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USE OF AGRICULTURAL WASTES AS SUBSTRATES FOR COST EFFECTIVE CITRIC ACID PRODUCTION

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Abstract

One of the organic acids that is utilized the most frequently is citric acid, which finds usage in the food industry, the pharmaceutical industry, and industrial activities. On the other hand, the high cost of production continues to be a key obstacle in the creation of its large-scale products. The purpose of this research is to investigate the possibility of using agricultural wastes as low-cost substrates for the synthesis of citric acid through the process of microbial fermentation. Utilizing *Aspergillus niger* as the microbiological agent, agro-industrial wastes such as sugarcane bagasse, corn stover, rice husk, wheat bran and fruit peels were examined for their potential as carbon sources in submerged and solid-state fermentation systems. Based on preliminary findings, it appears that these waste materials not only diminish the expenses associated with manufacturing but also make a contribution to the sustainable management of waste by recycling agricultural by-products. Citric acid production was dramatically increased as a result of the optimization of fermentation parameters, which included pH, temperature, substrate concentration, and nutrient supplementation. Sugarcane bagasse that had been treated with nitrogen sources had the best capacity for production efficiency among the substrates that were examined. The purpose of this study is to demonstrate that it is possible to utilize agricultural waste for the manufacturing of citric acid, therefore proposing a strategy that is not only cost-effective but also environmentally beneficial and sustainable for industrial applications. For the purpose of increasing production and determining whether or not it is economically viable at commercial levels, more study is needed.

Keywords : Citric acid***,*** *Aspergillus niger, agricultural, wastes, cost effective,*

Introduction

Citric acid is an important organic acid that has a wide range of uses in a variety of sectors, including the food industry, the pharmaceutical industry, and the biotechnology industry. Due to the fact that it functions as an acidulant, a preservative, and a stabilizing agent, it is one of the biochemicals that is in high demand all over the world. Conventionally, the synthesis of citric acid is dependent on the fermentation of sugar-based substrates. This fermentation process frequently involves significant costs connected with the raw materials, which in turn has an effect on the overall economic feasibility of the processes involved. Waste materials from agriculture, which are produced in large quantities all over the world, present a viable option as substrates that are also cost-effective. These by-products, which include sugarcane bagasse, wheat bran, rice husk, and fruit peels, are abundant in carbohydrates and other nutrients that have the potential to act as carbon sources for the development of microorganisms and the creation of metabolites.

Not only does their utilization cut down on manufacturing costs, but it also solves environmental problems that are associated with the disposal of trash and the sustainability of resources. The manufacture of citric acid is generally accomplished by the process of microbial fermentation. *Aspergillus niger* is the microbe that is utilized the most frequently due to its high yield potential, capacity to metabolize a wide variety of substrates, and industrial scalability. When it comes to optimizing the synthesis of citric acid, the selection of the substrate and the conditions of fermentation are extremely important factors. The purpose of this research is to examine the utilization of a variety of agricultural waste products as substrates for the synthesis of citric acid, with the end goal of developing a manufacturing method that is both environmentally friendly and economical. The findings highlight the importance of waste-to-wealth conversion tactics in the biotechnology industry, contributing to both economic and environmental advantages in the process. The solution that has been described is consistent with the ideas of circular economy and sustainable development since it makes use of agricultural leftovers. Through the process of valorizing these leftovers, which are typically thrown out or underutilized, it is possible to create high-value biochemicals such as citric acid, hence lowering dependency on standard sugar-based substrates. In addition, the use of agricultural wastes in fermentation processes helps to reduce the carbon footprint that is linked with waste management strategies that are popular in many countries, such as burning or landfilling. It is essential to select appropriate substrates and optimize fermentation parameters, such as pH, temperature, aeration, and nutrient supplementation, in order to get the highest possible output of citric acid. A substantial amount of variation may be seen in the composition of agricultural residues, particularly with regard to the amount of cellulose, hemicellulose, lignin, and other fermentable carbohydrates. The use of pre-treatment methods, such as hydrolysis or enzymatic degradation, is frequently necessary in order to boost the bioavailability of substrates and to promote the development and metabolism of microorganisms. The use of various agricultural waste materials for the synthesis of citric acid was investigated in this study using a methodical approach to determine whether or not it would be feasible to do so. Among the goals were the determination of the substrates that were the most efficient, the optimization of the circumstances under which fermentation took place, and the comparison of the production yields with those obtained using traditional substrates. In addition, the study investigated the ways in which the incorporation of agricultural waste valorization into citric acid manufacturing processes might have repercussions for both the economy and the environment. It is anticipated that the outcomes of this research will provide a sustainable paradigm for the bioprocessing sector, which will promote the twin benefits of cost reduction and environmental sustainability. Additionally, it underlines the potential for agricultural wastes to serve as renewable feedstocks for a variety of biotechnological applications, therefore furthering the area of environmentally friendly technologies and production systems that are highly efficient in terms of resource utilization.

The diverse applications of citric acid in a wide variety of sectors, including the food and beverage industry, the cosmetics industry, and the pharmaceutical industry, are driving the ongoing increase in the global demand for citric acid. Not only is it necessary to increase production in order to satisfy this expanding demand, but it is also necessary to make certain that the procedures are both economically feasible and ecologically sustainable. An innovative strategy for accomplishing this objective is to make use of waste products from agricultural production as substrates. The manufacture of citric acid using traditional techniques frequently involves the use of refined sugars as major carbon sources. Examples of such sugars are glucose and sucrose. These substrates are effective; nevertheless, they are costly and

compete with food supply chains, which raises ethical and economic considerations with regard to their use. The agricultural leftovers, on the other hand, are plentiful, renewable, and underutilized in the agricultural sector. Creating value from waste streams and reducing reliance on refined substrates are two goals that may be accomplished by industries through the transformation of by-products into valuable inputs for the synthesis of citric acid. When it comes to the manufacture of citric acid, microbial fermentation with *Aspergillus niger* is a process that has been studied extensively and proven to be dependable. Due to the fact that the fungus has shown extraordinary flexibility to a wide range of substrates and fermentation conditions, it is appropriate for the use of agricultural waste. On the other hand, due to the variety of agricultural wastes, appropriate pre-treatment and process optimization are required in order to maximize the availability of fermentable sugars and optimize metabolic efficiency. Increasing the appropriateness of a substrate is often accomplished by the use of several methods, including acid or enzymatic hydrolysis, steam explosion, or microbial pre-digestion. Through the investigation of a wide variety of agricultural wastes, the optimization of fermentation parameters, and the evaluation of the process's scalability, this research intends to expand upon the information that is already available. In addition, the study assesses the environmental effect of the suggested technique by calculating the reductions in waste and emissions of greenhouse gases in comparison to the standard manufacturing methods. An all-encompassing perspective on the viability of citric acid synthesis based on agricultural waste is made possible by the use of life cycle assessment (LCA) methodologies. In conclusion, this research highlights the twin benefits of economic and environmental sustainability that are afforded by the process of valuing agricultural waste. In addition to addressing critical difficulties in the citric acid business, it also contributes to worldwide efforts in waste management and sustainable development. This is accomplished by the transformation of low-value wastes into high-value products to achieve this transformation. It is anticipated that the discoveries would open up new paths for biotechnological breakthroughs, helping to encourage industry to adopt production practices that are both more environmentally friendly and more cost-effective.

MATERIALS AND METHODS

The soil samples were taken from rice and groundnut fields in at Parbhani and Gadchiroli and Central Market yard of both places, where damaged fruits were gathered. Samples were collected in sterile nylon bags and then sent to the tested. Gathered from Parbhani and Gadchiroli farmlands, wheat straw and rice straw were used to determine the substrate preference. Potato peel powder was purchased at the Hi-media. For each soil sample, five grammes were suspended in 35 ml of sterile distilled water before isolation. A bent glass rod was used to apply 0.1ml of the mixture to the surface of solid potato dextrose agar (PDA) plates so that the development of fungus could be observed. For five days, while being checked daily, the plates were left to incubate at room temperature (29±3°C). The resulting fungal colonies were subculture on fresh potato dextrose agar(PDA) in order to achieve pure cultures. Following their maintenance on slants of potato dextrose agar, the pure cultures were chilled to 48^0 C and stored for further use. The fungal pure cultures were inspected under a microscope for characteristics such exudates and colony diameter on days 4 and 7 of incubation. This made it possible to identify *Aspergillus niger* based on its appearance. To identify the hyphae and conidia types, the fungal isolates were mounted on grease-free glass slides, stained with lacto-phenol cotton blue, and examined under a microscope (at magnification X40). The next step was to capture images. Extensive fungal species identification was carried out in accordance with the protocols laid forth in the Manual about the Genus *Aspergilli* (Raper and Fennell, 1965; Domsch *et al*.,

1980). After then, the experiments that followed made use of the previously characterized *A. niger* isolates. Check for substrate preference: We thought about and used the method from Prashant *et al*. (2003) to find out what substrates people prefer. 10g of natural substrate, including rice straw, wheat straw, and potato peel powder, was filtered via a sieve. The next step was a three-minute immersion in distilled water for the organic substrate. It took the same amount of time to remove the strainer as it did to drain the excess water. It was necessary to repeat this procedure three times to eliminate dust and ensure adequate soaking. The 250 ml conical flasks were sterilized at 121°C for twenty minutes after the substrate was transferred to them. A sterile 10mm of water was added in an aseptic way to change the final moisture content. To inoculate each flask with *Aspergillus niger* isolates, a total of 1mm was added to each. The inoculated flasks were left at room temperature for five days. To ensure that the mycelia were properly dispersed throughout the solid particles, the flasks were gently shaken on the third day. In order to keep the incubator at a constant humidity level, water-filled beakers were placed within. Spore Extraction and Conidia Quantification Recovering information: The slants and flasks were each given 50 cc of sterile water and then mixed together. The flasks were vigorously shaken for fifteen minutes to collect the spores on the natural substrates before filtering them using filter paper. The solution, which was rather brown, was kept in the fridge for twenty-four hours. The quantity of conidia present was measured at a wavelength of 560 nm using a spectrophotometer. Finding a citric acid-producing strain in the *Aspergillus niger* population: Isolates of *Aspergillus niger* were quantitatively tested for citric acid production in a sterile liquid basal medium that contained soluble starch (10 gm/L), $(NH_4)_2SO_4$ (2.2 gm/L), K_2HPO_4 (1 gm/L), $MgSO_4.7H_2O$ (0.05 gm/L), and $CaCl_2$ (0.05 gm/L). Doing this necessitated maintaining a pH level of 6.0. Each of the 250 mL Erlenmeyer flasks contained 100 mL of fermentation medium, and the fermentation was conducted at room temperature. The content of citric acid in the fermentation medium was determined by titration analysis (AOAC, 1995). The analysis was carried out every 12 hours for a total of 72 hours. Improvements to the fermentation medium: The cultivation conditions were optimized to generate citric acid using an *Aspergillus niger* isolate. Two independent variables, temperature and pH, were considered in this optimization. An estimate of the citric acid synthesis was generated after 72 hours of incubation the contaminated media. To facilitate the production of citric acid by *A. niger*, a range of temperature conditions was selected, including 25^0–40°C and a pH range that started at 5.5, continued through 6.0, 6.5, 7.0, 7.5, and 8.0. The citric acid concentration in the fermentation media was determined by titrimetric measurement (AOAC, 1995). Application of *Aspergillus niger* in wheat straw fermentation. After washing and letting it air dry, the untreated wheat straw was heated to 70°C in a hot air oven for around 2-3 hours to speed up the drying process. Following that, the substrate was ground to a particle size of around 1-2 mm. Untreated wheat straw and distilled water were mixed to form a slurry with a weight-to-volume ratio of 1:10. Specialized wheat straw: Before being incubated in a water bath at 100°C for one hour, ground wheat straw was acid-treated by slurring it in 5 NHCl at a solid-liquid ratio of 1:10 (w/v). After letting it cool, the substrate was rinsed with distilled water and dried in a 100°C oven. Experimental flasks used for fermentation were 250 mL in volume and contained 4 grammes of crude wheat straw (untreated and treated). Next, the necessary amount of distilled water was added to the flasks until they reached a moisture content of 65 percent by weight. The substrate's initial sugar content was increased to fourteen percent (w/w) by adding sugar cane molasses; the moisture level was adjusted taking into account the molasses's water content. To get the substrate back to its original pH of 6.0, 2N NaOH was used. Once the flasks had cooled to room temperature following autoclaving at 121°C for fifteen minutes, 1ml of spore suspension was added to infect them. Over the course of five days, the flasks were maintained in an incubator set at 30°C. According to AOAC (1995) and Khosravi *et al*. (2008), the filtrate was subjected to titration analysis

following purification from the medium. The concentration of citric acid was ascertained by analysing the filtrate.

RESULTS AND DISCUSSION

Aspergillus niger may be identified based on its morphology. Isolates grown on PDA and SDA initially had white mycelia, but as they matured, the mycelia transformed into velvety black spores, and the conidial head became black as well. Table 1 and Figure 1 illustrate the results of the microscopic inspection, which showed a distinct conidiophore terminating in an enlarged vesicle that contained flask-shaped biseriate phialides.

Table 1: Microscopic characteristic of Aspergillus niger

Features	Matured	Young
conidiophore	Larger	Smaller
Hyphae	Sepetate brown transparent growing on substratum	Sepetate blue-black transparent growing on substratum
Head	Dark brown globular head on hyphae	Black globular head on hyphae

Citric acid production screening of geographically diverse *Aspergillus niger* isolates: Figure 2 shows the total citric acid production during 72 hours, measured at 12-hour intervals. Two independent runs of the experiment were carried out. At 72 hours, the highest mean value for the KW site isolate was 0.38 g/100 ml, the second highest for the CM location was 0.27 g/100 ml, and the lowest for the KSU and MD locations were 0.24 and 0.225 g/100 ml, respectively. For optimisation tests, *Aspergillus niger* KW was employed as it produced the greatest mean citric acid value compared to the other isolates.

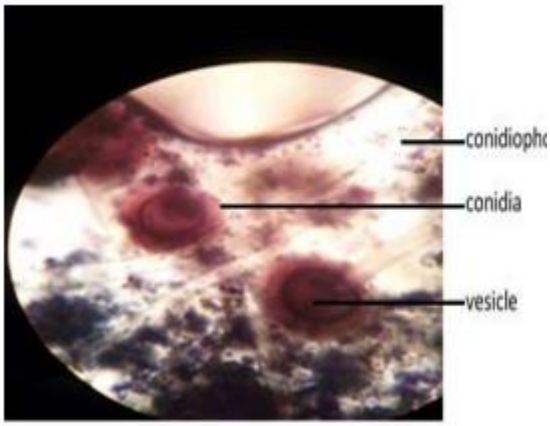

Fig 1: Viewed under a microscope, this is an adult Aspergillus niger (Magnification X40).

Submerged fermentation is sensitive to the medium's pH, which in turn influences citric acid synthesis. According to Figure 3, the ideal starting pH for citric acid synthesis in this investigation was 6.0. Citric acid had a concentration of 0.26g/100 ml at pH5.5, 0.4g/100 ml at pH6.0, and 0.27g/100 ml at pH 6.5. A significant drop of 0.09g/100 ml in citric acid output was observed with further increases to pH 8.0. Figure 4 shows the influence of temperature on citric acid generation by way of the incubation temperature. The average citric acid generation at various temperatures was found to be 0.4 g/100 ml at 25°C, 0.65 g/100 ml at 30°C, 0.12 g/100 ml at 35°C, and 0.06 gm/100 ml at 40°C, according to this study, which utilised a

pH of 6.0. Therefore, 30°C was determined to be the sweet spot for citric acid synthesis in this investigation.

Substrate Preference: Table 2 displays the results of *Aspergillus niger* isolates from four locations: KSU, CM, KW, and MD. Using wheat straw as a substrate resulted in the best percentage growth yield of 41%. Next came rice straw with a 26% yield, then potato peel powder with a 32% yield. Utilization of wheat straw in the synthesis of citric acid: Figure 5 displays the citric acid extracted from both untreated and processed wheat straw. On the fifth day of fermentation, the amount of citric acid generated by both the untreated and pretreated wheat straw was maximum. On day 5, the greatest value of untreated wheat straw by dry weight was 13.30g/kg, while on day 4, it was 12.20g/kg. The highest value of 25.6g/kg was achieved using pretreated wheat straw on day 3, followed by a value of 20.00g/kg on day 4.

Table 2: Natural substrate produced from *Aspergillus niger* isolates collected from several places

Substrate	Location				
	KW	MD	CM	KSU	% growth of isolates
Wheat straw	16.05	14.02	16.02	13.30	41
Rice straw	9.02	10.05	9.00	9.05	26
Potato peel powder	13.03	12.00	11.04	11.04	32

Key: CM-Central market, KSU- Kadam SU farm, MD Market Down and KW-KuptaWatur

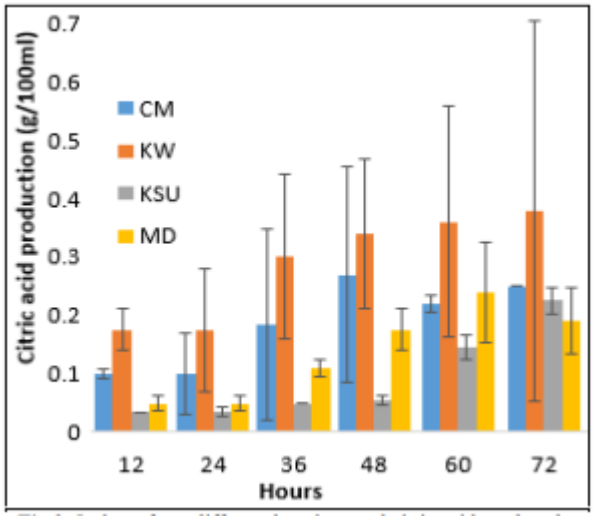

Fig 2: The production of citric acid and the isolation of samples from various places

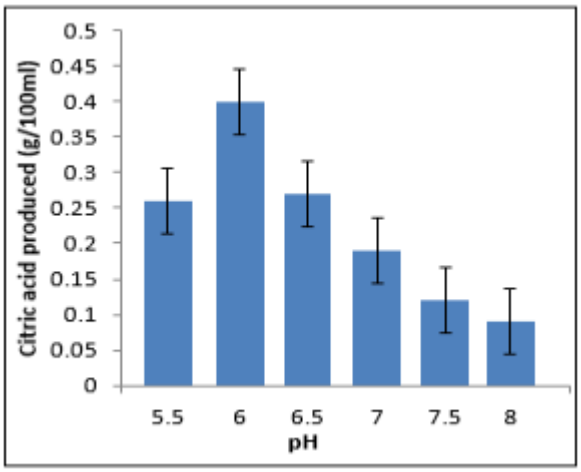

Fig 3: Different pH levels result in the synthesis of citric acid by the *A. niger* Kw isolate.

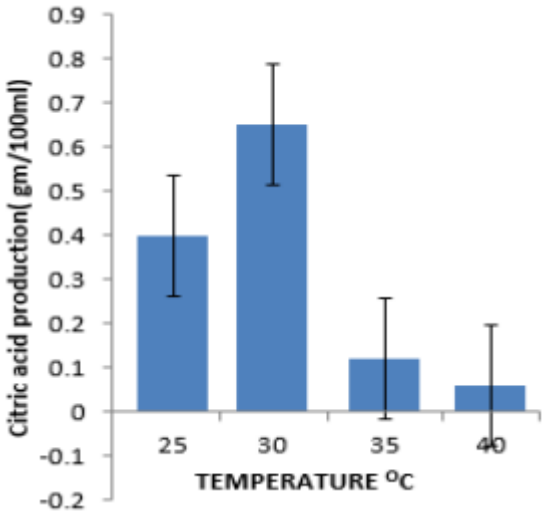

Fig 4: The synthesis of citric acid by the *A. niger* Kw isolate at a diverse range of temperatures

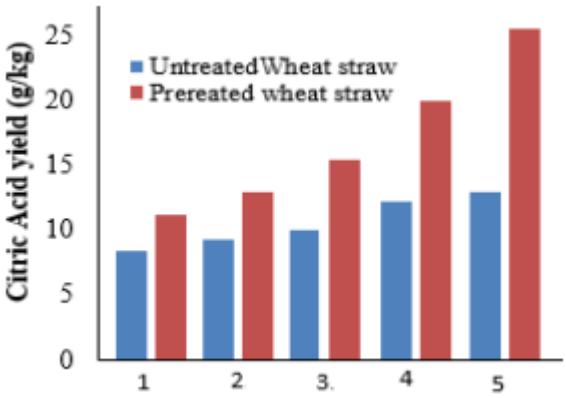

Fig 5: The use of wheat straw as a substrate for the synthesis of citric acid by the Kw isolate of A. niger

Utilising polymerase chain reaction (PCR) for the purpose of amplification of isolates obtained from various locations: Amplification and resolution of *A. niger* DNA were performed on agarose gel in order to identify the isolates with the anticipated band size of 500 base pairs. The amplicons that were recovered from each of the isolates were consistent with the band size that was anticipated.

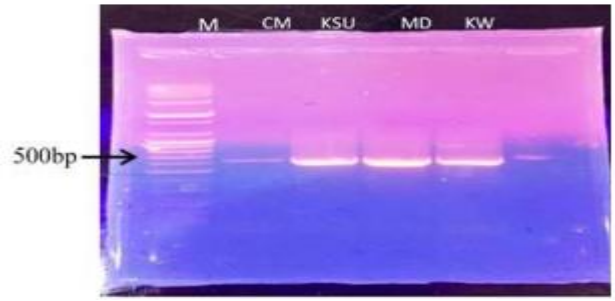

Fig 6: *Aspergillus niger* isolates from various locations were analysed by electrophoresis on an agarose gel after PCR amplification of the ITS region.

To learn more about isolate KW, which produced the most citric acid, we sequenced the PCR product from Kw (shown in Figure 6 above), and then we used that sequence to run a BLASTN search against the NCBI database, based on the sequence similarity of ITS-5.8s -ITS 2. The ITS 1-5.8s-ITS 2 gene of *Aspergillus niger* KW displays sequence similarity to several *A. niger* sequences in the NCBI database, as shown in Table 3. Various fungal isolates were identified using cultural and morphological traits from soil samples collected from cowpea and groundnut farms, spoilt fruits, and trash sites in the Parbhani metropolis. Through molecular characterisation, the 5.8SrRNA gene and two intergenic spacers (ITS1–5.8–ITS2) were amplified and sequenced, further confirming the identification of the KW isolate that generated significant quantities of citric acid. Previous work by Accensi *et al*. (1999) sequenced the DNA encoding the 5.8S gene of the ribosomal RNA and the two intergenic spacers ITS1 and ITS2 of the two type strains of *Aspergillus niger* that have been proposed for the aggregate. The study's nucleotide sequence of *Aspergillus niger* KW is similar to referenced sequences in the NCBI database with the following accession numbers: KX363462.1, KY962978.1, KY698415.1, KY657577.1, KP670427.1, KP748369.1, KT002562.1, KP329617.1, KP329655.1, and KT315445.1. The discovery of *A. niger* in the waste site is consistent with previous findings from other studies in India that have isolated this fungal species from various places (Obire *et al*., 2002; Williams and Hakam, 2016; Evangeline *et al*., 2017). After *Saccharomyces*, which was found in 42.8% of the dumpsite soil samples, Obire *et al*. (2002) found that the genus *Aspergillus* was present in 25.3% of the samples. *Aspergillus niger* isolates' natural substrate preferences showed that, at an optical density (OD540) of 16.0, wheat straw produced the maximum growth value (41%). The 540 nm wavelength that was utilised in this investigation has been previously documented by Dannaoui *et al*. (1999), Shiradiyi (2011), and Llop *et al*. (2000). The findings from the study by Prashant *et al*. corroborate the natural substrate preference (2003). Among the *Aspergillus niger* isolates tested, the one from Kupta watur produced the most citric acid (0.38g/100 ml). Based on their screening of 8 *Aspergillus* spp. cultures for citric acid synthesis, Ipsita and Baruah (2015) reported a little lower value of citric acid than what this study found. *Aspergillus niger* S-6 was the most productive of these cultures, yielding 0.33 g/100 ml of citric acid. In addition, Ipsita and Baruah (2015) also found that citric acid synthesis was most effective at a pH of 6.0.

Table 3: Fungal isolate identification using Aspergillus niger KW ITS sequence analysis

Isolate code	Morphological identification	Gene bank(BLAST) Result	E-value	Identity	Query cover	Accession
KW	*Aspergillus niger*	*Aspergillus niger* isolate 6029	0.0%	100%	100%	KX363462.1
		Aspergillus niger strain X-5	0.0%	100%	100%	KY962978.1
		Aspergillus niger strain UPMZ01	0.0%	100%	100%	KY698415.1
		Aspergillus niger strain SS4	0.0%	100%	100%	KY657577.1
		Aspergillus niger strain An0314M	0.0%	100%	100%	KP670427.1
		Aspergillus niger strain TA01-24	0.0%	100%	100%	KP748369.1
		Aspergillus niger isolate K7	0.0%	100%	99%	KT002562.1
		Aspergillus niger strain DTO:129-E9	0.0%	100%	100%	KP329617.1
		Aspergillus niger strain DTO:132-A5	0.0%	100%	100%	KP329655.1
		Aspergillus niger strain DTO 132-C7	0.0%	100%	100%	KP329662.1
		Aspergillus niger strain DTO:133-E8		100%		

Shetty (2015) Also, at pH 2.5, they measured a citric acid output of 10.4 mg/ml when they tested *A. niger* in a molasses-containing medium. Based on the results of the temperature optimisation process, it was shown that *A. niger* KW produced the highest output of citric acid (0.65g/100 ml) at 30°C. The results of this study are in agreement with those of Kareem *et al*. (2010), who found that 30°C was the sweet spot for citric acid synthesis. The impact of fermentation medium temperature on citric acid generation from solid-state fermentation of agricultural wastes was highlighted by Hang and Woodams (1986). The high temperature difference may be due to the substrate utilised, as it contradicts the findings of Helen *et al*. (2014), who found that *Aspergillus niger* produced the most citric acid when cultured on Parkia biglobosa fruit pulp at 55°C. Because it produced a high percentage of fungal growth in the substrate preference experiment, wheat straw was chosen as the natural substrate for citric acid synthesis in this investigation. Since wheat straw only contains around 4% sugar, we had to supplement it with 14% (w/w) sugarcane molasses to make up for it. This was in contrast to the 14-22% starting sugar concentration that Rohr *et al*., (1998) found to be ideal for commercial fermentations. Fatemi and Shojaosadati (1999) highlighted the significance of this variable on citric acid synthesis using *A. niger*, however the exact amount of sugar needed at the start might vary according on the species and/or strain. On the fifth day of fermentation, wheat straw treated with 5N HCl yielded 25.60g/kg, while untreated wheat straw yielded 13.00g/kg. Pretreatment is necessary to transform agro-industrial leftovers into a more metabolisable state, increasing the utilisation by microorganisms. This may explain why treated wheat straw produced more citric acid than untreated wheat straw (Khosravi *et al*., 2008). Khosravi *et al*., (2008) made a similar point when they compared the concentration of citric acid produced from pretreated and untreated wheat straw; they found that the concentration was higher when 5N HCl was applied to the straw. Our findings on the production of citric acid from wheat straw are in line with previous research that has explored the use of various agricultural byproducts as inexpensive and viable substrates for solid state fermentation of citric acid. These include, but are not limited to, pineapple waste, sugar beet cosset, kiwi fruit peel, African star apple peel, cassava bagasse, coffee husk, apple pomace, sugar beet pulp, etc.

Conclusion

Citric acid may be produced by microbial fermentation using agricultural wastes, according to this study, which is both sustainable and inexpensive. Sugarcane bagasse, rice husk, wheat bran, and fruit peels are examples of leftovers that can be used to save production costs and tackle the environmental problems caused by agricultural waste. The results show that standard refined substrate yields may be matched or exceeded by optimizing fermentation conditions, such as substrate preparation and nutrient supplementation, leading to citric acid yields. Sugarcane bagasse, when pre-treated and supplied with

appropriate nutrients, proved to be the most effective substrate among the agricultural leftovers that were evaluated. Citric acid manufacturing that incorporates agricultural waste valorization has environmental sustainability and economic viability built right in. This method is in line with worldwide initiatives to decrease industrial waste and increase resource efficiency, and it also reduces manufacturing costs. In addition, by recycling materials into useful bioproducts, it bolsters the circular economy. The environmental effect of commercial-scale manufacturing may be evaluated through life-cycle evaluations, process scaling, and improved pre-treatment technologies. Future study should concentrate on these areas. Promoting these initiatives will hasten the bioprocessing industry's transition to citric acid made from agricultural waste, which will aid in environmental preservation. Innovation in waste management and industrial bioproduction may be fostered by this effort, which sets the groundwork for biotechnological processes that are greener and cheaper.

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