Screening and Characterisation of Yeast Species for Citric Acid Production Using Glycerol and Agro-waste

Afolabi F. T.* **and Adeoye A. E.**
Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Nigeria.
* Corresponding author: folakeojo1@yahoo.com

Abstract: The present study was aimed to isolate and screen yeast species for the production of citric acid using glycerol and agro-waste. Fruit samples (orange, lime, lemon and pineapple) were collected from Bodija market in Ibadan. The samples were subjected to microbiological and physicochemical analyses. A total of 43 yeast isolates were recovered from the fruit and 17 isolates had the potential to produce citric acid after screening. Yeast isolates were identified as: *Candida tropicalis*, *Meyerozyma guilliermondii* and *Candida tropicalis*2. The best incubation period for citric acid production by *Candida tropicalis* PiB10 was 120 hours (18.02 g/l)**,** highest citric acid production (33.53 g/l) at pH 5.0, maximum citric acid production (20.84 g/l) was at 30°C, Glucose was the best carbon source, yielding 18.00 g/l of citric acid. Yeast extract was the best nitrogen source with citric acid production of 19.20 g/l and the highest production of 19.58 g/l at 200 rpm from glycerol. *Meyerozyma guilliermondii* LeB1 showed the highest citric acid production at 120 hours of incubation, yielding 17.02 g/l. The best pH was 5.5, yielding 35.90 g/l of citric acid. The best temperature was 30°C, with a production of 15.80 g/l, Glucose was also the preferred carbon source for this isolate, with a production of 20.13 g/l. Yeast extract was the best nitrogen source for *Pichia guilliermondii* LeB1, yielding 18.85 g/l. At 200 rpm from glycerol, the highest production was 22.37 g/l. This study demonstrated that *Candida tropicalis*, *Meyerozyma guilliermondii* and *Candida tropicalis*2 yielded high amount of citric acid using glycerol and agro-industrial wastes as substrates.
Key word: Citric acid**,** *Candida tropicalis*, Glycerol, *Meyerozyma guilliermondii*

INTRODUCTION

Citric acid, also known as a weak tribasic organic acid (2-hydroxy-propane-1,2,3-tricarboxylic acid), is an intermediary in the tricarboxylic acid cycle (TCA), a key metabolic pathway abundant in aerobic organisms and integral to microbial cellular metabolism (Adeoye and Lateef, 2022). Its status as a commodity chemical is substantial and it reflects its high demand across different industries. It is recognized by the Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives as generally regarded as safe (GRAS) and has widespread application in pharmaceutical and food sectors (Dutta *et al.*, 2019). Approximately 70% of citric acid production caters for the food industry, with 12% set aside for pharmaceutical use and the remaining 18% allocated across other sectors (Sato and Kawaguti, 2016). The complex utility of citric acid in food arises from its attributes which include flavour enhancement, non-toxicity, antioxidative properties, acidification, flavoring, preservation, and emulsification (Csutak and Sarbu, 2018).

Citric acid is produced universally by plants, animals, chemicals, and microorganisms. Microorganisms have significant contributions to citric acid production and also have industrial potential, ease of cultivation and handling, readily available and scalability (Behera, 2020). *Aspergillus niger*, is one of the industrial-scale producer of citric acid. Citrus fruits stand out for their elevated levels of citric acid. Lemons, oranges, limes, and grapefruits are mostly abundant sources and natural reservoirs of citric acid (Tahjib-Ul-Arif *et al.*, 2021).

Citric acid is one of the organic acids manufactured in large quantities via microbial processes. Fermentation contributes over 90% to global citric acid production (Cavallo *et al.*, 2017). Authors have reported the fact that citric acid can be chemically and synthetically produced from acetone or glycerol (Haq *et al.*, 2004), microbial fermentation has gained significant attention owing to its suitability for large-scale production. Among the array of microorganisms capable of citric acid

production, fungi are prominently employed in commercial production of the organic acid (Kareem and Rahman, 2011).

The various biodegradable organic waste materials abundantly generated in food, beverage, milk, and sugar processing industries, including; sugarcane bagasse, grape, apple and pineapple pomace, rice bran, coconut husk, banana peels, citrus peels, whey, and decaying fruits, are viable fodder and feedstock for biogas production (Hamdy, 2013; Sawant *et al.,* 2018). These nutrient-rich and cost-effective wastes are utilized as substrates by microorganisms to produce citric acid as a value-added product (Afolabi and Akanbi, 2022).

The environmental impact associated with chemically produced citric acid is a major concern. Chemical synthesis methods besides being expensive involve harsh chemical, high energy consumption and emission of pollutants. It is therefore necessary to explore more sustainable production methods such as biological fermentation by yeast using cheap substrate like glycerol and agro-industrial waste. This study was designed to isolate and screen yeast species for the production of citric acid using glycerol and agro-waste.

MATERIALS AND METHODS

***Sample collection*:** The samples, comprising orange, lime, lemon, and pineapple fruits were collected from Bodija market in Ibadan, Nigeria. The agro-industrial waste (cassava peel, rice bran) and glycerol were collected from agro-allied industry also within Ibadan. The samples were carefully placed in sterile polythene bags to prevent contamination during transportation. Upon arrival at the Microbiology laboratory of the University of Ibadan, the samples were processed immediately under sterile conditions to maintain their microbiological quality.

***Yeast isolation*:** One gram of the sample was mixed with 9mls sterile distilled water and subjected to serial dilution, resulting in dilutions up to 10^{-5}. Portions of the diluted samples were then plated onto Yeast Extract Agar (LAB M) supplemented with glycerol and streptomycin to inhibit bacterial growth. These plates were then incubated at 25°C for 48-72 hours to allow yeast colonies to develop (Iris *et al.,* 2020).

Screening of citric acid producing yeast: Pure cultures of yeast isolate obtained were initially screened qualitatively to determine the presumptive positive citric acid producing yeast. The presumptive positive isolates were then screened quantitatively to investigate citric acid production titre by each of the isolates (Finogenova *et al.,* 2008; Shaikh and Qureshi, 2013)

Qualitative screening of citric acid producing yeast using acid indicator medium (AIM) containing bromocresol purple: All yeast isolates were primarily screened for organic acid production by determining the acid unitage (AU) values using a basal medium containing (g/l): $(NH_4)_2SO_4$ 1.0; KH_2PO_4 1.0; K_2HPO_4. $3H_2O$ 0.16; $MgSO_4 \cdot 7H_2O$ 0.70; NaCl 0.50; $Ca(NO_3)$. $4H_2O$ 0.40; bromocresol green 0.40; glucose 20.0; agar 20.0 and 1 litre of distilled water (Finogenova *et al.,* 2008). A loopful of yeast colony was inoculated on agar plates and incubated at 25˚C for 48 h. Formation of a yellow halo around the colonies indicated the ability of isolates to produce organic acid. (Shaikh and Qureshi, 2013).

Quantitative screening of citric acid producing yeast using titrimetric method: The isolates showing the highest levels of acid production were screened again for citric acid production using submerged cultures (Karasu-Yalcin *et al.,* 2010). The inoculum was prepared by incubation of the isolates at 25°C, 150 rpm for 48 hours in a Yeast Extract Agar medium. Fermentation Medium and Conditions Fermentation were carried out in the laboratory in 250 ml Erlenmeyer flasks as small-scale laboratory fermentor containing 100 ml of fermentation medium in each flask. The production medium used contained (g/l): Glucose 30, $(NH_4)_2SO_4$ 0.5, yeast extract 0.5, KH_2PO_4 7, Na_2HPO_4 2.5, $MgSO_4.7H_2O$ 1.5, $CaCl_2$ 0.15, $FeCl_3.6H_2O$ 0.15, $ZnSO_4.7H_2O$ 0.02,

$MnSO_4.H_2O$ 0.06 (Papa Nikolaou *et al.,* 2002). The isolates were cultivated at 30˚C in a rotary shaker at 180 rpm speed for 7days. After incubation, the fermentation medium was centrifuged at 4000rpm for 15minutes.

Optimisation of fermentation medium

***Effect of incubation period on citric acid production*:** The effect of incubation days on citric acid production was studied over range of 24 hours to 168 hours. The production medium containing (g/l): Glucose 30, $(NH_4)_2SO_4$ 0.5, yeast extract 0.5, KH_2PO_4 7, Na_2HPO_4 2.5, $MgSO_4.7H_2O$ 1.5, $CaCl_2$ 0.15, $FeCl_3.6H_2O$ 0.15, $ZnSO_4.7H_2O$ 0.02, $MnSO_4.H_2O$ 0.06 was sterilized and inoculated with pure culture of the yeast isolate. The best incubation period was determined after citric acid quantitative assay using titrimetric method (Mahmoud *et al.,* 2015).

***Effect of temperature on citric acid production*:** The production medium containing (g/l): Glucose 30, $(NH_4)_2SO_4$ 0.5, yeast extract 0.5, KH_2PO_4 7, Na_2HPO_4 2.5, $MgSO_4.7H_2O$ 1.5, $CaCl_2$ 0.15, $FeCl_3.6H_2O$ 0.15, $ZnSO_4.7H_2O$ 0.02, $MnSO_4.H_2O$ 0.06 was sterilized and inoculated with pure culture of the yeast isolate. The temperature ranges selected for incubation were; 25°C, 30°C, 35°C, 40°C; and the fermentative media were allowed to produce citric acid using these temperature ranges. The best temperature for production was determined after citric acid quantitative assay (Afolabi and Akanbi, 2022).

***Effect of pH on citric acid production*:** The production medium containing (g/l): Glucose 30, $(NH_4)_2SO_4$ 0.5, yeast extract 0.5, KH_2PO_4 7, Na_2HPO_4 2.5, $MgSO_4.7H_2O$ 1.5, $CaCl_2$ 0.15, $FeCl_3.6H_2O$ 0.15, $ZnSO_4.7H_2O$ 0.02, $MnSO_4.H_2O$ 0.06 The pH of the fermentation medium was varied using citrate - phosphate buffer. pH; 4.5, 5.0, 5.5, 6.0, 6.5 and 7 were the pH range selected for citric acid production by the yeast isolates. The best pH was determined after citric acid quantitative assay using titrimetrically as previously described (Afolabi and Akanbi, 2022).

***Effect of different carbon sources on citric acid production*:** Different carbon sources were varied during optimization and assayed for citric acid production. The selected carbon sources were based on the sugars fermented by the yeast isolates. The carbon sources included; glucose, sucrose, mannitol, fructose and glycerol. The citric acid produced was determined titrimetrically as previously described (Afolabi and Akanbi, 2022).

***Effect of different nitrogen sources on citric acid production*:** Different nitrogen sources were varied during optimization and assayed for citric acid production. The selected nitrogen sources were based on the nitrogen source fermented by the yeast isolates. The nitrogen sources included; Yeast extract, Sodium nitrate, Peptone, Ammonium sulphate and Urea. After 7 days, the citric acid produced was determined titrimetrically as previously described (Afolabi and Akanbi, 2022).

***Effect of agitation on citric acid production*:** The effect of agitation on the citric acid production was determined by incubating a set up containing the fermentation medium and the yeasts at different rpm (100, 120, 140, 160, 180 and 200rpm). Thereafter, the yield of citric acid was determined by titrimetric method as previously described (Afolabi *et al.*, 2018)

***Statistical analysis*:** The data obtained were analysed using Duncan's Multiple Range Test (DMRT) with a statistical significance in difference set when $p< 0.05$.

RESULTS

Forty-three (43) yeast isolates were screened qualitatively and quantitatively for citric acid production. Out of the 43 yeast isolates, 17 were positive for citric acid production. The 17 presumptive isolates were further screened quantitatively to determine the amount of citric acid produced. When compared to isolate PiB2, which had production of 3.74±0.07 (g/l), isolate LeB1: had highest production of 11.10±0.07 (g/l) as shown in Table 1 and Table 2. Isolates PiB10, LeB1, OrB6, LiB1 and LiB2 were selected for further studies. Table 3 shows the molecular

identity of the selected yeast isolates. The yeast isolates were identified as: *Candida tropicalis* and *Meyerozyma guilliermondii*

The effect of incubation period showed that. *Meyerozyma guilliermondii* LeB1 had highest production of citric acid of 17.02 g/l at 120 hours. After which there was a gradual decline to 168 hours with production of 11.73 g/l. It was observed for *Candida tropicalis* PiB10 that the highest production was 120hours with the production of 18.02 g/l. as shown in Figure 1.

The effect of pH on citric acid production is shown in Figure 2. *Meyerozyma guilliermondii* LeB1 produced the highest citric acid at pH 5.5 (35.90 g/l), *Candida tropicalis* PiB10 had the highest production at pH 5.0 (33.53 g/l). For all the yeast isolates, there was gradual increase in the production of citric acid from pH 4.5 - 5.5 and started declining at pH 6 till 7. pH had profound and significant effect on citric acid production by yeast species.

The effect of different temperature on citric acid production revealed that *Meyerozyma guilliermondii* LeB1 produced the highest citric acid at 30°C (15.80 g/l) and *Candida tropicalis* PiB10 had the highest production at 30°C (20.84 g/l). The lowest production of citric acid for the five selected yeast isolated was observed at 40°C. There was an increase in production of citric acid from 25 to 30°C. There was gradual increase in citric acid production at temperature of 25°C while at 30°C citric acid production was higher by most of the yeast isolates while at 35°C to 40°C the production decline (Figure 3).

The effect of different carbon sources was examined to determine the most preferred carbon source. As shown in figure 4, *Meyerozyma guilliermondii* LeB1, *Candida tropicalis*, *Candida tropicalis* 2, *Pichia* sp. and *Candida tropicalis* PiB10 were able to utilize glucose as their carbon source with the value (20.13 g/l, 15.53 g/l, 20.65 g/l, 16.49 g/l and 18.00 g/l respectively). The second most utilized carbon source was glycerol with citric acid production of (13.37 g/l, 9.21 g/l, 14.10 g/l, 13.20 g/l and 15.31 g/l respectively) while fructose had the lowest production (4.45g/l)

Figure 5 showed the effect of different nitrogen sources on citric acid production by the selected isolates. *Meyerozyma guilliermondii* LeB1, *Candida tropicalis*, *Candida tropicalis* 2, *Pichia* sp. and *Candida tropicalis* PiB10 all preferred yeast extract as the best nitrogen source with the production of (18.85 g/l, 18.00 g/l, 19.17 g/l, 19.26 g/l and 19.20 g/l respectively). The second most preferred nitrogen source was ammonium sulphate with citric acid production of (10.56 g/l, 8.74 g/l, 10.19 g/l, 8.08 g/l and 9.92 g/l respectively) while urea had the lowest production (3.23g/l).

The citric acid production increases with increase in agitation from static, 100 rpm, 150 rpm and 200 rpm. Glycerol was the substrate utilized for the highest production of citric acid, followed by rice bran and cassava peel had the lowest production of citric acid as shown in Figure 6a.

Candida tropicalis PiB10 had an increase in production of citric acid as the agitation increases with the highest production of 19.58 g/l at 200 rpm from glycerol. This was followed with 18.19 g/l, observed at 200 rpm from rice bran while the lowest was 6.69 g/l from cassava peel (static). *Meyerozyma guilliermondii* LeB1 had an increase in citric acid production as the agitation increases with the highest production of 22.37 g/l at 200 rpm from glycerol. The result is presented in Figure 6b.

Table 1: Qualitative screening for citric acid production by yeast isolates on acid indicator medium (AIM)

Isolate code	Source	Zone of clearance (mm)
OrB1	Orange	52.1±0.23
OrB2	Orange	21.2±0.17
OrB6	Orange	56.0±0.11
LiB1	Lime	50.1±0.15
LiB2	Lime	62.1±0.10
LiB6	Lime	44.0±0.05
LeB1	Lemon	52.1±0.15
LeB3	Lemon	25.1±0.11
LeB4	Lemon	46.1±0.10
LeB5	Lemon	45.0±0.05
PiB1	Pineapple	50.1±0.15
PiB2	Pineapple	71.0±0.05
PiB3	Pineapple	62.0±0.05
PiB5	Pineapple	57.0±0.05
PiB6	Pineapple	42.1±0.15
PiB9	Pineapple	60.1±0.10
PiB10	Pineapple	52.0±0.05

*****Values are means of three replicates with the Standard Error indicate

Table 2: Quantitative screening for citric acid production in submerged fermentation medium

Isolate code	Citric acid produced (g/l)
OrB1	8.51±0.12
OrB2	2.94±0.06
OrB6	10.40±0.16
LiB1	10.47±0.12
LiB2	10.02±0.03
LiB6	4.77±0.09
LeB1	11.10±0.41
LeB3	3.33±0.06
LeB4	9.34±0.06
LeB5	8.13±0.19
PiB1	1.76±0.03
PiB2	3.74±0.09
PiB3	10.08±0.16
PiB5	2.59±0.03
PiB6	8.26±0.06
PiB9	8.38±0.64
PiB10	10.67±0.12

Table 3: NCBI Blast of the sequence identity of the yeast isolate

Sample ID	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Identity	Accession
PiB10	*Candida tropicalis*	893	893	99%	0	99.79%	PP751510
LeB1	*Meyerozyma guilliermondii*	893	893	99%	0	99.19%	PP751511

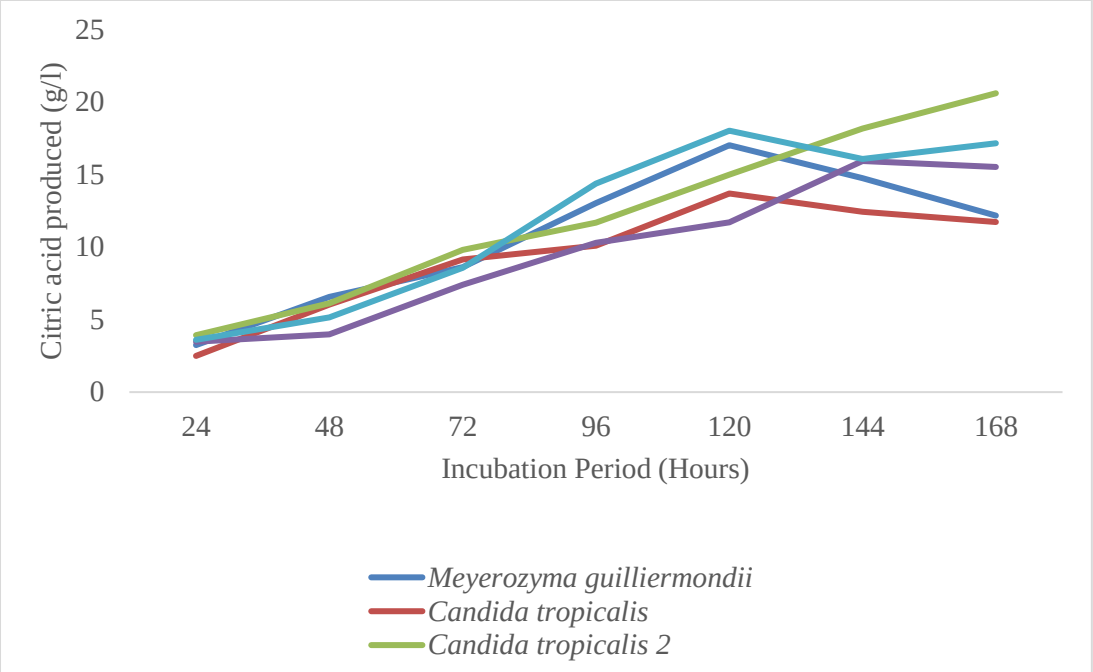

Figure 1: Effect of incubation period on citric acid production by the selected yeast isolates obtained from different fermented fruits

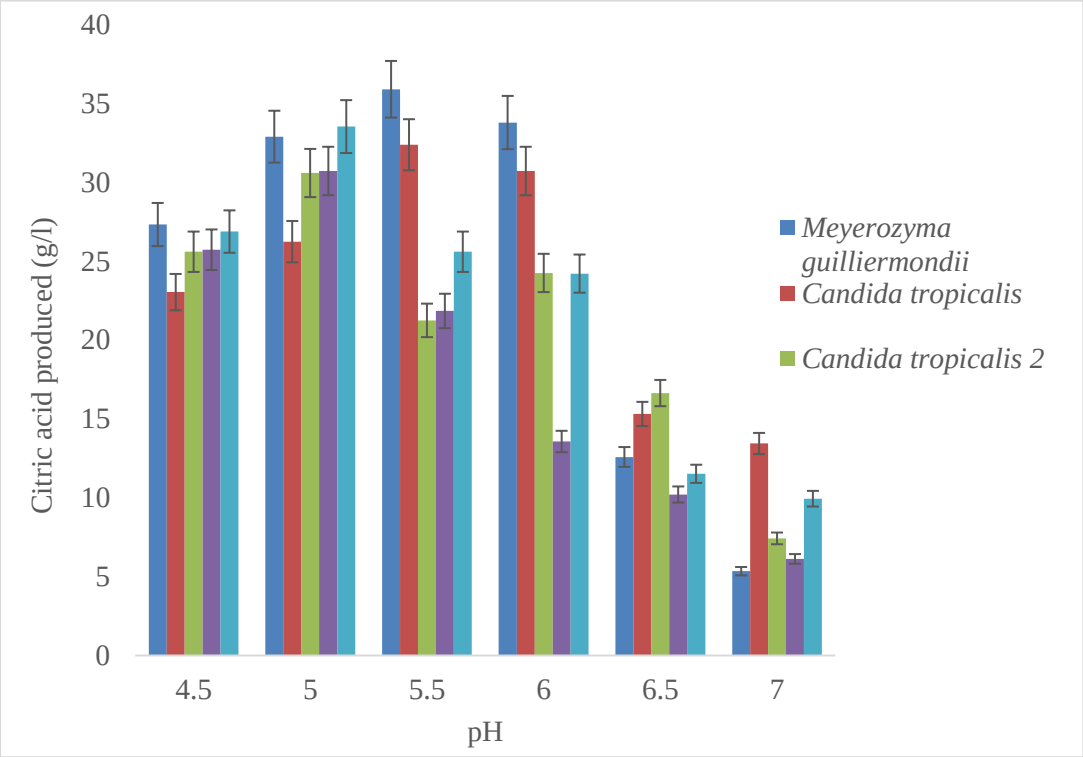

Figure 2: Effect of pH on citric acid production by the selected yeast isolates obtained from different fermented fruits

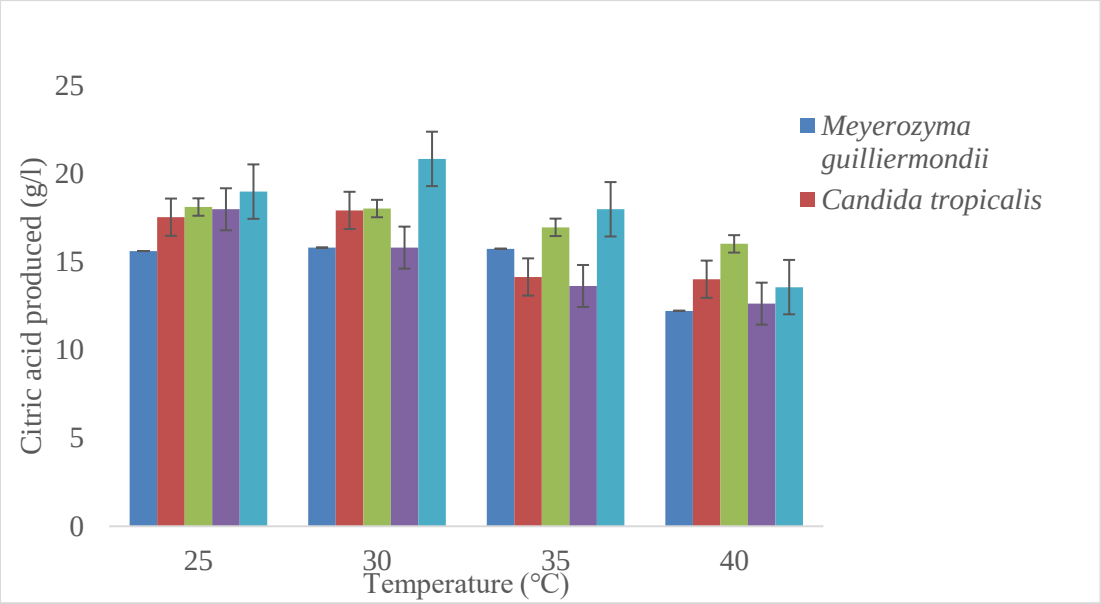

Figure 3: Effect of temperature on citric acid production by the selected yeast isolates obtained from different fermented fruits

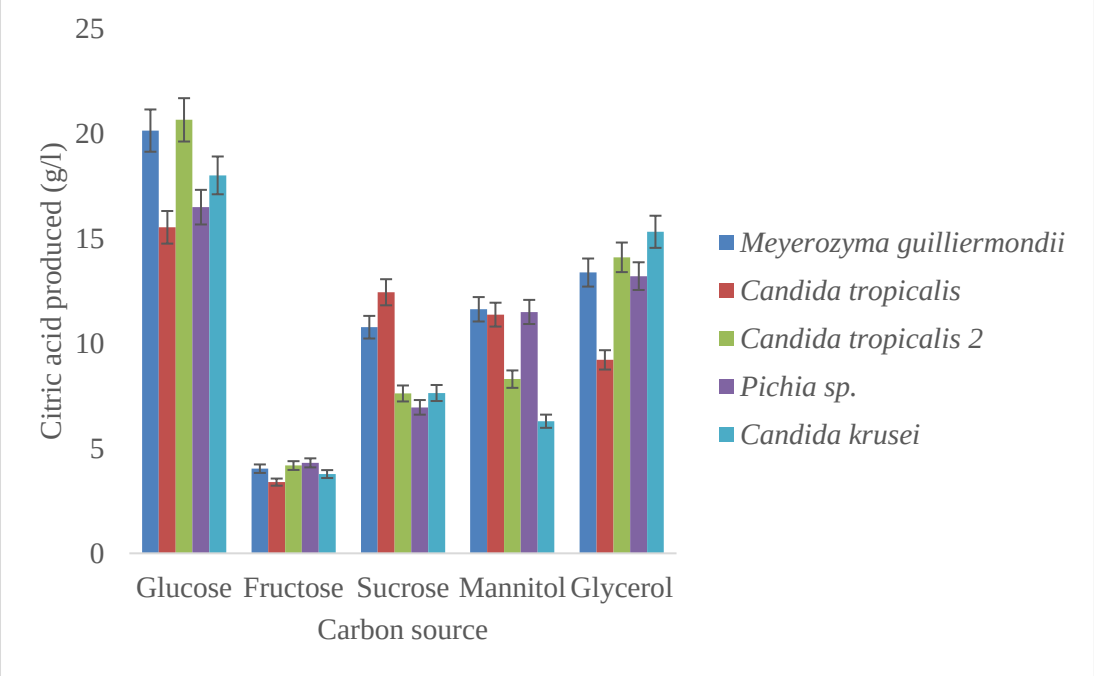

Figure 4: Effect of different carbon sources on citric acid production by the selected yeast isolates obtained from different fermented fruits.

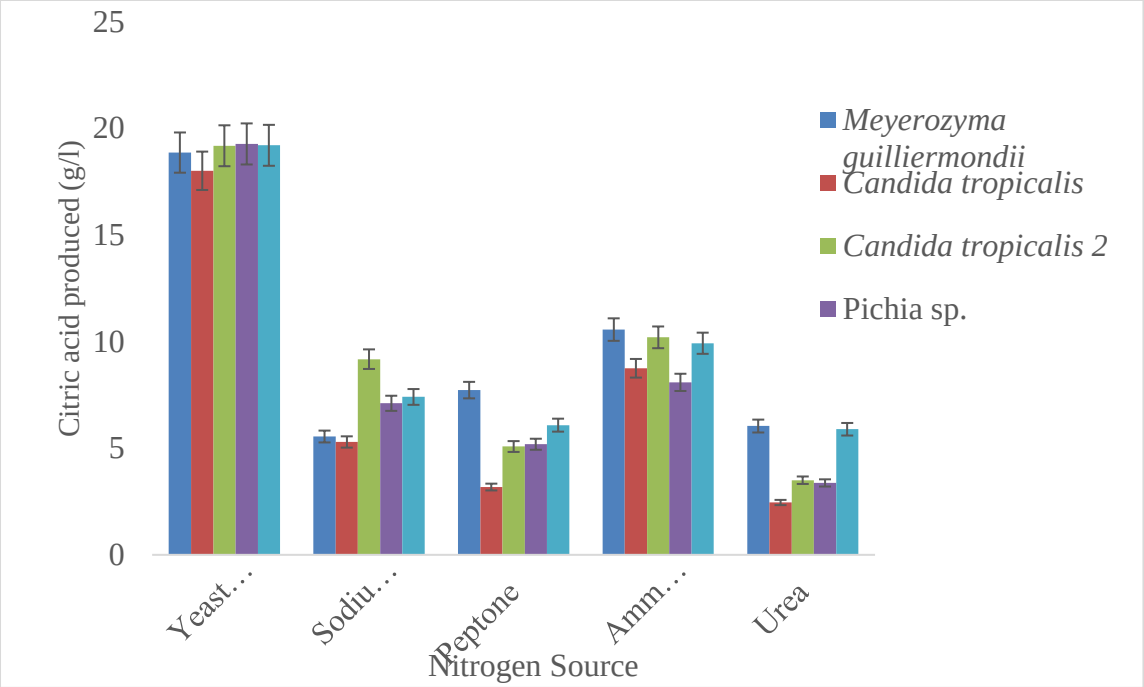

Figure 5: Effect of different Nitrogen sources on citric acid production by the selected yeast isolates obtained from different fermented fruits.

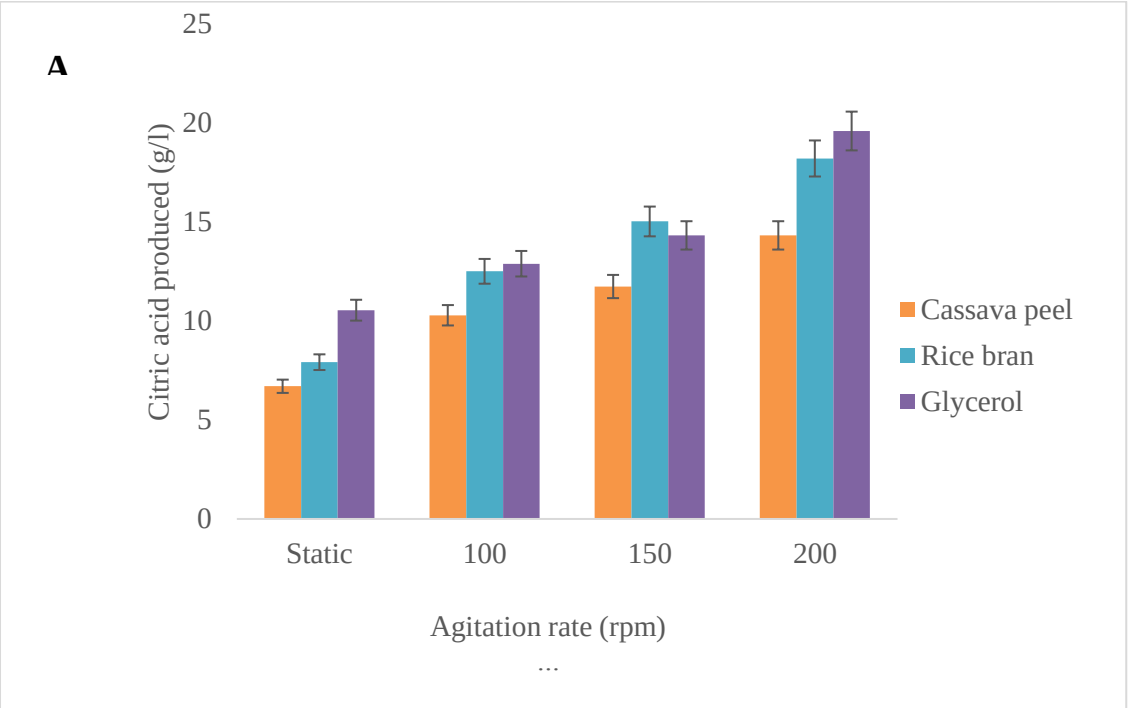

Figure 6a: Effect of agitation rate on citric acid production on cassava peel, rice bran and glycerol using *Candida tropicalis* PiB10.

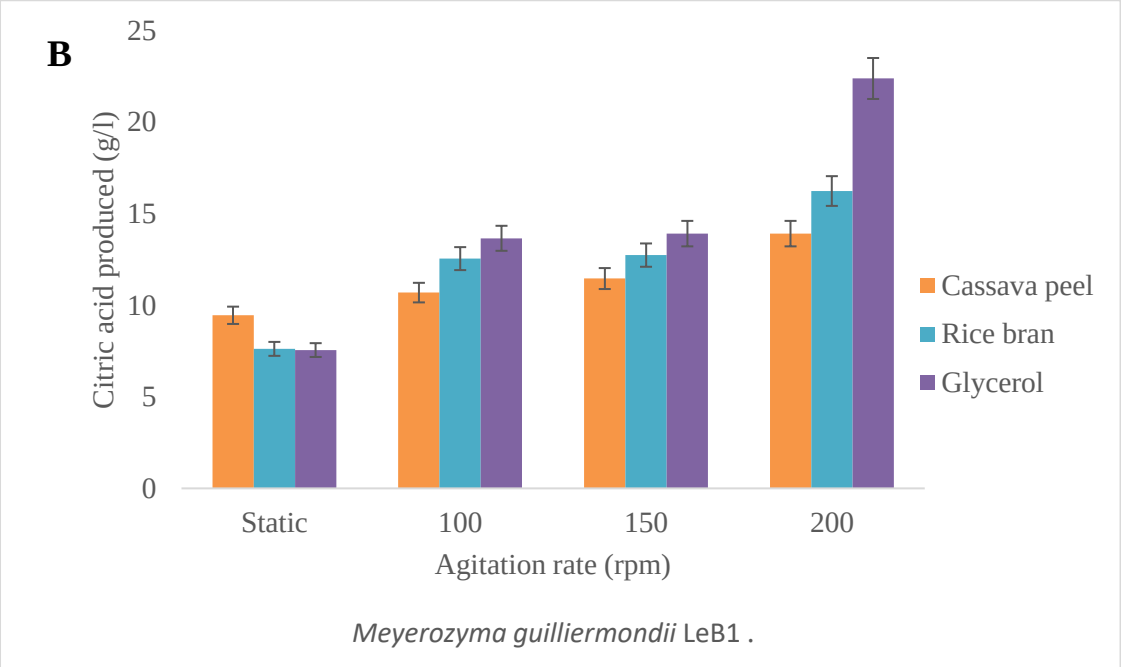

Figure 6b: Effect of agitation rate on citric acid production on cassava peel, rice bran and glycerol using *Meyerozyma guilliermondii* LeB1.

DISCUSSION

A total of 43 yeast isolates were obtained from the fruit samples (orange, pineapple, lime and lemon) and 17 of the isolates had the potential to produce citric acid after screening. The yeast isolates were identified as: *Candida tropicalis*, *Meyerozyma guilliermondii* and *Candida tropicalis*2. The isolate LeB1 exhibited the highest citric acid production of 11.10 ± 0.07 g/l, significantly higher than the reference isolate PiB2 (3.74 ± 0.07 g/l). A similar finding was also recorded by (Emeka *et al.*, 2012). The ability of these yeast isolates to produce citric acid is not surprising, as many yeast species are known for their capability to synthesize organic acids, including citric acid, under appropriate conditions (Rymowicz *et al.*, 2009; Kareem *et al.*, 2010; Kołożyn-Krajewska and Dolatowski, 2012; Yadav *et al.*, 2014).

The incubation period plays a vital role in citric acid production, as it influences the growth and metabolic activity of the microorganism. This research showed that the optimal incubation period varied among the selected yeast isolates. *Meyerozyma guilliermondii* LeB1 exhibited the highest citric acid production of 17.02 g/l at 120 hours, while *Candida tropicalis* PiB10 reached its maximum production of 20.60 g/l at 168 hours. These findings are consistent with previous studies that have reported optimal incubation periods ranging from 96 to 192 hours (4 to 8 days) for citric acid production by various yeast strains (Kareem *et al.*, 2010; Ali *et al.*, 2020). The variation in the optimal incubation period among the isolates could be attributed to differences in their growth rates, metabolic activities, and nutrient utilization patterns. It is noteworthy that after reaching the maximum production, a gradual decline in citric acid levels was observed with further incubation. This decline could be due to the depletion of nutrients, accumulation of inhibitory byproducts, or the degradation of citric acid by the yeast cells themselves.

In this study, the optimal pH for citric acid production varied among the selected yeast isolates, with most isolates exhibiting maximum production at pH 5.0 or 5.5. Specifically, *Meyerozyma guilliermondii* LeB1 produced the highest citric acid level of 35.90 g/l at pH 5.5, while *Candida tropicalis* PiB10 exhibited maximum

production at pH 5.0 (33.53 g/l). These findings were in agreement with previous reports that have identified the optimal pH range for citric acid production by yeasts to be between 4.5 and 6.0 (Kareem *et al.*, 2010; Ali *et al.*, 2020). The acidic pH range is generally favored for citric acid production as it enhances the activity of the enzymes involved in the citric acid cycle and inhibits the activity of enzymes involved in the tricarboxylic acid cycle, thereby promoting the accumulation of citric acid (Yadav *et al.*, 2014). It was noted that the optimal pH varied among different yeast strains due to their inherent physiological characteristics and metabolic pathways (Kołożyn-Krajewska and Dolatowski, 2012).

The effect of temperature on citric acid production showed that optimal temperature for citric acid production varied among the selected isolates, with the yeast isolates exhibiting maximum production at either 25°C or 30°C. *Meyerozyma guilliermondii* LeB1 and *Candida tropicalis* PiB10 produced the highest citric acid levels at 30°C (15.80 g/l and 20.84 g/l, respectively), These findings were consistent with previous studies that have reported optimal temperatures ranging from 25°C to 30°C for citric acid production by various yeast strains (Kareem *et al.*, 2010; Kołożyn-Krajewska and Dolatowski, 2012; Yadav *et al.*, 2014). This temperature range is suitable for the growth and metabolic activities of mesophilic yeasts, which are the predominant citric acid producers. It was observed that at higher temperatures (35°C and 40°C), there was a decrease in citric acid production by the selected isolates. This reduction in production could be attributed to the thermal stress experienced by the yeast cells, leading to impaired growth and metabolic activities, as well as the possible denaturation of enzymes involved in the citric acid biosynthetic pathway.

The choice of carbon source is a crucial factor in citric acid fermentation, as it serves as the primary energy source for the growth and metabolic activities of the microorganisms, as well as the precursor for the biosynthesis of citric acid. All the selected yeast isolates preferred glucose as the best carbon source, with citric acid production ranging from 15.53 g/l to 20.65 g/l. These findings are consistent with previous studies that have reported glucose as the preferred carbon source for citric acid production by various yeast strains. Glucose is a readily metabolizable sugar and serves as the primary carbon and energy source for many microorganisms, including yeasts (Show *et al.*, 2015).

The ability of the selected isolates to utilize glycerol as a carbon source for citric acid production is noteworthy, as glycerol is a by-product of biodiesel production and has gained attention as a potential substrate for microbial fermentation processes. The use of glycerol as a carbon source can contribute to the valorization of this by-product and promote sustainable bioprocessing (Papanikolaou *et al.*, 2002). The relatively lower citric acid production observed with fructose, sucrose, and mannitol as carbon sources could be attributed to the metabolic preferences and capabilities of the selected yeast isolates, as well as the potential inhibitory effects of these substrates on the citric acid biosynthetic pathway as reported in *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol (Papanikolaou *et al.*, 2002).

All the selected yeast isolates preferred yeast extract as the best nitrogen source, with citric acid production of 18.00 g/l (*Candida tropicalis*). Ammonium sulfate was the second most preferred nitrogen source of 10.56 g/l (*Meyerozyma guilliermondii* LeB1).This was in agreement with previous studies that have reported yeast extract and ammonium salts as suitable nitrogen sources for citric acid production by various yeast strains. Yeast extract is a complex nitrogen source that provides essential nutrients, vitamins, and growth factors, which can support the growth and metabolic activities of the yeast cells (Hamdy, 2013). Ammonium salts, such as ammonium sulfate, are inorganic nitrogen sources that can be readily assimilated by yeasts and

serve as the primary nitrogen source for citric acid biosynthesis. The relatively lower citric acid production observed with other nitrogen sources, such as urea and sodium nitrate, could be due to the metabolic preferences and capabilities of the selected yeast isolates, as well as the potential inhibitory effects of these compounds on the citric acid biosynthetic pathway.

The effects of agitation showed that the citric acid production increased with increasing agitation rates, with the highest production observed at 200 rpm for all the selected isolates and carbon sources. When glycerol was used as the substrate, *Meyerozyma guilliermondii* LeB1 exhibited the highest citric acid production of 22.37 g/l at 200 rpm, followed by *Candida tropicalis*2 (21.33 g/l) and *Candida tropicalis* PiB10 (19.58 g/l). With rice bran as the carbon source, the highest citric acid production was observed for *Candida tropicalis* PiB10 (18.19 g/l) at 200 rpm, followed by *Meyerozyma guilliermondii* LeB1 (16.23 g/l) and *Candida tropicalis* (15.21 g/l). When cassava peel hydrolysate was used, the citric acid production was relatively lower, with the highest levels observed under static conditions (6.69 g/l for *Candida tropicalis* PiB10, 7.77 g/l for *Meyerozyma guilliermondii* LeB1, and 6.33 g/L for *Candida tropicalis*). The positive effect of agitation on citric acid production can be attributed to several factors. Agitation improves the distribution of nutrients, oxygen transfer, and removal of metabolic by-products, which can enhance the growth and metabolic activities of the yeast cells. The higher citric acid production observed with glycerol as the carbon source could be due to the ability of the selected yeast isolates to efficiently metabolize this substrate and channel the carbon flux towards the citric acid biosynthetic pathway. Several studies have reported the successful utilization of glycerol for citric acid production by various yeast strains, including *Candida tropicalis* and *Yarrowia lipolytica* (Mandenius and Brundin 2008; Ali *et al.*, 2020).

The use of agro-industrial wastes, such as rice bran and cassava peel, as alternative carbon sources for citric acid production is an attractive approach from an economic and environmental perspective. These materials are abundant, renewable, and cost-effective, and their valorization through microbial fermentation processes can contribute to sustainable bioprocessing and waste management. The relatively lower citric acid production observed with cassava peel hydrolysate could be due to the presence of inhibitory compounds or the lack of essential nutrients in the hydrolysate. Pretreatment methods and supplementation with additional nutrients may be required to enhance the citric acid yield from cassava peel hydrolysate (Kareem *et al.,* 2010; Yadav *et al.*, 2014; Prado *et al.*, 2016).

CONCLUSION

This study has demonstrated that the selected yeasts namely: *Candida tropicalis* PiB10, *Meyerozyma guilliermondii* LeB1 and *Candida tropicalis*2 yielded high amount of citric acid using glycerol and agro-industrial wastes as substrates. The optimal incubation period for maximum citric acid production varied among the yeast isolates, ranging from 120 to 168 hours. The optimal pH was found to be in the moderately acidic range of 5.0 to 5.5, while the optimal temperature was between 25°C and 30°C. Among the carbon sources evaluated, glucose and glycerol were the preferred substrates for citric acid production by the yeast isolates. Yeast extract was identified as the best nitrogen source for citric acid production, followed by ammonium sulfate. Agitation rate was found to have a positive effect on citric acid yield, with the highest production levels observed at 200 rpm for all the selected yeast isolates. This study demonstrated that *Candida tropicalis, Meyerozyma guilliermondii* and *Candida tropicalis*2 yielded high amount of citric acid using glycerol and agro-industrial wastes as substrates and this represents an attractive approach in agro-waste economy.

REFERENCES

Adeoye, A. O. and Lateef, A. (2022). Improving the yield of citric acid through valorization of cashew apple juice by *Aspergillus niger*: Mutation, nanoparticles supplementation and Taguchi technique. *Waste and Biomass Valorization*, 13(4): 2195-2206.

Afolabi, F. T., Adeyemo, S. M. and Balogun, H. O. (2018). Fermentation conditions and process optimization of citric acid production by yeasts. *The International Journal of Biotechnology*, 7(1): 51–63.

Afolabi, F. T. and Akanbi, A. A. (2022). Production and optimization of citric acid by yeasts isolated from kola nut pod husk. *Annals of Microbiology and Research*, 6(10):142-148.

Ali, S., Rehman, A., Khalid, S., Iqbal, S. and Haq, I. U. (2020). Optimization of process parameters for enhanced citric acid production from date syrup by *Candida tropicalis*. *Biocatalysis and Agricultural Biotechnology*, 25: 101593.

Behera, B. C. (2020). Citric acid from *Aspergillus niger:* a comprehensive overview. *Critical Reviews in Microbiology*, 46(6): 727-749.

Cavallo, E., Charreau, H., Cerrutti, P. and Foresti, M. L. (2017). *Yarrowia lipolytica*: A model for citric acid production. *FEMS Yeast Research*, 17:8.

Csutak, O. and Sarbu, I. (2018). Genetically modified microorganisms: Harmful or helpful? In A. M. Holban and A. M. Grumezescu (Eds.), *Genetically Engineered Foods*. 6th ed. pp: 143-175.

Dutta, A., Sahoo, S., Mishra, R. R., Pradhan, B., Das, A. and Behera, B. C. (2019). A comparative study of citric acid production from different agro-industrial wastes by *Aspergillus niger* isolated from mangrove forest soil. *Environmental and Experimental Biology*, 17: 115-122.

Emeka, N., Ogbonna, J., Ominyi, M., Nwagu, K. and Gibson, U. (2012). Isolation of citric acid-producing fungi and optimization of citric acid production by selected isolates. *Global Journal of Bio-Science and Biotechnology*, 1(2): 261-270.

Finogenova, T., Puntus, I., Kamzolova, S., Lunina, Y. N., Monastyrskaya, S., Morgunov, I., and Boronin, A. (2008). Mutant *Yarrowia lipolytica* strains producing citric acid from glucose. *Applied Biochemistry and Microbiology*, 44(2): 197-202.

Hamdy, H. S. (2013). Citric acid production by *Aspergillus niger* grown on orange peel medium fortified with cane molasses. *Annals of Microbiology*, 63(1): 267–278.

Haq, I., Ali, S., Qadeer, M. A. and Iqbal, J. (2004). Citric acid production by mutants of *Aspergillus niger* from cane molasses. *Bioresource Technology*, 93(2): 125-130.

Iris, L., Antonio, M., Antonia, B. M. and Antonio, S. L. J. (2020). Isolation, selection, and identification techniques for non-*Saccharomyces* yeasts of oenological interest. In *Biotechnological progress and beverage consumption* pp: 467-508.

Karasu-Yalcin, S., Tijen Bozdemir, M., and Yesim Ozbas, Z. (2010). Effects of different fermentation conditions on growth and citric acid production kinetics of two *Yarrowia lipolytica* strains. *Chemical and Biochemical Engineering Quarterly*, 24: 347–360.

Kareem, S. O., Rahman, R. A., Mahmood, N. A. N., Salleh, M. S. M., and Ariff, A. B. 2010. Optimisation of fermentation conditions for citric acid production from oil palm empty fruit bunches by *Candida tropicalis*. *New Biotechnology*, 27.6: 540-546.

Kareem, S. O., and Rahman, R. A. 2011. Utilization of banana peels for citric

acid production by *Aspergillus niger*. *Agricultural Biology Journal of North America*, 4.4: 384-387.

Kołożyn-Krajewska, D. and Dolatowski, Z. J. (2012). Citric acid production from glycerol by *Yarrowia lipolytica* and *Candida guilliermondii*. *Chemical Papers*, 66(9): 894-900.

Mahmoud, N. H.,Abou-Arab A.A. and Abu-Salem F.M. (2015). Effect of some different drying methods on the chemical analyses of citrus by-products. *Research Journal of Pharmaceutical, Biological, and Chemical Sciences*. 6(6):104-115.

Mandenius, C. F. and Brundin, A. (2008). Bioprocess Optimization Using Design-of-Experiments Methodology. *Biotechnology Progress*, 24(6): 1191–1203.

Papanikolaou, S., Munoz, R., Chevalot, I., Aggelis, G. and Marc, I. (2002). *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. *Journal of Applied Microbiology*. 92(4): 737-744.

Rymowicz, W., Rywińska, A. and Żarowska, B. (2009). Biosynthesis of citric acid from glycerol by acetate mutants of *Yarrowia lipolytica* in fed-batch fermentation. *Chemical Papers*, 63(2): 156-162.

Rzechonek, D. A., Dobrowolski, A., Rymowicz, W. and Mirończuk, A. M. (2019). Aseptic production of citric and isocitric acid from crude glycerol by genetically modified *Yarrowia lipolytica*. *Bioresource Technology*, 271: 340-344.

Sato, H. and Kawaguti, H. (2016). No Biotechnological production of organic acids. In Journal M. Maróstica, and G. Pastore (Eds.), Biotechnological Production of Natural Ingredients for Food Industry. *Bentham Science Publishers.* 1st ed., pp: 164-206.

Sawant, O., Mahale, S., Ramchandran, V., Nagaraj, G. and Bankar, A. (2018). Fungal Citric acid production using waste materials: A mini-review. *Journal of Microbiology, Biotechnology and Food Sciences*, 8(2): 821–828.

Shaikh, Z. and Qureshi, P. (2013). Screening and isolation of organic acid producers from samples of diverse habitats. *International Journal of Current Microbiology and Applied Sciences*, 2:39-44.

Show, P. L., Oladele, K. O., Siew, Q. Y., Aziz Zakry, F. A., Lan, J. C. W. and Ling, T. C. (2015). Overview of citric acid production from *Aspergillus niger*. *Frontiers in Life Science*, 8(3): 271-283.

Tahjib-Ul-Arif, M., Zahan, M. I., Karim, M. M., Imran, S., Hunter, C. T., Islam, M. S., Mia, M. A., Hannan, M. A., Rhaman, M. S., and Hossain, M. A. (2021). Citric Acid-Mediated Abiotic Stress Tolerance in Plants. *International Journal of Molecular Sciences*, 22.13: 7235.

Yadav, R. L., Shastri, N. V., Yadav, N. B. and Rao, K. V. (2014). Optimization of culture conditions for production of citric acid from glycerol by *Candida tropicalis* using response surface methodology. *Preparative Biochemistry and Biotechnology*, 44(3): 259-272.