid moisture was obtained at 255 g/kg wheat straw, 270 g/k g sugarcane wastes, and 292 g/kg mixed substrate, respectively. The results showed that the ideal moisture content for the synthesis of citric acid was the blended substrate at pH 6.5 for 70% (w/v). The greatest citric acid moisture was obtained at 255 g/kg wheat straw, 270 g/kg sugarcane waste, and 292 g/kg mixed substrate, so the mixed substrate is the preferred yield of citric acid by (70%w/v) moisture content. The number of citric acids its production declined as the moisture content rose by 80%. These amounts were expected and are within the range of previous studies (El-Sohaimy and Hafez, 2010, Pereira et al., 2017, Gourchala et al., 2015)



(Figure 7). Citric acid production by *A. Niger* mixed substrate at different moisture content

3.3.5. Effect of Different Substrate Preference on Citric Acid Production

Citric acid obtained from different substrates is shown in Figure 8. At 300c, *A. niger* produced more citric acid. On substrates made of 7 grams of sugarcane waste and 7 grams of wheat straw, there has been a greater synthesis of citric acid. This indicates that 14% of the substrate may promote the synthesis of citric acid, but an additional 14% would raise the concentrations of several sugars in the mixture that impede the synthesis of citric acid. The blended yield with the highest percentage growth was 54%. Then came sugarcane waste, which produced a 43% yield; using wheat straw as a substrate produced a 31% yield. Citric acid production was studied by mixed substrate as a good substrate for the synthesis of citric acid. The amount of citric acid produced was highest in 192 Hrs of fermentation (Figure 8). The wheat straw and sugarcane waste in the mixed substrate had the highest value of citric acid production yield (34.3g/kg of dry weight), followed by 27.2g/kg and 30.6g/kg, respectively. Strains of *A. niger* using agricultural wastes to produce citric acid. The filamentous fungus *A. niger* is most frequently used to produce citric acid because it is simple to handle, has a well-developed enzymatic system that allows it to employ a variety of substrates, can ferment a wide range of inexpensive raw materials, and produces a high output of citric acid several studies (Książek, 2023; Auta et al., 2014; Femi-Ola and Atere, 2013). Even though they are costly, they can be swapped out for a variety of readily available, inexpensive substrates, such as byproducts or agro-industrial wastes (Prasad et al., 2014). Citric acid can be produced effectively using a variety of source materials. Nevertheless, there are a few important considerations that must be made when selecting the type of substrate, such as pricing or the requirement for pretreatment (Soccol et al., 2006). After 129 hours of growing cultures in which 7 grams of specific raw materials were used, the goal of this section was to embrace the use of specific local raw materials as a low-cost medium for the manufacture of citric acid by *A. niger* (wheat straw, sugarcane wastes, and mixed or wheat straw and sugarcane wastes) was added either carbon-free or carbon- and nitrogen-free to the fermenting media. By employing molasses as a carbon source, the results demonstrated that the mixed substrate was considerably superior to the wild strain for *A. niger* generation of citric acid (1.6 mg/100 ml) These results agree with (Munshi et al., 2013).



(Figure 8). Different substrates to produce citric acid by A. Niger

4. Conclusions

According to the study's results, using mixed as a substrate for *A. niger* production of citric acid may be a practical way to reduce mixed substrate disposal issues while simultaneously creating a less costly, economically important organic acid. The findings also demonstrated that farm soil contains strains of *A. niger* that may generate respectable levels of citric acid. Few microbiological sources are known to be suitable for the commercial production of citric acid, although numerous are accessible for its manufacture. Bio technologists from all over the world have taken notice of them due to their spectrum of action and great diversity. Not much work is being done in Ethiopia right now to use microorganisms to make citric acid. Using locally available and less expensive materials, such as agricultural waste and byproducts, as a substrate for the synthesis of this

valuable organic acid and its subsequent application in the food and pharmaceutical industries, may be the best option when cost-effectiveness is considered. As a result, the strategy for further studies has been prepared to use *Aspergillus niger* to manufacture citric acid.

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Conceptualization, K.A. and T.M.; Methodology, K.A.; Data analysis, K.A.; Investigation, K.A.; Resources, K.A.; Writing – original draft, K.A.; Writing – review & editing, K.A,N.B. and T.M.; Visualization, K.A,N.B and T.M.; Supervision, T.M.and N.B; Funding acquisition, K.A. All authors have read and agreed to the published version of the manuscript.
Ethical approval and consent
Ethical approval and consent were not required.

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