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Isolation, Identification, and Screening of Citric Acid-Producing Fungi from Fermented Orange, Pawpaw, and Banana Peels

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ABSTRACT

The high demand for citric acid in diverse industries is increasing annually. Fruit peels which serve as wastes can be utilized as valuable substrates for cultivating fungi that can be used to produce citric acid through fermentation. The present study was conducted to isolate, identify, and screen citric acid-producing fungi from selected fruit peels. Orange, pawpaw, and banana peels were obtained from Ipata market, Ilorin, Kwara State, Nigeria. They were rinsed, dried, and pulverized. Submerged fermentation of the peels was carried out for 14 days to isolate naturally occurring fungi. The peels were fermented singly and combined as pawpaw and orange, pawpaw and banana and banana and orange. Isolation was performed using the pour plate method, identification was made using macroscopic and microscopic characteristics of the isolates, while screening for citric acid was done using Czapek-dox agar and bromocresol green as indicator. The highest fungal count was obtained from fermented pawpaw peels ($49.0 \pm 2.83 \times 10^3$ cfu/g), while banana peels had $43.00 \pm 0.71 \times 10^3$ cfu/g and orange peels $40.50 \pm 0.71 \times 10^3$ cfu/g. Fermented banana and orange peels had the highest fungal count of $38.00 \pm 1.41 \times 10^3$ cfu/g, while fermented pawpaw and orange peels had a fungal count of $28.00 \pm 1.41 \times 10^3$ cfu/g. A total of five fungi were isolated and identified as *Aspergillus niger, Fusarium* sp, *Aspergillus flavus, Mucor* sp, and *Penicillium* sp. Four of the fungal isolates screened positively with yellow halos surrounding the colonies except for *Fusarium* sp. which screened negatively. *Aspergillus niger* had the highest zone of yellow coloration with a diameter of 41.00 mm, *followed by Penicillium* sp. (33.00 mm), *Mucor* sp (29.00 mm), and *A. flavus* (0 mm). It can be concluded that orange, pawpaw, and banana peels are potential substrates for cultivating citric acid-producing fungi and *A. niger* screened best for citric acid production.

Keywords: Citric acid, Fruit peels, Submerged Fermentation, Fungal Isolates.

RESUME

La forte demande d'acide citrique dans diverses industries augmente chaque année. Les écorces de fruits qui servent de déchets peuvent être utilisées comme substrats précieux pour la culture de champignons pouvant être utilisés pour produire de l'acide citrique par fermentation. La présente étude a été menée pour isoler, identifier et sélectionner les champignons producteurs d'acide citrique à partir d'écorces de fruits sélectionnés. Des écorces d'orange, de papaye et de banane ont été obtenues sur le marché d'Ipata, Ilorin, État de Kwara, Nigeria. Elles ont été rincées, séchées et pulvérisées. La fermentation immergée des écorces a été réalisée pendant 14 jours pour isoler les champignons naturels. Les écorces ont été fermentées seules ou combinées sous forme de papaye et d'orange, de papaye et de banane et de banane et d'orange. L'isolement a été réalisé à l'aide de la méthode de la plaque coulée, l'identification a été effectuée à l'aide des caractéristiques macroscopiques et microscopiques des isolats, tandis que la recherche de l'acide citrique a été effectuée à l'aide de la gélose Czapek-dox et du vert de bromocrésol comme indicateur. Le nombre de champignons le plus élevé a été obtenu à partir des écorces de papayes fermentées ($49,0 \pm 2,83 \times 10^3$ ufc/g), tandis que les écorces de banane en présentaient $43,00 \pm 0,71 \times 10^3$ ufc/g et les écorces d'orange $40,50 \pm 0,71 \times 10^3$ ufc/g. Les écorces de banane et d'orange fermentées avaient le nombre fongique le plus élevé de $38,00 \pm 1,41 \times 10^3$ ufc/g, tandis que les écorces de papaye et d'orange fermentées avaient un nombre fongique de $28,00 \pm 1,41 \times 10^3$ ufc/g. Au total, cinq champignons ont été isolés et identifiés comme étant *Aspergillus n*iger, *Fusarium* sp, *Aspergillus f*lavus, *Mucor* sp et *Penicillium* sp. Quatre des isolats fongiques ont été dépistés positivement avec des halos jaunes entourant les colonies, à l'exception de *Fusarium* sp. qui a été dépisté négativement. *Aspergillus niger* avait la zone de coloration jaune la plus élevée avec un diamètre de 41,00 mm, suivi de *Penicillium* sp. (33,00 mm), *Mucor* sp (29,00 mm) et *A.*

flavus (0 mm). On peut conclure que les écorces d'orange, de papaye et de banane sont des substrats potentiels pour la culture de champignons producteurs d'acide citrique et que *A. niger* est le mieux sélectionné pour la production d'acide citrique.

Mots clés : Acide citrique, écorces de fruits, fermentation immergée, isolats fongiques.

1. INTRODUCTION

Citric acid is a metabolite of both plants and animals, it is known as 2-hydroxy-1,2,3-propane tricarboxylic acid ($C_6H_8O_7$). It is the most adaptable organic acid and is utilized by both the pharmaceutical business (12%) and the food industry (70%) (Ajala *et al.,* 2020; Behera *et al.,* 2021) in equal amounts. There are countless additional uses for it in various fields. Seven hundred and thirty-six thousand (736,000) tons of citric acid is reportedly produced annually worldwide (Hu *et al.,* 2019), fully through fermentation. Its utilization is steadily increasing (3.5–4%) annually, indicating the requirement for new substitutes to be discovered in its manufacture (Vasanthabharathi *et al.,* 2021). The chemical reaction to produce citric acid through this fermentation process can be summarized as follows:

$$C_6H_8O_7 \text{ (sucrose)} + 3O_2 \rightarrow 3CO_2 + 3H_2O + C_6H_8O_7 \text{ (citric acid)}$$

In this process, the fungus consumes sugar (usually sucrose) and oxygen and produces citric acid, carbon dioxide, and water as byproducts. During citric acid fermentation, the metabolic processes of the microorganism are orchestrated by a series of enzymatic reactions. These enzymes are responsible for converting substrates such as sugars into citric acid through metabolic pathways like glycolysis and the citric acid cycle. The activity of these enzymes directly influences the efficiency of citric acid production.

To produce citric acid, a variety of microorganisms have been used, including yeasts and bacteria like *Candida tropicalis* (Hesham *et al.,* 2020), *Arthrobacter paraffinens, Bacillus licheniformis,* and *Corynebacterium sp.*, as well as fungi and bacteria like *Aspergillus niger* (Dienye *et al.,* 2018), *Aspergillus aculeatus, Aspergillus carbonarius, Aspergillus awamori*. However, the bulk of them are unable to deliver economically viable yields because citric acid is a byproduct of energy metabolism and only accumulates in significant amounts under conditions of extreme imbalances such as specific genetic disorders or metabolic imbalances. *A. niger* produces more citric acid per unit of time than the other fungi mentioned therefore, it has remained the strain of choice for commercial production. The main advantages of using A. *niger* are its ease of handling, adaptability in the fermentation of inexpensive raw materials, and high yields. There aren't many industrial strains that produce commercial amounts of citric acid, and just a handful may be found in foreign culture collections (Behera, 2020).

Citric Acid is a multifunctional, non-toxic, ready to use and economical monomer used for pharmaceutical applications (Salihu *et al.*, 2021). This is a multipurpose natural monomer and plays a crucial role in the control of metabolism, mineralization, neuronal excitations, and renal stone prevention (Ma *et al.,* 2018; Chysirichoete, 2020). Agricultural residues are considered one of the most prominent substrates in renewable energy production and carbon source content due to their availability as a cheap renewable energy feedstock (Chilakamarry *et al.,* 2022; West, 2023). In recent times, the use of fruit waste to produce citric acid is increasingly becoming more attractive to researchers because it can reduce the cost of producing organic acid (Dutta *et al.,* 2019; Hussain, 2019). The process known as microbial fermentation uses microorganisms to break down complex organic compounds into smaller ones (Roukas and Kotzekidou, 2020). Vitamins, vital amino acids, antinutrients, proteins, food appearance, taste, and scent can all be improved naturally through fermentation. Fruit peels can be harnessed as a substrate for obtaining potential

producers of citric acid, hence leading to substantial citric acid production by naturally occurring microbes. This research aimed at isolating, identifying, and screening citric acid-producing fungi from orange, pawpaw, and banana peels.

2. METHODS

2.1. Collection and Processing of Samples

Orange, pawpaw, and banana fruits were bought from Ipata market, Ilorin, Kwara State. The fruits were washed and peeled with a clean knife. The peels were collected into sterilized plastic bags that had been washed and rinsed with 70% alcohol and were labeled properly. The peels were conveyed to the Microbiology Laboratory at Clenixx diagnostic laboratory where they were subjected to further processing. The fruit peels collected were dried in an oven at 40 ^{o}C for 2½ hrs and were later pulverized with an electric blender to increase the surface area for microorganism attachment.

2.2. Isolation of Naturally Occurring Fungi from Fermented Peels

Two hundred (200) grams of the grounded peels were measured into a clean plastic container and 500 ml of distilled water was added to the samples (orange, pawpaw, banana peels, 100 grams each of pawpaw and banana, pawpaw, and orange and banana and orange peels were combined respectively) and was left for natural fermentation at 28 ^{0}C for 3,7,10 and 14 days.

Plate 1: Banana, Orange, and Pawpaw Peels

For the enumeration of fungi, serial dilution of the fermented substrate was done at 3-14 days. One (1) ml of the sample was withdrawn from the 10^{-3} dilution tube and spread on duplicate potato dextrose agar (PDA) plates each. Incubation of all plates was carried out at 28^{0}C for 5 days after which visible colony growth was counted and further analysis was carried out. Pure cultures of isolates were obtained by repeated sub-culturing on PDA.

2.3. Characterization and Identification of Isolates

The fungal morphology was studied macroscopically by observing the colony features (color, shape, size, hyphae) and microscopically using a compound microscope with a digital camera at x40 magnification with a lactophenol cotton blue stained slide mounted with a small portion of the mycelium (Domsch *et al.,* 2020).

2.4. Screening of the Fungal Cultures

The Fungal cultures were screened qualitatively for citric acid production (Ajiboye and Sani, 2015). Sterile Czapek-dox agar medium incorporated with Bromo-cresol green dye was poured into individual sterile Petri dishes and allowed to cool at room temperature. A sterile cork borer of 5 mm was used to borehole at the center. One (1) ml of the spore suspension of the individual fungal isolate was transferred to each hole of the Petri plates. The plates were incubated at 28°C for 5 days. The fungal isolates with the yellow zone were measured in mm and recorded.

3. RESULTS

3.1. Total Fungal Count from Fermented Fruit Peels

The highest Fungal count was obtained on day 14 from fermented pawpaw peels with the value 49.00 ± 2.81 x 10^{-3} cfu/g while the lowest was obtained on day 3 with a count of 8.00 ± 1.41 x 10^{3} cfu/g. The highest Fungal count obtained from banana and orange peels was 43.00 ± 0.70 x 10^{3} cfu/g and 40.50 ± 0.70 x 10^{3} cfu/g on day 14 respectively as shown in table 1.

Table 1: Total Fungal Count Obtained from Selected Samples

Fruit Peel Samples	Fermentation (Days)	Total Fungal Count 10^{3} (cfu/g)
Pawpaw peels	3	8 .00 ± 1.41
	7	19.50± 2.12
	10	32 .00± 1.41
	14	49.00± 2.81
Banana peels	3	11 .00± 2.83
	7	16.00 ± 1.41
	10	21.00 ± 2.82
	14	43.00± 0.70
Orange peels	3	8.50 ± 3.54
	7	19.50 ± 4.94
	10	38.00± 1.41
	14	40.50 ± 0.70

3.2. Total Fungal Count from Combined Fermented Peels

The highest fungal count from combined pawpaw and banana peels was obtained on the 10^{th} day with the value 31.50 ± 14.14 x 10^{-3} cfu/g shown in Table 2 while the lowest was obtained from the sample site on the third day with a value of 8.50 ± 3.53x 10^{-3} cfu/g with three fungal isolates. From combined pawpaw and orange peels, the highest Fungal count, 28.00 ± 1.41 x 10^{-3} cfu/g was obtained on day 3 while the lowest fungal count of 15.50 ± 4.94 x 10^{-3} cfu/g was obtained on day 14 (Table 2). From combined banana and

orange peels, the highest Fungi count, 38.00 ± 1.41 x 10^{-3} cfu/g was obtained on day 10 while the lowest fungal count of 7.00 ± 1.41 x 10^{-3} cfu/g was obtained on day 3 (Table 2).

Table 2: Total Fungal Count Obtained from Fermented Fruit Peel Samples

Fruit Peel Samples	Fermentation (Days)	Total Fungal Count 10^3(cfu/g)
Pawpaw and Banana peels	3	8.50 ± 3.53
	7	20.50 ±3.53
	10	31.50 ± 14.14
	14	9.00 ±16.97
Pawpaw and Orange peels	3	28.00 ± 1.41
	7	19.50 ±4.94
	10	27.50 ± 2.82
	14	15.50 ±4.94
Banana and Orange peel	3	7.00 ± 1.41
	7	14.50 ±2.12
	10	38.00 ± 1.41

3.3. Characterization and Identification of the Isolates

The morphological properties of the isolates include the presence of sporangia, pale brown to white greenish spore colour, radiate conidia head, etc. as presented in Table 3.

Table 3: Identification of Fungal Isolates from Fruit Peel Samples

Fruit Peel Sample	Fungal Isolates	Colonial Appearance	Microscopic Characteristics	Probable Isolate
Pawpaw Peels	F1	White to grey and fast growing with some black sporangiospores	The sporangiophores have terminal sporangia containing round sporangiospores and columella was well developed with non-septate hyphae	*Mucor* sp
Banana Peels	F2	Colonies with loose white mycelium rapidly becoming dark brown to black on the development of conidia	The conidiospores are large with septate hyphae	*Aspergillus niger*
Orange Peels	F3	Yellowish green growth, with yellow spores	Powdery masses of yellowish-green spores on the upper surface and reddish-gold on the lower surface	*Aspergillus flavus*

Fruit Peel Sample	Fungal Isolates	Colonial Appearance	Microscopic Characteristics	Probable Isolate
Pawpaw and Banana Peels	F4	Green fluffy mycelia with some white sporangiospores	Septate hyphae with filamentous structure	*Penicillium* sp.
Pawpaw and Orange Peels	F5	Colorless, white-creamy with dark brown zonation	Cottony mycelium with pale brown to brown, brown-greenish to white-greenish aerial mycelium	*Fusarium* sp
Banana and Orange peels	F6	White to grey and fast growing with some black sporangiospores	The sporangiophores have terminal sporangia containing round sporangiospores and columella was well developed with non-septate hyphae	*Mucor* sp

F1 = *Mucor sp,* F2= *Aspergillus* sp, F3= *Aspergillus flavus,* F4= *Penicillium* sp, F5= *Fusarium* sp, F6= *Mucor* sp

3.4. Screening Reaction for Citric Acid Production from Pawpaw Peels Samples

Three of five isolates were positive in the production of citric acid, which is *Aspergillus niger*, *Mucor* spp, and *Penicillium* spp with diameters of 41.0, 29.0, and 33.0 mm, respectively, while *Fusarium* spp and *Aspergillus flavus* is negative in production of citric acid.

Table 4: Screening Reaction of Fungal Isolate for Citric Acid Production

Fungal Isolates	Citric Acid Screening Reaction	Zone of Yellow coloration (mm)
Mucor sp	Positive	29.00
Aspergillus niger	Positive	41.00
Aspergillus flavus	Negative	0.00
Penicillium sp	Positive	33.00
Fusarium sp	Negative	0.00

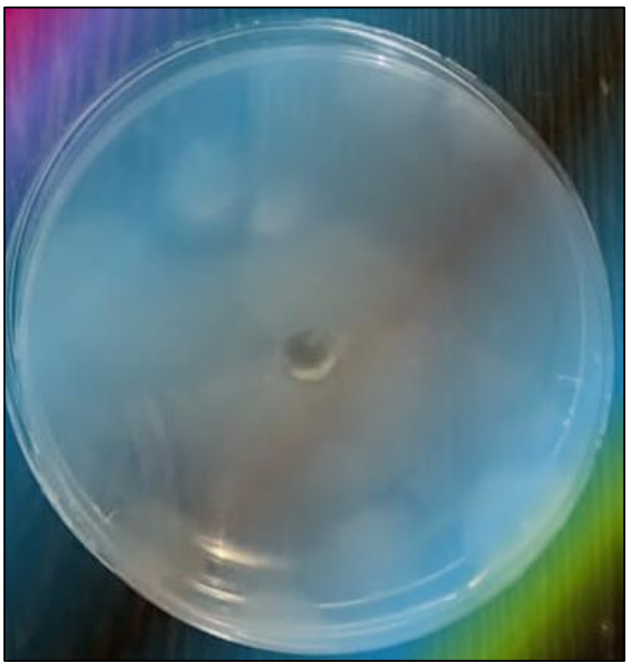

Plate 2: Negative for citric acid production-*Fusarium* sp

Plate 3: Positive for citric acid production- *Aspergillus* sp

4. DISCUSSION

The potential of using pawpaw, orange, and banana peels as a substrate for citric acid-producing fungi was investigated for 14 days. Five different fungi were isolated from the fruit peel samples and were differentiated based on colonial morphology which were *Mucor* sp, *Aspergillus niger, Aspergillus flavus, Penicillium* sp, and *Fusarium* sp. The successful isolation of these organisms from fermented fruit peels can be as result of the natural occurrence of these organisms in the environment including fruits and vegetables. The fermentation process could also provide favorable conditions for the growth of fungi such as *Mucor* sp, *Aspergillus niger,* and *Penicillium* sp which are known to thrive in fermentative environments where organic matter is present. These fungi genera isolated were similar to the report of Ajiboye and Said (2023), which mentioned the presence of similar organisms in fermented agricultural wastes.

The presence of a high fungal count in fermented pawpaw compared to orange and banana on day 14 could be due to several possible reasons which are the natural fungal composition in pawpaw may be more than orange and banana, environmental conditions in which the different fruits were grown, sugar contents in the fruits also the interactions between different microorganisms present during fermentation of the fruit peels can also impact fungal growth. This was in agreement with the report of Martgrita *et al*. (2022).

The ability of three of the isolates to produce citric acid as presented in Table 4 is attributable to the fact that fungi carry out enzymatic degradations leading to the release of diverse organic acids. *Mucor* sp, *Aspergillus niger,* and *Penicillium* sp generated a yellow zone of coloration typical of citric acid producers. Conversely, *Aspergillus flavus* and *Fusarium* sp were negative indicating their unsuitability with the substrate for citric acid production. *Mucor* sp, *Aspergillus niger,* and *Penicillium* sp have been reported for their ability to efficiently produce citric acid because of their metabolic pathways and enzymatic

capabilities to effectively convert substrates into citric acid. This was similar to the study of Behera *et al*. (2021).

5. CONCLUSION

This study highlights the potential of using indigenous microorganisms and banana, orange, and pawpaw peels as a substrate for citric acid production, contributing to the interest in converting waste materials into valuable products for various applications. This could help solve the problems of waste of fruits and waste management strategies by adding value to peels and converting waste to wealth. Hence, indirectly reducing the health hazards faced due to the haphazard dumping of waste and concurrently producing organic acids of valuable importance for food and pharmaceutical industries.

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CONFLICT OF INTEREST. No conflict of interest.

AUTHOR CONTRIBUTIONS. Ajiboye A.E. conceived the idea, supervised the research work, and edited the manuscript. Yusuf N.A. carried out the research, collected the data and analysed it. Adesokan T.E. wrote the manuscript.

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