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New Insights into Valorization of Agro-Industrial Wastes for Production of Citric Acid: Effects of Mutation and Optimization – A Review

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Abstract. Agro-wastes have been found useful for the production of different important metabolites through fermentation process. Notable metabolite of industrial importance is citric acid, which has wide applications in many areas of human activities. The huge amount of waste generation from agricultural and industrial activities causes pollution and discomfort. The wastes pose colossal threat to human health due to indiscriminate disposal. Valorization of these wastes will invariably amount to converting wastes to wealth, which is in line with sustainable development goals of United Nations. Citric acid is a good natural preservative; it serves as acidulant in many processes because of its low pH and less toxicity compared to other known acidulants. Citric acid is suitably applied in many areas of human activities. Its industrial applications are in cosmetics, pharmaceuticals, chemicals, textiles and electroplating industries. As an antimicrobial and preservative agent, it has found applications in many areas of food processing and preservation to inhibit the growth of spoilage organisms. The demand for citric acid is increasing ditto the cost of production due to increase in energy cost and raw materials. Hence, there is need for cheaper means of production. This review gives insights into the innovative raw materials, application of strain improvement, and optimization technique that have been of tremendous benefits in the production of citric acid.

Keywords: agro-wastes, citric acid, cashew apple, fermentation, optimization, nanoparticles

Introduction

The waste generated through agro-industries is such a huge quantity of biodegradable solid or liquid wastes that consist of organic field residues or industrial processed wastes (Adeoye *et al.*, 2015; Nayak & Bhushan, 2019, Elegbede *et al.*, 2021). As per the study conducted by Baiano (2014), current increase in agricultural and industrial practices have led to the generation of large amounts of various low-value or negative cost crude raw materials. These agro-wastes are often disposed indiscriminately because of cost addition in treatment and high technicality involved their valorization (Elegbede *et al.*, 2021). However, agricultural residues are rich in bioactive compounds and can be used as alternative source of energy and substrates for the production of different products like citric acid, biogas, bioethanol, enzymes, mushroom, and organic fertilizer making them potential raw materials in various investigations with good industrial applications (Lateef *et al.*, 2008; Lateef *et al.*, 2010; Lateef *et al.*, 2012; Lateef *et al.*, 2015; Bertolo *et al.*, 2021; Dhanya, 2022).

Agro-wastes often have serious disposal problems (Rodríguez-Couto, 2019; Maji *et al.*, 2020). For example, the juice industries produce huge amount of wastes as peels, the coffee industry produces coffee pulp as a waste, and cereal industries produce husks (Santos *et* al., 2020; Nogueeira *et al.*, 2020). All over the world, there is increase in agricultural productivities, consequently, an increase in agro-waste generation (Elegbede *et al.*, 2021). Mohlala *et al.*, (2016) estimated annual global waste biomass generation from various agricultural processes

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to be about 140 billion metric tons. Also, about 709.2 and 673.3 million metric tons of wheat straw and rice straw residues were estimated to be generated, respectively (Belewu & Babalola, 2009). Another agro-waste of high potentials is cashew apple. In Nigeria, cashew nuts are produced on a large scale. The harvesting and processing of cashew nut generate a lot of wastes, which include cashew apple pomace, shell, press cake and cashew nut shell liquid (Sawadogo *et al.,* 2018; Felix *et al.,* 2019; Mgaya *et al.,* 2019; Sharma *et al.,* 2020; Elegbede *et* al., 2021; Adeoye & Lateef, 2022).

The use of agro-industrial wastes such as sugar cane bagasse, corncob and rice bran, have been widely investigated via different fermentation strategies for the production of enzymes (Elegbede *et* al., 2021; Sanchez *et al.*, 2021). Solid-state fermentation holds much potential in some specific area compared with submerged fermentation methods for the utilization of agro-based wastes for enzyme production, however there are limitation to its application in high moisture laden fruits and vegetables, unlike submerged fermentation (Adeoye & Lateef 2022). When agro-waste is used as raw material, it can help to reduce the production cost of substrate for to produce enzymes, citric acid and single cell protein. Also, it reduces the pollution load from the environment which destroys aesthetic value of the landscape, pollution of surface and ground water, emission of carbon monoxide to the atmosphere through burning and creation of breeding grounds for pest when it is converted in to useful raw material (Adeoye *et al*., 2015; Bonkkhaew *et al*., 2022; Wongsirichot *et al.*, 2022).

Groups of Agro-wastes

Agricultural residues can be grouped into two: field residues and process residues. The field residues are residues that are left on the field after the harvest of crops. These include the leaves, stalk, seeds, pods and stems. The process residues are left even after the crops have been processed into tangible valued resources. These residues include molasses, pomace, husks, bagasse, straw, shell, pulp, stubble, peel, and roots. Some of these residues have been employed in the production of animal feeds, soil improvement, biogas and organic fertilizers manufacturing. Control application of these agro-wastes can be enhanced by biotechnological process of fermentation to produce citric acid through hydrolysis of the wastes and microbial fermentation.

According to the study conducted by Baiano (2014), it is estimated that approximately 26% of food wastes are generated from the drinks industry, followed by the dairy industry (21%), fruit/vegetable production and processing (14.8%), cereal processing and manufacturing (12.9%), meat product processing and preservation (8%), manufacturing and processing of vegetable and animal oils (3.9%), fish product processing and preservation (0.4%) and others (12.7%). Thousands of tons of organic residues and effluents are produced every year through the food processing industries such as juice, chips, meat, confectionary and fruits industries. Organic wastes with high level of components like cellulose, hemicellulose, lignin, moisture, ash, nitrogen and other nutrients have the potentials as good resources for microbial fermentation for citric acid production and other primary and secondary metabolites of fermentation processes; biogas, biofuel or bioethanol and other commercially useful products (Negro *et al.*, 2016; Ruiz *et al.*, 2016).

Citric Acid Production Using Different Agro-wastes

Different substrates from agro-wastes have been employed in citric acid production using different fermentation methods (Dashen *et al*., 2014). Valencia orange peel was employed as raw materials for the production of citric acid by Solid-State Fermentation (SSF) of *A. niger* CECT-2090 (ATCC 9142, NRRL 599) in Erlenmeyer flask (Torrado *et al*., 2011). Moreover, additional experiments were done by adding methanol or water in different proportions and ways. The optimal condition for citric acid production was found to be an inoculum of 0.5 ×

10^6 spores/g dry orange peel, a bed loading of 1.0 g of dry orange peel and a humidification pattern of 70%. Maximum water retention capacity (MWRC) at the beginning of the incubation with posterior addition of 0.12 ml H_2O per g dry orange peel (corresponding to 3.3% the MWRC) every 12 h starting from 62 h. The addition of methanol was detrimental for the citric acid production. Under these conditions, the SSF ensured an effective specific production of citric acid (193 mg CA/g dry orange peel). The result demonstrated the viability of the citric acid production by solid state fermentation from orange peel without addition of other nutrients, and this could be of interest to possible future industrial application (Baker *et al.*, 2008; Torrado *et al.*, 2011; Bajar *et al.*, 2020).

In strain improvement study by Mahin *et al.* (2007), starchy substrates of pumpkin and cane molasses were used for citric acid production by fermentation. The work employed gamma ray to induce mutation, to obtain mutant strains of 14/20 and 79/20 of *A. niger* under surface culture condition. Citric acid production was shown to be different with various fermentation media by *A. niger.* Mixed substrates prepared with molasses and pumpkin media was proved to be of good potential for citric acid production as demonstrated (Ezejiofor *et al.*, 2014; Nadeem *et al*., 2014).

An investigation carried out by Ali *et al.* (2002) dealt with the kinetics of submerged fermentation by *A. niger* using blackstrap molasses as the basal fermentation medium. A laboratory scale stirred fermentor of 15-L capacity having working volume of 9-L was used for cultivation process and nutritional analysis. Among the 10 stock cultures of *A. niger*, the strain GCBT7 was found to have higher citric acid production. Maximum citric acid production (99.56 ± 3.5 g/l) was achieved by *A. niger* GCBT7, the specific production rate and growth coefficient revealed the hyper-producibility of citric acid using mutant GCBT7.

In another study, local isolate of *Aspergillus niger* MTCC 281 strain was employed for the production of citric acid from the citrus fruit wastes. A total of four substrates; sweet lime pulp, orange pulp, sweet lime and orange peels were selected. The citric acid production was carried out by simultaneous saccharification and fermentation. This process was carried out for a period of eight days at 30 ^oC with two types of inocula i.e. *A. niger* spores and *A. niger* mycelium in separate flasks and the production of citric acid in the two systems was compared. Higher yield was observed in intact mycelium than the spores and peels were found to be more suitable than pulp (de Moraes Barros *et al.*, 2012). The use of cassava waste was demonstrated by Adeoye *et al.* (2015) with appreciable production of citric acid from submerged fermentation of cassava peels.

Nigeria produces a large amount of agricultural products and this result in large amount of organic wastes which may be used as substrates (Suoware and Amgbari, 2022). Among the wastes in this group are the fruits' wastes which include the peels, seeds kernels and part of the pulp. Even though fruits' wastes are rich sources of vitamins, minerals, essential nutrients and fibre, they can constitute environmental problem when not utilized maximally. This is due to high digestibility and biodegradability, hence, may negatively contribute to conventional solid waste disposal problem (Ire *et al.*, 2016). One of the wastes in this category is the cashew apple which is often allowed to rot away after collection of the cashew nut and contribute in no small measure to greenhouse gas emission (Santos *et al.*, 2020). The alternative means of maximizing the economic value of cashew as cash crop, as it is being produced in Nigeria on a large scale, is to convert the cashew apple juice into citric acid by fermentation (Mgaya *et al.*, 2019; Adeoye & Lateef, 2022).

Citric acid is a weak tribasic organic acid (2-hydroxy-propane- 1, 2, 3-tricarboxylic acid) (Figure 1). It is an organic acid with high demand as a commodity chemical (Betiku *et al.*, 2010; Adeoye *et al.*, 2015; Adeoye & Lateef, 2022). It occurs naturally in citrus fruits and as an intermediate product in the citric acid cycle which occurs in the metabolism of all aerobic organisms. It exists in greater than trace amount in a variety of fruits and vegetables, mostly in

citrus fruits and especially lemons and lime, where they occur in larger concentration (Penniston *et al*., 2008; Shankaran & Sivakumar, 2016). Citric acid major source of production are from fruits extraction, chemical process and by fermentation as shown in Figure 2.

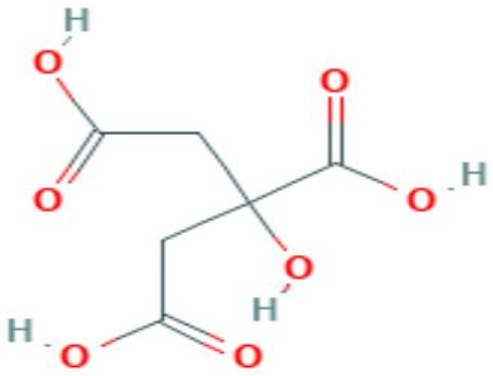

Figure 1: Chemical structure of Citric acid ($C_6H_8O_7$) (Chem.oline.org, 2013)

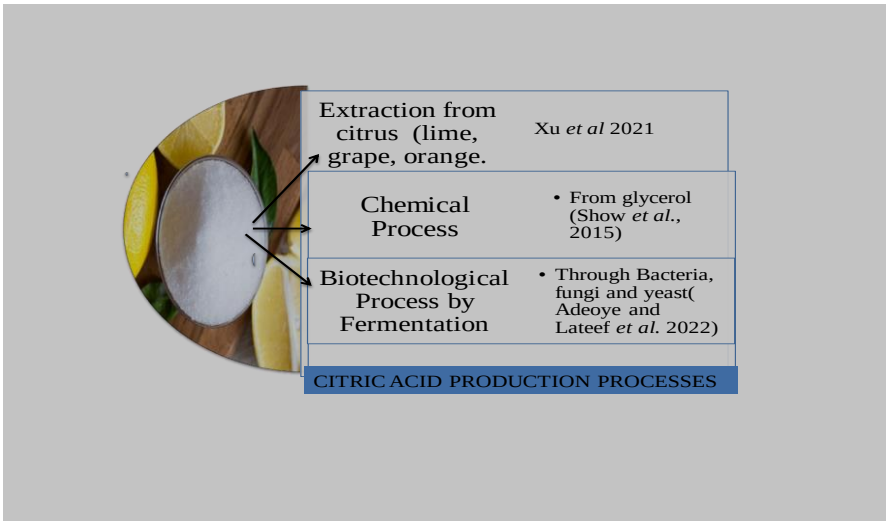

Figure 2: Citric acid sources

Citric acid is a good natural preservative; it serves this purpose as acidulant because of its low pH and less toxicity compared to other acidulants. It finds its various applications in many areas of human activities such as cosmetics, pharmaceuticals, chemicals, textiles and electroplating industries. It is regarded as sour salt and it is used as table salt for those that are allergic to common salt. Citric acid is useful as preservatives, which is either used in isolation or simultaneously combined like citrol®; an antimicrobial preservative combining synergistic action of two components to inhibit the development of molds. The product citrol is an alcoholic solution of citric acid (E330) and sorbic acid (E200) (Lofty *et al*., 2007; Silva & Lidon, 2016).

World-wide commercial production of citric acid is now accomplished by fermentation processes using various carbon sources as raw materials and *Aspergillus niger* as the fermenting organism (Table 1) (ECAMA, 2014). Fermentation can be carried out in deep tanks (submerged fermentation being the most commonly used) or shallow pans (surface fermentation). Shallow pan fermentation is preferred in some instances because of its relatively lower energy cost. However, both methods required regular agitation (Soccol & Vandenbergbe, 2000; Soccol *et al*., 2006; Show *et al*., 2015). The global production of citric acid is largely

achieved by the saprophytic filamentous fungus, *Aspergillus niger*. *Aspergillus* comprises a diverse group of species based on morphological, physiological and phylogenetic characters, which significantly impact biotechnology, food production, indoor environments and human health (Samson *et al.*, 2014).

Table 1: Some agro-wastes involved in citric acid production and fermentation methods

Agro-waste substrate	Type of fermentation	Organism for bioconversion	Yield of Citric acid	References
Cashew apple	SmF	*Aspergillus niger*	92.60%	Adeoye and Lateef (2022)
Cassava peel	SmF	*A. niger*	1.93-9.4 g/l	Adeoye *et al*. (2015)
Inulin	SmF	*Yarrowia lipolytica*	200 g/dm^3	Rakicka *et al.* (2019)
Apple pomace	SmF	*A. niger*	33.81 g/l	Sekoai *et al. (*2018)
Coconut husk	SSF	*A. niger*	-	Sharma *et al.* (2018)
Rice straw	SSF	*A. niger*	50.23 g/kg	Ali *et al.* (2012)
Sugarcane bagasse	SmF	*A. niger*	0.46%	Dutta *et al.* (2019)
Banana peel	SSF	*A. niger*	82 g/kg	Karthikeyan *et al.* (2010)
Banana peel	SmF	*A. niger*	0.51%	Dutta *et al.* (2019)
Wheat bran	SSF	*A. niger*	-	Pandey *et al.* (2000)
Apple pomace	SSF	*A. niger*	-	Dhillon *et al*. (2011)
Orange peel	SSF	*A. niger*	192.2 g/kg	Torrado *et al.* (2011)
Brewery waste	SSF	*A. niger*	0.19-0.22%	Pathania *et al.* (2018)
Coffee husk/pulp	SSF	*A. niger*	Citric acid	Pandey *et al.* (2000)
Banana peels	SSF	*A. niger*	-	Abbas *et al.* (2016b)
Sugar bagasse	SSF	*A. niger*	75.45 g/kg	Alam *et al.* (2011)
Sugarcane waste	SSF	*Trichoderma reesei*	Citric acid	Bastos and Riberio (2020)
Pineapple waste	SSF	*A. foetidus*	5.25 g/kg	Tran and Mitchell (1995)
Biodiesel waste	SSF	*A. niger*	350 g/kg	Schneide *et al.* (2014)
Biodiesel waste	SSF	*Yarrowia lipolytica*	-	Morgunov *et al.* (2018)
Olive mill waste-water	SmF	*Yarrowia lipolytica* ACA-DC5029	-	Sarris *et al.* (2019)
Sugarcane molasses	SmF	*A. niger* (FQW)	256.94 mg/ml	Almousa *et al.* (2018)

Aspergillus niger is found globally and exhibits a great diversity in phenotype. *A. niger* is cultured for the production of many substances; various strains of *A. niger* are used in the industrial preparation of citric acid, oxalic acid and gluconic acid. Citric acid has been assessed in daily intake by World Health Organization (Lofty *et al*., 2007; Dhillon *et al*., 2010; Samson *et al*., 2014). *Aspergillus niger* fermentation is generally recognized as safe (GRAS) by the United States Food and Drug Administration under Federal Food Drug and Cosmetic Act (Yigitoglu, 1992; Schuster *et al*., 2002; Van Dijck *et* al., 2003; Shankar & Sivakumar, 2016). Other fungi such as *Aspergillus wenchi, A. awamori, A. foetidus* as well as some strains of *Penicillium* such as *P. simplicissinum* and *P. restricium* (Angumeenal & Venkappayya, 2013; Adeoye *et al*., 2015; Hu *et al*., 2016; Sawant *et al.*, 2021) are used for citric acid production. Yeasts such as *Candida lipolytica, C. intermidia, Yarrowia lipolytica, Candida tropicalis, Pichia kluyveri* and *Saccharomyces cerevisiae* (Cavallo *et al.*, 2017; Carsanba *et al.*, 2019; Hesham *et al.*, 2020; Behera *et al.*, 2021), and some bacteria such as *Bacillus licheniformis, B. subtilis, Corynebacterium* spp. (Kareem *et al*., 2010) have also been used to produce citric acid (Figure 3).

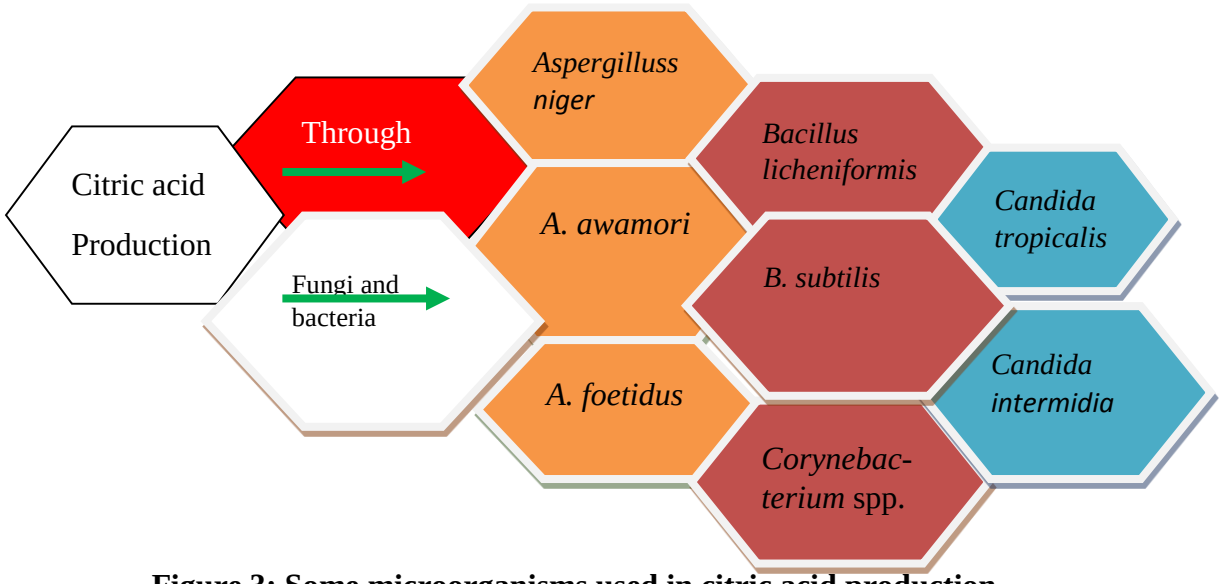

Figure 3: Some microorganisms used in citric acid production

The usefulness and various applications of citric acid have a great influence on its demand. The demand rate is estimated to be increasing at 3.5-4.0 % annually (Pandey *et al*., 2000; Finogenova *et al*., 2005; Adeoye & Lateef, 2022). Commercial importance of industrial enzymes production was estimated at market value of about US $5 billion in 2009, of which filamentous fungi account for almost half of the production. The remarkable demand for citric acid stimulated researchers and industrialists to look for, and modify an economic production process to reduce the cost of production and additives (Ali *et al*., 2012). Carbon sources employed by the world largest producer like China are sugar cane molasses and starch (Pazouki *et al*., 2000; MRR, 2019; Adeoye & Lateef, 2021).

The entire demand for citric acid in Nigeria is satisfied by importation (Betiku *et al*., 2010; Adeoye *et al*., 2015). This has been source of drain on Nigerian economy. There is need to search for economic means by which the need of this important commodity can be met, using the local raw materials for its production, especially agro-industrial waste (Adeoye & Lateef, 2022). Nigeria may be able to compete well in citric acid production, with the use of agro-wastes which have economic and comparative advantages. Such agro-wastes include cassava waste, cashew apple juice and sugarcane bagasse among others. Earlier, Adeoye *et al.* (2015) demonstrated a feasible process of producing citric acid using cassava peel. Nigeria produces large amounts of agricultural products and these results in large amount of organic wastes which may be used as substrates. One of the wastes in this category is the cashew apple which is often allowed to rot away after collection of the cashew nut. The alternative means of maximizing the economic value of cashew as cash crop, as it is being produced in Nigeria on a large scale, is to convert the cashew apple juice into citric acid by fermentation (Adeoye & Lateef, 2022).

Fermentation Process for Citric Acid Production

Different methods of fermentation are employed for citric acid production. The different methods of fermentation that can be used in citric acid production using *Aspergillus niger* are (i) surface fermentation, (ii) submerged fermentation and (iii) solid state fermentation (Meers & Milson, 1987*;* Pintado *et al.*, 1998). In any method employed for the production, the spores of the organism are used as inocula. This is done in form of spores inoculated to substrate and the growth may result in the production of different metabolites. To have maximum yield of the desired product, the organism must be grown under precise cultural condition at a particular growth rate. If a microorganism is introduced into a nutrient medium that support its growth,

the inoculated culture will pass through a number of stages, the lag phase, the exponential phase, stationary phase, death and decline phase (Stanbury *et al*., 2013).

Surface Fermentation

Surface fermentation system is the first system employed for the production of citric acid on an industrial scale. In surface fermentation, *Aspergillus nige*r forms a mycelium layer on the liquid surface of the stainless steel trays which are staked in fermentation trough supplied with filtered air which serves both to supply oxygen and to control the temperature of fermentation process (Meers & Milson, 1987, Adeoye *et al.,* 2015). When maximum mycelium is separated from the fermentation medium by filtration, the citric acid contained in the solution is precipitated as calcium citrate (Kapoor *et al*., 1983; Adeoye & Lateef, 2022).

Surface fermentation is easy to control and to implement, its application is not as elaborate as submerged fermentation, a little extensively used than solid state fermentation as It needs no aeration or agitation of the fermentation trough, the separation of citric acid from mycelium is easy because the microorganism is not dispersed into the fermentation medium. In this method of fermentation, only the temperature, pH and concentration of substrate are monitored. The expenses and losses during recovery are minimal. However, the disadvantages of surface fermentation are the initial cost of equipment and facilities; also, it involves long fermentation time and therefore reduces productivities (Kapoor *et al*., 1983; Adeoye *et al*., 2015).

Submerged Fermentation

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Many industrial fermentation processes adopt this method. This involves carefully selected microorganisms in closed vessels containing a rich broth of nutrients and a high concentration of oxygen. As the microorganisms breakdown the nutrients, they release the desired products. Submerged fermentation is carried out in shake flask, aerated stainless steel tanks, agitated reactor, sparged towers, a bioreactor, a bubble column, a tower fermentor, a disk fermentor and a rotating disk contactor of trickle flow fermentor (Sakurai & Imai, 1992; Rodríguez-Couto, 2019).

Submerged fermentation is favored over surface fermentation for the following reasons: higher yield of citric acids, low cost of equipment and facilities, improved process control, reduced fermentation time, lower labor cost, simpler operations, and easier maintenance of aseptic condition on an industrial scale (Sodeck *et al.,* 1981). However, submerged fermentation has some disadvantages compared to surface fermentation in that it requires filtration of culture medium to separate the mycelium and the process is vulnerable to infections (Kapoor *et al.,* 1983; Reihani & Khosravi-Darani, 2019).

Solid State Fermentation

Solid state fermentation (SSF) is defined as the growth of microorganism in a low water activity environment on an insoluble material that acts both as a physical support and as a source of nutrients (Pintado *et al.,* 1998; Sadaf *et al.,* 2021). Solid state fermentation is a biomolecule manufacturing process used in the food, pharmaceutical, cosmetic, fuel and textile industries. These biomolecules are mostly metabolites generated by microbial growth on a solid support selected for this purpose. This technology for the culture of microorganism is an alternative to liquid or submerged fermentation. Solid state fermentation has existed for several centuries. It has been used in Asia (Japan), where it is referred to as "Koji" fermentation (Qaiser *et* al., 2008; Shruthi *et al*., 2019). This process consists of depositing a solid culture substrate, such as rice or wheat bran, on flatbed after seeding it with microorganisms; the substrate is

then left at room temperature for several days. It implies the use of a solid but porous matrix with a large surface area per unit volume (Qaiser *et al.*, 2008; Singh *et al.*, 2020).

The solid substrate material can be biodegradable but must provide a suitable support for fungal growth and exchange of gases. Typical example of solid state fermentation method is the koji process which is the fermentation of steamed rice by *Aspergillus oryzae* to produce soy sauce and the composting of lignocelluloses fibers which are naturally contained by bacteria, mould and *Streptomyces* spp. Solid state fermentation offers many advantages: water requirement is very low, energy consumption is very low, low waste treatment cost, limited contamination risk because of a lower moisture level and simplified nutrient media composition as compared with submerged fermentation process. The disadvantages include: difficulty in controlling process parameters such as pH, heat and nutrient conditions and high cost of product recovery (Qaiser *et al.*, 2008). More so, during a fermentation process methods are required for the routine determination of the microbial population, cell number and biomass in order to monitor fermentation progress, these may be difficult while employing solid state fermentation method (Shi *et al*., 2019).

Factors Affecting Citric Acid Production by Fermentation

Production of citric acid has been majorly by fermentation process, many factors have been identified as barrier in maximizing yield through these biotechnological processes (Show *et al.*, 2015). To improve productivity, solving those identified problem will enhance maximum production. Factors that affect citric acid production by fermentation are subdivided into two which are chemical and physical factors (Adeoye *et al.*, 2015; Adeoye & Lateef, 2021).

Chemical Factors Affecting Citric Acid Fermentation

Citric acid production by *A. niger* is influenced by a number of fermentation parameters. There are significant variations in fermentation environments of different substrates that are used in citric acid production. To achieve high production of citric acid, it is essential that the study of influence of physical and chemical environments on citric acid production is checked (Jianlong & Ping, 1998; Soccol *et al*., 2006; Max *et al.*, 2010; Rattanaporn *et al.*, 2018).

Medium Composition

Growth of microorganism and production of metabolites are strongly affected by the medium composition such as concentration of carbon, nitrogen, phosphorus, potassium, trace element and stimulators. Thus, citric acid productivity by *A. niger* can be improved by optimizing the medium composition. The choice of raw materials (medium composition) for developing citric acid biotechnology is determined by factors such as renewability, ability of the producer organism to assimilate the substrate with a high conversion rate, consumption value and cost/ price of the target product. To carry out fermentation processes, in addition to very expensive food raw materials, such as glucose which is simple sugar, other form of carbohydrates like starch may be used. The problem in using such medium is the cost, however, sugarcane bagasse, orange pulp and other industrial-wastes may be cheaper but there is need for purification and pre-treatment because of chemical contamination (Costal *et al.*, 2018; Gomes *et al.*, 2020). Much cheaper substrates which are waste products of various industries, such as glycerol-containing waste of the biodiesel industry, glucose-containing wood hydrolysates (Morgunov *et al.*, 2018), olive mill waste-water (Sarris *et al.*, 2019), and inulin (Rokicka *et al.*, 2019) are better alternatives. It is important to note that most established chemical and physical pre-treatment techniques are expensive and most often corrosive and detrimental to the equipment (Bostos & Ribero, 2020).

Stimulators

Several reports have shown the stimulatory effect of additives on fungal citric acid accumulation and secretion (Pando, 1996; Goes & Sheppard, 1999; Papadaki & Mantzouridou, 2019). To improve citric acid production, stimulators have been used, such as organic solvent, phytate and lipids (Ping & Jianlong, 1998; Show *et al.*, 2015; Wang *et al*., 2016, 2017).

Organic Solvents

Higher citric acid production levels can be obtained by applying stimulators, such as methanol, ethanol, phytate, vegetable oil, oximes, n-dodecane, fluroacetate and chelating agent (Navarathan *et al.*, 1996; Kareem *et al.*, 2010; Almousa *et al*., 2018; Amato *et al.*, 2020). Organic solvents including ethanol and methanol stimulate the production of citric acid by increasing the permeability of the cell membrane (Jianlong 1998). The decreasing of cell growth or changing the activity of key enzymes associated with the TCA cycle increases the activity of citrate synthetate and reduces the activities of aconitate. In addition, ethanol can improve citric acid production by being converted to acetyl-coA and assimilated by *A. niger* as an alternative carbon source (Hang & Woodams, 1995; Adeoye *et al.*, 2015; Show *et al.*, 2015). However, the stimulating effect of methanol is not clear. It is proposed that the addition of methanol increases the permeability of the cell membrane and increase the transfer of nutrients, which in turn increases the excretion of citric acid across the cell membrane (Jianlong & Ping, 1998; Souza *et al.*, 2014; Amato *et al*., 2020).

The enhanced citric acid production with ethanol addition can be attributed to slow degradation of citric acid due to reduction in aconitase activity. Addition of ethanol also resulted in the slight increase in the activity of other TCA cycle enzymes. There is also a possibility that ethanol might be converted to the acetyl-CoA, a metabolic substrate required for citric acid formation. Methanol is not metabolized nor assimilated by *Aspergillus niger,* and so its exact role in enhancing citric acid formation is not much clear (Dhillon *et al*., 2013; Yu *et al*., 2017).

However, addition of methanol might increase cell permeability to citrate. There have been reports that the addition of methanol to the fermentation medium remarkably depressed the cellular protein synthesis without affecting nitrogen uptake, thus causing an increase in amino acids, peptides and low molecular weight protein pooled in the mycelium during the early stage of cultivation (Kanti & Sudiana, 2017). Also, methanol addition also slightly changed the activity of some TCA cycle enzymes favoring citric acid accumulation. The stimulatory effect of methanol on citric acid yield can be explained in terms mycelia morphology as well as pellet shape and size (Dhillon *et al*., 2011; Napitupulu *et al.*, 2019). Methanol has a direct effect on mycelia morphology and it promotes pellet formation. It also increases the cell membrane permeability to provoke more citric acid production from mycelia cells. It has been generally found that addition of methanol, ethanol, isopropanol, and methyl acetate was found to enhance citric acid production (Nadeem *et* al., 2010; Show *et al.*, 2015).

Phytates

Citric acid production using *A. niger* is very sensitive to the concentration of trace element. A presence of trace element in media can cause considerable negative effect on citric acid production (Adham, 2002; Yu, 2020). Phytate acid acts as a metal chelating agent, chelating free trace element (Wang, 1998b; Haq *et al.*, 2002; Viera *et al*., 2018). Phytate may also improve citric acid production by *A. niger*, by controlling key enzymes involved in the TCA cycle (Jianlong, 1998; Wang, 1998a,b; Kareem *et al.*, 2010; Maly *et al.*, 2017; Yu *et al.*, 2017; Kanti & Sudiana, 2017; Singh *et al.*, 2020).

Fatty Acids

Vegetable oils with high level of unsaturated fatty acids, such as olive, maize and sunflower oil, are stimulators for citric acid production, when using both submerged and solid substrate fermentation by *A. niger*. The stimulating mechanism of natural oil was investigated by Sawant *et al*. (2021). In some previous reports, it was suggested that unsaturated lipid play a role of alternative hydrogen acceptors to oxygen. Vegetable oil can be converted to acetyl-coA and assimilated by *A. niger* as an alternative carbon source (Souza *et al.*, 2014; Lu *et al*., 2018; Sawant *et al.*, 2021). Therefore, the addition of vegetable oil also increases citric acid production; fat and vegetable oils act as carbon source and are degraded to glycerol and fatty acid. Glycerol enters directly in the TCA cycle by the formation of acetyl-coA and fatty acids enter via glycolysis (Kareem *et al.*, 2010; Behera, 2020). Lipids as stimulator will increase the citric acid yield when added to the fermentation unit with no effect on the biomass of the microorganism (Adham, 2002; Souza *et al.*, 2014; Xie *et al.*, 2018; Khairan *et al.*, 2019).

Physical Factors Affecting Citric Acid Production

Fermentation Temperature

Optimum fermentation temperature must be maintained despite the large amount of heat (14 MJ/kg of substrate dry matter) generated by the metabolic activity of microorganisms (Miller, 1999; Sadh *et al.*, 2018; Lee *et al.*, 2020). A solid substrate with a poor heat transfer coefficient result in localized temperature build-up and non-homogeneous fermentation conditions, especially for large scale fermentation (Napoothiri *et al*., 2003; Kola *et al*., 2017). Cultivation under temperature other than ideal, result in enzymes denaturation and inhibition of growth, excess moisture losses and growth arrest, while lower temperature lead to lower metabolism (Adinarayan *et al.*, 2003; Almousa *et al*., 2018; Behera *et al.*, 2021). Although most filamentous fungi are mesophilic and require optimal temperature between 25 and 35 ^{o}C, some species thrive at 50 ^{o}C (Reid, 1989; Suresh & Chandrasekaran, 1999; Auta *et al*., 2014; Sharma *et al.*, 2017). A temperature of 40 ^{o}C was identified as optimum for metabolite production and sugar utilization by *A. niger* (Fawole & Odunfa, 2003; Kareem *et al*., 2013; Almousa *et al.*, 2018).

Aeration Effect in Fermentation

It has been established that variation in the rate of aeration can have a detrimental effect on performance and yield of citric acid. If the aeration is too high, the partial pressure of dissolved CO_2 in the fermentation broth may be too low. Carbon dioxide is important as substrate for pyruvate carboxylase that replenishes the supply of oxaloacetate for citrate synthase. Sufficient carbon dioxide is produced by the reaction catalyzed by pyruvate decarboxylase, but excessive aeration lead to low yield. On the other hand, high level of carbon dioxide in the medium is detrimental for the final concentrations of citrate and biomass (Max, 2010; Wang *et al.*, 2020).

In major fermentation processes, aeration is essentially important; because aerobic organisms involved, hence it required the provision of oxygen (Show *et al*., 2015). In fermentation, for the organism involved to generate energy for growth and yield metabolite of interest, it requires oxidation of substrates, for example glucose or any other carbohydrate. The oxidation of glucose may be represented as:

$$C_6H_{12}O_6 + 6O_2 \text{ — } 6H_2O + 6CO_2 + \text{energy}$$

It is not possible to provide a microbial culture with all the oxygen it will require for the complete oxidation of glucose in one addition. Therefore, in the process of fermentation there is need for constant supply of oxygen to the microbial culture during growth at a rate that will meet the requirements of the cultured organism for proper growth and yield. Because of the

enormous need of the oxygen during fermentation, many devices are incorporated to agitate and aerate the fermentation broth. Therefore, for fermentation to be effective and productive, oxygen availability is a critical factor (Nongkynrih & Pawar, 2014; Zhang *et al.*, 2017)

The Effect of pH

The metabolic activity of a fungus is very sensitive to pH level of the medium (Adeoye *et al.*, 2015; Sawant *et al.*, 2018). The pH of the medium is very important in two stages of the process, fermentation start from spores and their germination required high pH (> 5), the utilization of the substrate through *A. niger* metabolism lowers the pH. The initial pH of the substrate was found to have an impact on citric acid production by *A. niger* growth on the peat moss (Kim *et al*., 2004; Malik *et al*., 2018; Elsayed *et al.*, 2021). In addition, the type of buffer used in the nutrients solution is a key factor governing citric acid production by *A. niger* (Roukas, 2000; Xie *et al.*, 2018). Most filamentous fungi are observed to grow well under slightly acidic conditions ranging from 3 to 6, but some fungi are able to grow at a pH below 2 to better compete against bacteria (Kristiansen & Sinclair, 1979; Fawole & Odunfa, 2003; Adeoye *et al*., 2015). James Curries was the first to observe the ability of some strains of *A. niger* to grow at very low pH (2.5 – 3.5). This acid environment would inhibit growth of most other strains of bacteria (Kristiansen & Sinclair, 1979; Sawant *et al.*, 2018). Wang *et al.*, (2020) stated that pH is an important parameter for fungal growth and for the functioning of their enzymes. Hence, *Aspergillus niger* is sensitive to low pH below pH 3, therefore, optimum pH for acid production is slightly above 2.5 (Dashen *et al*., 2014; Elsayed *et al.*, 2017; Wu *et al.*, 2020, Adeoye & Lateef, 2022).

Inoculum Density

A higher inoculum density leads to population over-crowding, high nutrients competition and rapid exhaustion of nutrients (Uyar & Baysal, 2003; Show *et al*., 2015; Wang *et al*., 2020). Up to a specific limit, metabolic production generally increases with inoculum density (Kota & Sridhar, 1999; Kola *et al.*, 2018; Lee *et al*., 2020). At lower inoculum density, metabolite production drops and contamination risks increase due to an insufficient cell population. Existing literature has shown that an inoculum density between 1×10^4 to 1×10^9 spores/ml was found to be suitable for citric acid production by *A. niger* (Falvela-Torres *et al.*, 1998; Adham, 2002; Angumeenal & Venkappayya 2013; Soccol *et al.*, 2017).

Carbon Sources

Citric acid accumulation is strongly influenced by the type and concentration of carbon source. The carbon source can be varied according to the microorganism used. Substrate profile of *Aspergillus niger* and yeast used for citric acid production can be extremely different from each other (FAO, 1990; Max *et al*., 2010; Nielsen *et al*., 2017).

For *A. niger*, sucrose is the most favorable substrate among the easily metabolized pure carbohydrates; followed by glucose, fructose and galactose (Yalcin *et al.*, 2010; Wang *et al.*, 2020). Molasses is often used as raw materials for citric acid production by *A. niger*. However, in citric acid production process by *A. niger*, many distinct substrates could be used beside simple carbohydrates like sucrose. Citric acid accumulation is strongly influenced by the type and concentration of carbon source. Many of the carbohydrate polymers (compound carbohydrate) can be utilized by using enzymes to break the higher polymer down to simpler form of sugar (monosaccharides). The presence of easily metabolized carbohydrates has been found essential for good production of citric acid (Hossain *et al*., 1984; Hamad *et al.*, 2014; Hou and Boa, 2018). Glucose, sucrose, fructose and galactose are favourable carbon sources. Other sources of carbon such as sorbose, ethanol, cellulose, mannitol, lactic acid, and malic acid allow a limited growth and low production (Yokoya, 1992; Auta *et al*., 2014; keekan *et*

al., 2017; Muddanna *et al.*, 2019; Behera, 2020). Kovats (1960) reported that the initial sugar concentration was critical for citric acid production and other organic acids produced by *A. niger* (Papadaki & Mantzouridou, 2019). Xu *et al*. (1989) also reported that *A. niger* strain needed an initial sugar concentration of 10 – 14% as optimal as no citric acid was produced at sugar concentration of less than 2.5% (Behara, 2020).

Several raw materials can be used for citric acid production but the critical constraints are the cost and pretreatment requirements (Dienye *et al.*, 2018; Sawant *et al.*, 2018; IIgin *et al*., 2020). The most widely used carbon sources in industrial fermentation are glucose syrups from starch hydrolysis, sugar beet molasses and low quality sugarcane by-products. The last two substrates are generally heavily contaminated by cations from previous processes. Cations usually come from insoluble residues formed by precipitation with potassium ferrocyanide and the contamination lead to very expensive and complex pretreatment which results to low desirability of the carbon source from industrial by-products (Max *et al.*, 2010; Yesser *et al*., 2012; Cunha *et al.*, 2014; Sawant *et al*., 2018).

Nitrogen Sources

The effect of nitrogen sources on citric acid production has been intensively studied in solid substrate and submerged fermentations. Nitrogen constituent has a profound effect on citric acid production because nitrogen is not only important for metabolic rate in the cells but it is also a basic part of the cell protein (Show *et al*., 2015). Ammonium-chloride, ammonium-sulphate, ammonium-nitrate, peptone and yeast extract were the most suitable nitrogen sources for production of citric acid by fungi (Abou-zeid & Ashy, 1984; Gueguim-Kana *et al.*, 2012; Francisco *et al*., 2018; Xie *et al*., 2018). The limitation or starvation of nitrogen during the fermentation results in the limited growth of *A. niger* and reduce enhancement of citric acid production (Miriminachi et *al*., 2002; Angumeenal & Venkapayya 2013; Papadaki & Mantzouridou, 2019). However, very high nitrogen concentration increases the growth of biomass and high consumption of sugar but low citric acid production (Auta *et al.*, 2014; Adeoye & Lateef, 2022).

Some complex media such as molasses are rich in nitrogen and rarely need to be supplemented with nitrogen source. The highly pure media used in laboratory scale research are usually supplemented with ammonium salts which lead to low pH. Hence, it enhances or favors fermentation (Wang *et al.*, 2020). The nitrogen concentration conditions that will be specifically favorable to the citric acid production by fermentation are low nitrogen supply. This will reduce biomass, and the nitrogen is better to be in ammonium salt form than to be nitrate form. The presence of nitrogen in form of ammonium salt will inhibit iron utilization as stimulant and does not have obvious metabolic effect on the process. But when nitrogen is present as nitrates, iron has an obvious effect as stimulating agent, it increases the mycelium biomass (Yalcin *et al.*, 2010; Show *et al.*, 2015; Almousa *et al.* 2018).

Phosphorus Sources

Beside nitrogen, phosphorus is another very critical element that is a very important component of cell that is critical in cell development (Show *et al.*, 2015). The concentration of exogenous phosphorous in medium had significant effect on cell multiplication and metabolite production (Almousa *et al.*, 2018; Tong *et al.*, 2021). Presence of phosphate in the medium has a great effect on the yield of citric acid. Potassium di-hydrogen phosphate has been reported to be the most suitable phosphorous source (Kubicek & Rohr, 1986).

Shu and Johnson (1998) reported that phosphorous at concentration of 0.5 to 1.0 g/l was required by the fungus in a chemically defined medium for maximum production of citric acid (Show *et al.*, 2015; Almousa *et al.* 2018)). Phosphate is known to be essential for the growth and metabolism of *A. niger* (Shankaran & Lonsane, 1984; Sharma, *et al.*, 2018; Titilayo *et al.*,

2018). Low levels of phosphate favor citric acid production, however, the presence of excess of phosphate is believed to lead to the formation of certain sugar acids, a decrease in the fixation of CO_2, and the stimulation of growth. Phosphates act at the level of enzyme activity and not at the level of gene expression (Show *et al*., 2015; Titilayo *et al.*, 2018). It is interesting to note that different strains require distinct nitrogen and phosphorous concentrations in the medium. In fact, nitrogen and phosphorous limitation is a crucial factor in citric acid production as there is an interaction between them. Consequently, the study of their combined effect is necessary (Chen, 1994; Pintado *et al*., 1998; Dhillon *et al.*, 2010; He *et al*., 2014; Gopinadh *et al.*, 2015; Tong *et al*., 2021).

Pintando *et al.* (1998) reported how the culturing modality conditions, the behavior of the microorganisms referring to the tendencies of production as a function of the levels of nitrogen and phosphorus. The authors used as first order, an empirical model based on rotatable design to study the effects of both nutrients. As predicted, for the two studied strains, a similar behavior was noticed, showing an improvement towards low levels of nitrogen and phosphorus in submerged culture and towards high levels in solid state culture, and with higher productions for the solid state culture (Behera, 2020). Roukas *et al.* (2020) affirmed low citric acid yield in solid state culture and this was the consequence of lower diffusion rate of nutrients and metabolites, which occurs in low water activity conditions (Torrado *et al.*, 2011; Adeoye *et al*., 2015).

Consequently, strain with large requirements of N and P seems to be disfavored, due to the restriction of accessibility to the nutrients in the medium. KH_2PO_4 and K_2HPO_4 proved to be the best phosphorus sources. Francisco *et al*. (2020) reported that phosphorus limitation induced higher citric acid production and yield, while Patel and Pandya, (2017) reported higher citric acid production and yield with phosphorus in the range of 0.5 to 5.0 g/l. (Miriminachi *et al.*, 2002; Behera, 2020; Fejes *et al.*, 2020; Francisco *et al.*, 2020).

Substrates for Citric Acid Production

Although citric acid is mostly produced from starch or sucrose-based media using liquid fermentation, a variety of raw materials such as molasses and several starchy materials and hydrocarbons have also been employed. Rohr *et al*. (1983) classified raw materials used for citric acid production in to two groups: (i) with a low ash content from which the cations could be removed by standard procedures (e.g. cane or beet sugar, dextrose syrup and crystallized dextrose); and (ii) raw materials with a high ash content and high amounts of other non-sugar substances (e.g. cane and beet molasses, crude unfiltered starch hydrolysates) (Wang *et al.*, 2013).

Several attempts have been made to produce citric acid using molasses, which is preferred due its low cost and high sugar content (40-55%). The composition of molasses depends on various factors, e.g. the kind of beet and cane, methods of cultivation of crops and fertilizers and pesticides applied during cultivation, conditions of storage and handling (e.g. transport, temperature variations), and production procedures. Both cane and beet molasses are suitable for citric acid production. However, beet molasses is preferred due to its lower content of trace metals. Generally, cane molasses contains calcium, magnesium, manganese, iron and zinc, at proportion which have a retarding effect on the synthesis of citric acid. Consequently, some pre-treatment is required for the removal/reduction of trace metals. Despite that, cane molasses poses difficulties in achieving good fermentation yields.

Various other agro-industrial residues such as apple pomace, cassava bagasse, coffee husk, wheat straw, pineapple waste, sugar beet cosset, and kiwi fruit peel among others have been investigated with solid state fermentation techniques for their potentials to be used as substrates for citric acid production (Pandey & Soccol, 1998; Pandey *et al*., 1999, Vandenberghe *et al*., 1999a, b, c). In fact, these residues are very well adapted to solid-state

cultures due to their cellulosic and starchy nature. However, despite the fact that these solid residues provide rich nutrients to the microorganisms, and are good substrates for growth and activity of microorganisms, much remains to be done for developing commercially feasible process utilizing these residues (Pandey, 1992, 1994).

A. niger has a metabolic system which is composed of the cytoplasm, mitochondria, and peroxisome. Incorporated in this system are carbohydrate metabolism and amino acid metabolism which take place in both anabolic and catabolic reactions. Different reactions and pathways are used wherever *A. niger* consumes a substrate. Production of citric acid is effected by many factors which include fermentation time, pH, temperature, dissolved oxygen tension, nutritional composition of the media, environmental conditions and influence of types and concentrations of sugar (Hossain *et al*, 1984). With the use of different strains of microorganisms, *Aspergillus niger* has been used intensely with different substrates. Citric acid has been produced commercially using mutant strains of *Aspergillus niger* and with a significant amount by *Saccharomycopsi lipolytica* (Good *et al*., 1985) and *Penicillium simplicissinum* (Franz *et al.*, 1993).

Many carbohydrates and wastes that have been used for citric acid production experimentally include inulin (Drysdale & Mckay 1995), date fruit syrup (Roukas & Kolzekidom, 1997), sugarcane molasses (Gupta, 1994), Soy whey (Khare, 1994), and cheese whey (Hossain *et al.*, 1984; El-Samragy *et al.*, 1996). There is great world-wide demand for citric acid due to its low toxicity when compared with other acidulants used mainly in the pharmaceutical and food industries. Other application of citric acid can be found in detergents and cleaning products cosmetic and toilet-tries. Global production reached 1.4 million tones as at 2004 and there is annual growth of 3.5-4% in demand /consumption of citric acid (Adeoye *et al.*, 2015; Adeoye & Lateef, 2022).

Low Cost Substrate for Citric Acid Production

The search for inexpensive substrates is necessary to reduce the production cost of citric acid. Considerable interest has been shown in using agricultural wastes for citric acid production because it reduces waste disposal problems, especially in developing countries. Current emphasis is on biological conversion of agricultural wastes into value added products. For this purpose, different agro-industrial residues such as cashew apple juice, grape pomace, apple pomace, banana peels, sugar-beets, jack fruits carpel and kiwi fruits peels have been investigated (Khare & Gandhi, 1995; Hang & Woodams, 1987; Singh *et al.*, 2020). Many by-products and residues of the agro-industries can be used in the production of citric acid. A cost reduction in citric acid production can be achieved by using less expensive substrates. The use of agro-industrial residues as supports in solid state fermentation and submerged culture is economically important and minimize environmental problem. Other perspective for citric acid production sector are the improvement of citric acid producing strains which is been carried out by mutagenesis and selection (Carlos *et al.*, 2006).

Cashew Apple Juice as Substrate

Cashew apple is a promising and economically viable pseudo-fruit. Cashew is produced in 32 countries in the world. Brazil is one of the leading cashew apple producing countries. The production figure for the year 2010, based on the food and agriculture organization was 104,342 tonnes (FAO, 2010). However, only 18% of the production is exploited for obtaining various products, from concentrate juice to deserts, and 80% of the pulp is wasted (Pinheiro *et al.*, 2007; Olife *et al.*, 2013). The cashew apple also called cashew fruit is the fleshy part of the cashew fruit that is attached to the cashew nut as shown in Figure 4. The top end of the cashew apple is attaché to the stem that comes off the tree. The cashew apple is a soft fruit, rich in nutrients, and contains five times more vitamin C than an orange. It can be eaten fresh, cooked

in curries, or fermented into vinegar, as well as alcoholic drinks. It is also used to make preserves, chutneys and jams in some countries such as India and Brazil due to high sugar content. In many countries particularly in South America, the cashew apple is used to flavor drinks both alcoholic and nonalcoholic. Cashew apple juice however, may be used for processing of blended juice (Stephanie, 2014).

Figure 4: Cashew fruit

Cashew apple has a sweet but astringent taste traced to the skin that contains a chemical, urushiol (Adeoye & Lateef, 2022). The cashew apple juice has been used as substrate for the fermentation of alcohol production. In Goa, (India) the cashew apple, accessory fruits is mashed and the juice extracted and kept for fermentation for few days, fermented juice then undergoes a double distillation process, the resulting beverage called Femi or Fenny has about 40 – 42% alcohol. The single distillation version is called Urrac, which is about 15% alcohol. In the south region of Mtwara, Tanzania, the cashew apple is dried and stored, later it is reconstituted with water and fermented into strong liquor. An alcohol had been distilled in the early 20th century from the juice of the fruits and was manufactured in the West Indies (Stephanie, 2014).

Economic Importance of Cashew Juice

The apple and nut fall together when both are ripe and in commercial nut plantations. By twisting off the nut, the apple is left on ground for later grazing by cattle or pigs. But, where labor costs are very low, the apple may be gathered up and taken to market or processing plants. In Goa (India), the apple juice are extracted manually using foot, by trampled the apple by foot to extract the juice for locally distilled liquor production (Oduwole *et al.*, 2001; Olife *et al.*, 2013; Das & Aroro, 2017; Emovon & Aibuedefe, 2020).

In the cashew plantations, great heaps are displayed by fruit vendors, and the juice is used as a fresh beverage and for wine. In the field, the fruit are picked up and chewed for refreshment, the juice swallowed and the fibrous residue discarded. In the home and in a limited way for commercial purposes, the cashew apple is preserved in syrup glass jars, because fresh apple are highly perishable. Various species of yeast and fungi cause spoilage after the first day at room temperature. Food technologists in India have found out that good condition can be maintained for 5 weeks at 32 ^{o}F – 35 ^{o}F (0 ^{0} -1.67 ^{o}C) and relative humidity of 85-90%. The juice is astringent due to the presence of tannin and urushiol up to 35%, the tannin is higher in the red apple than the yellow. To reduce the astringent taste, the fruit is pressure-steamed for 5-15 minutes before candling or making into jam, chutney or extracting the juice for carbonated beverages, syrup or wine. Efforts are made to retain as much as possible of the ascorbic acid (Gyed-Akoto *et al.*, 2010).

The lack of awareness on the economic potential of cashew has been a problem to the development of the capacity of the cashew crop economically. Cashew kernels are a high value

luxury commodity with sales growing steadily at an annual rate of 70%, with every expectation that the market will remain strong (Azam-Ali *et al.*, 2001). Besides, there is substantial potential to exploit cashew by-product, such as cashew apple juice fermentation to produce citric acid, cashew nut shell liquid (CNSL) and vitamin C rich juice of cashew apple among others. Therefore, there is need for promotion of awareness and research in to potential of cashew apple juice fermentation for the production of citric acid (Adeigbe *et al*., 2015).

Cashew (*Anacardium occidentale* L.), a tropical nut tree crop, is a source of food, income, industrial raw materials and foreign exchange for many countries of Asia and Latin America. In Nigeria, current cashew trading and exports is worth 24 billion naira ($160 million) and over one million people depend on the industry. Commercial cultivation of cashew in Nigeria dated back to more than 60 years, while research and development into its production, processing and marketing started in 1972. The past four decades ware marked with introduction of exotic cashew genotypes selection, cultivation and production from local and exotic varieties. Much discrepancies exist in yield records, cultivation and production of raw nuts is estimated at 836,500 metric ton on 366,000 hectares with an average yield of 2, 286 Kg/ha.

Local juice extractor/processor that produces cashew apple juice adaptable for use on cottage industry scale has been fabricated and found economically viable (Akinwale *et al*., 2001; Oduwole *et al*., 2001). Developed cashew meal from the kernel including bread, candy, cake, and biscuits coated with chocolate with good and acceptable organoleptic properties. Cashew nut shell has been incorporated into fertilizer composition and hydraulic paints. There has been development of improved technique for processing cashew apple into wine, jam and non-alcoholic beverage of high nutritional value with vitamin C content of 170-180 mg/100 ml juice.

Production of Citric Acid from Cashew Apple Juice

The cashew apple is about 60-80% liquid and the extraction can be done using juice extractor. The collection of matured apple fruits and separation of the nuts from the apples is the first stage (Figure 5). The extracted juice, according to Adeoye and Lateef (2021), must be conditioned to suitable concentration for microbial fermentation for citric acid recovery. In a report by Adeoye and Lateef (2021), the extracted cashew juice was adjusted to 12 ^{o}brix by addition of 10% sucrose and the pH adjusted to 6.5 using 0.1 M NaOH. The fermentation process of the cashew apple juice was done using *A. niger*. In this process, the choice of submerged fermentation was the option, because the substrate was in liquid form. The inoculum of *A. niger* was introduced into a batch system, the choice of continuous system may be adopted in an industrial scale production (Adeoye & Lateef, 2022). Citric acid fermentation temperature varies from one process to another; many authors adopted 30 ± 2 ^{o}C (Adeoye *et al.*, 2015; Adeoye & Lateef, 2021; Adeoye & Lateef, 2022). The fermentation days also depends on the system adopted, the substrate concentration and ability of strain of the organism used to resist low pH. The recovery process, which is the downstream process involve filtration, centrifugation, precipitation and separation as shown in Figure 6.

Strain Improvement/Supplementation for Citric Acid Production

The diversity of microorganisms may be exploited still by searching for strains from the natural environment that is able to produce products of commercial value. The first stage in strain improvement is isolation, in obtaining either pure or mixed cultures. The cultures obtained needed to possess traits desired, and the culture must be adaptable for desired reaction or product. The screening may involve both primary and secondary screening process (Ozdal & Kurbanoglu, 2019). To improve the process productivity and yield of citric acid, either physical or biological parameters require modification. In this respect, strain improvement has become an important activity. The improvement of citric acid producing strains has been

carried out by mutagenesis and selection. The most employed technique has been by inducing mutations in parent strains using mutagens. Among mutagens; γ-irradiation, UV irradiation and chemical mutagens are often used. It is reported that UV treatment can frequently be combined with some chemical mutagens (Adeoye *et al.*, 2015; Mores *et al.*, 2020). Successive treatments with physical and chemical mutagens followed by testing a large number of colonies will be necessary before strains with improved performance can be isolated (Yalcin *et al.*, 2010).

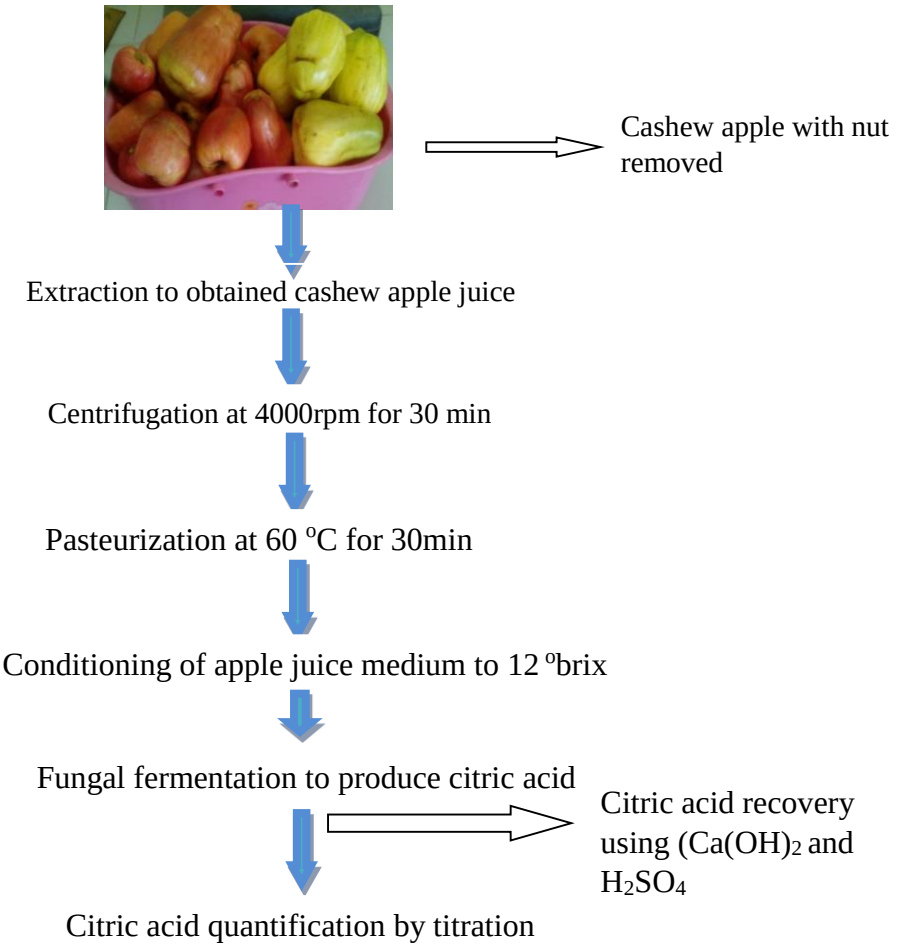

Figure 5: Citric acid production process from cashew apple juice

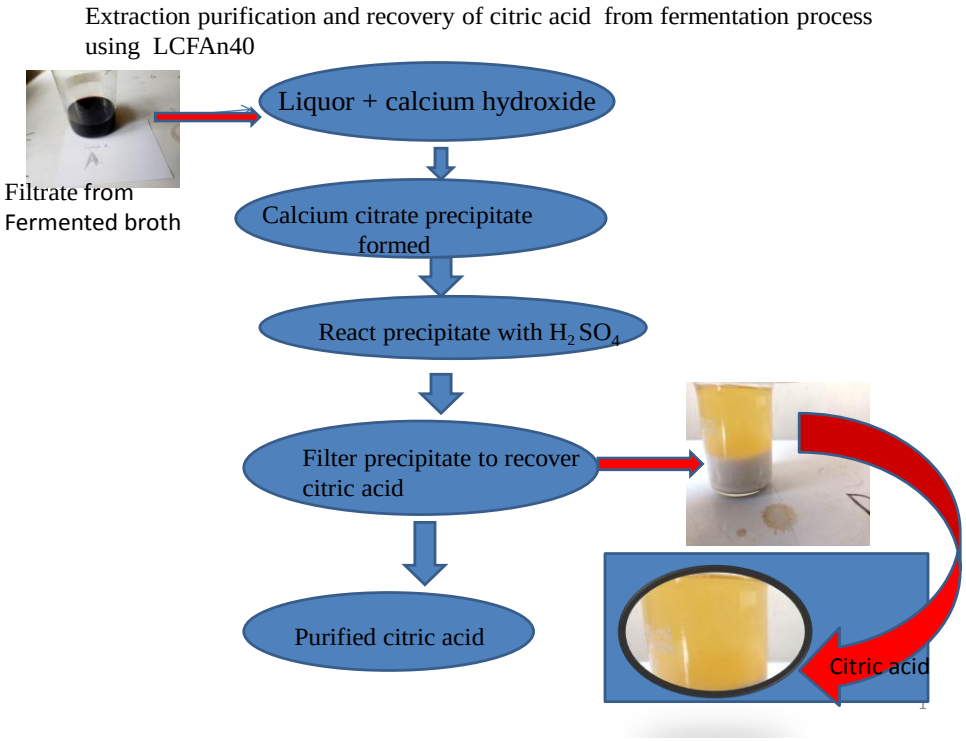

Figure 6: Extraction, purification and recovery of citric acid (Adeoye & Lateef, 2022)

Improvement of Microbial Strains

Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes (Sawant *et al.*, 2021). Conventionally, strain improvement has been achieved through mutation, selection or genetic recombination. Overproduction of primary or secondary metabolites is a complex process and successful development of improved strains required knowledge of physiology, pathways regulation and control and the design of creative screening procedure (Kola & Goli, 2012; Fiedurek *et al.*, 2017; Tong *et al.*, 2019).

Mutation as a means of strain improvement may be achieved through induced, physical agents, biological agents or chemical agents. For instance, UV-radiation generates cyclobutane dimers, usually thymine dimers, between adjacent pyrimidines which may lead to different gene sequence that may express new protein production. Other examples are ionizing radiation, chemicals and other biological agents (Willy *et al.*, 2011; Rajesh *et al.*, 2010; Fiedurek *et al.*, 2017). The effects of a mutation can be described at the protein expression level and in terms of observed phenotypes. In all cases, the impact is readily noticeable only if it produces a change in phenotypes. In general, the more prevalent form of gene and its associated phenotype is called the wild type. A mutation from wild type to a mutant form is called a forward mutation (Willy *et al.*, 2011; Fiedurek *et al.*, 2017). In addition, strain improvement with the use of mutagens required good knowledge of fermentation process for each new fine-tuning of process conditions (Willy *et al.*, 2011; Tong *et al.*, 2019). The improvement of citric acid producing strains has been carried out by mutagenesis and selection. The most employed technique has been by inducing mutations in parental strains using mutagens (Adeoye *et al.*, 2015). Mutants of *A. niger* have been used for the commercial production of citric acid (Jianlong, 2000). Among mutagens, irradiation by UV radiation and chemical mutagens are prevalent with the single-spore technique and the passage method are the principal methods of selecting strain (Rajesh *et al.*, 2010; Steiger *et al.*, 2019).

Mutagenesis

Mutagenesis is a process by which the genetic information of an organism is changed in a stable manner resulting in a mutation. It may occur spontaneously in nature or as a result of exposure to mutagens. It can also be achieved experimentally using laboratory procedures (Chatterjee & Walker, 2017). DNA may be modified, either naturally or artificially by a number of physical, chemical and biological agents, resulting in mutations. Mutation is the permanent alteration of one or more nucleotides at a specific site along the DNA strand. The strain that harbors the mutation is called a mutant strain. Mutations may be associated with the change of a single nucleotide (point mutation); it may be through substitution, deletion or rearrangement of one or more nucleotide base pairs in the chromosome. Mutation may also occur as a result of faulty re-union of DNA. Most mutations often occur at low frequency at any point along the gene (10^{-5}-10^{-10}/generation) (Willy *et al.*, 2011; Chatterjee and Walker, 2017). In many cases mutations are harmful, but certain mutation occur that make the organism better adapted to its environment and improve its biocatalytic performance. The potential for a microbe to mutate is an important property of DNA since it creates new variation in the gene pool. The major constraint is in selection process to isolate those strains which are true mutants with desired quality with benefiting products (Bernstein & Bernstein, 2013).

Mutagenesis may occur endogenously, for example through spontaneous hydrolysis or through normal cellular processes that can generate reactive oxygen species and DNA adducts or through error in replication and repair (Park & Pursell, 2019). Mutagenesis may also arise as a result of the presence of environmental mutagens that induces changes to the DNA. The mechanism that induces changes to the DNA varies according to the causative agent, the mutagen-involved. Most mutagens act either directly or indirectly via mutagenic metabolites on the DNA producing lesions some of which may affect the replication or chromosomal partition mechanism and other cellular processes (Bernstein & Bernstein, 2013).

Ultra-violet radiation promotes the formation of a cytobutyl ring between adjacent thymine, resulting in the formation of pyrimidine dimers. Some alkalating agents may produce crosslinking of DNA. Some naturally occurring chemicals may also promote cross-linking, such as psoralens after activation by UV radiation and nitrous acid (Chatterjee and walker, 2017; Rajesh *et al*., 2010; Pfeifer 2020). To obtain mutants of fungal strains, the use of ultraviolet light is employed and it has been established to have better result (Adebayo *et al.*, 2012). The ultraviolet radiation is more employed than other methods because of its simplicity, inexpensive nature and its availability as an effective germicidal tools and it has been preferred to X-rays. The germicidal effect and the absorption maximum of nucleic acid at 260 nm is relative (1 nanometer = 10 angstroms) (Rajesh *et al*., 2010; Elango *et al.*, 2019), which is within the range of the wavelength intensity exerted by germicidal lamp. In fact, most germicidal lamp delivered a broader range of wavelength, but experimentation demonstrated that 260 nm is the effective wavelength. This wavelength has also been shown to increase mutation frequency. There are usually germicidal and mutagenic effects which lead to covalent bonds between adjacent pyrimidine nucleotides, generally thymine and sometimes cytosine dimers. These linked pyrimidine nucleotides are referred to as dimers, which be thymine or cytosine dimers depending on the specific nucleotides that are bond together (Adebayo *et al.*, 2012). The result of dimer formation is an inhibition of normal DNA synthesis. Though there are repair mechanisms that can repair the UV-induced damages, the repair mechanism require re-exposure to wavelengths in the approximate range of 360-480 nm. Light in this rage has the effect of activating enzymes that split the dimer, thus permitting return to normal DNA synthesis and loss of the new coding strand. The light induced repair is called photo reactivation (Adebayo *et al.*, 2012).

In experimental work in which UV is used to obtain mutants, it is important that cell should not be exposed to the range of light intensity that will cause or induce photoreactivity.

This is accomplished by using yellow light illumination of the work area to prevent photo reactivation (Adebayo *et al.*, 2012; Elango *et al.*, 2019). When there is dimer formation as a result of mutation by UV exposure, it is followed by new coding enzymes by rRNA and change in DNA replication. The dimer is responsible for the gaps in DNA strands that are synthesized. This method of mutagenesis is different from the mode of action of ionization radiation like X-rays which causes alteration in the bases of the DNA strands. Ultraviolet radiation has been reported as one of the best physical methods for strain improvement for better yield performance and the method has been employed in improving enzyme production in *A. niger* (Adebayo *et al.*, 2012).

Optimization of Citric Acid Production

The need for sustainable production to meet the market requirements in a cost effective manner has put forward a challenging demand. To overcome this challenge, optimization, strain improvement and genetic manipulation has been utilized. Various microorganisms have been reported to produce an array of primary and secondary metabolites, but in a very low quantity. In order to meet the market demand, several high yielding techniques have been discovered in the past and have been successfully implemented in various processes. Optimization technique that has been used commonly include: One-factor-at-a-time (OFAT), factorial method, response surface methodology (RSM), artificial neural intelligence (ANI) and Taguchi optimization technique. The applications of these various techniques have improved the yield. Table 2 shows examples of the methods and the improvement in the yield; Adeoye *et al.* (2015) used RSM method to improve the citric acid yield from 0.193 g/100 ml to 8.873 g/100 ml through optimization. In the work reported by Adeoye and Lateef, (2021) using optimization by Taguchi technique, there was improvement in yield from 3.20 g/100 ml (wild strain) to 16.67 g/100 ml**.**, Betiku and Adesola through response surface methodology improved the yield from 45.0 g/l to 83.01 g/l. Bari *et al.* (2009) obtained an improved citric acid production in solid state fermentation of oil palm EFB. Imandi *et al.* (2007) obtained improvement in citric acid production from 60.85 to 77.39 through medium optimization. Some of the examples of applications of optimization in the production of citric acid are presented in Table 2. Medium optimization strategies are used to improve production which has been applied in chemical, food pharmaceutical and engineering design with objectives of increasing the yield and activities of the desired product (Singh *et al.*, 2017).

Table 2: Optimization techniques and the effect on citric acid production

Reference	Techniques	Substrate	Organism	Effect of Optimization		
				Initial	Optimization	%
Imandi *et al.* (2007)	RSM	Glycerol	*Yarrwia lipolytical*	60.85 g/l	77.39 g/l	78.63 %
Imandi *et al.* (2008)	RSM	Pine apple waste	*Yarrwia lipolytical*	199.89g/l	202.35g/l	98.78 %
Alam *et al.* (2008)	CCD	Palm oil mill effluent	*A. niger*	1.5g/l	5.37g/l	27.93 %
Barrinton and Kim (2008)	CCD	Dry Peat Moss	*A. niger*	-	354.8	2.7 fold
Bari *et al.* (2009)	OFAT	Oil palm fruit spent bunches	*A. niger*	128.9 g/kg	218.6 g/kg	58.96 %
Dhillon *et al.* (2011)	RSM	Apple Pomace Sludge	*A. niger*	-	44.9 g/ 100g	49.9 %
Dhillon *et al.* (2011)	RSM	Apple Pomase	*A. niger*	248.42 g/kg	342.40	72.55 %
Betiku and Adesola (2013)	RSM	Sweet potatoe starch hydrolysate	*A. niger*	45.0 g/l	83.01g/l	45.20 %

Adeoye *et al.* (2015)	RSM	Cassava peel	*A. niger*	0.193 g/100ml	8.873g/100ml	2.2 %
Adeoye and Lateef (2021)	Taguchi	Cashew apple	*A. niger*	3.20 g/100ml	16.67 g/100ml	19.20 %

Note: RSM; Response Surface Methodology, OFAT; One- Factor- At- A- Time

Industrial Production of Citric Acid and Applications

Citric acid production by microbial processes takes about 99% of world production, through majorly surface or submerged culture fermentation processes. The product is sold as anhydrous or monohydrate acid, and about 70% of the total production of between 1.5 – 1.7 million (Nielsen *et al.*, 2017; Wang *et al*., 2020) is used in food and beverage industry as an acidulant or antioxidant to preserve or enhance the flavor and aromas of fruits juices, ice creams, and marmalades. Pharmaceutical industry uses about 20% in drugs as antioxidant to preserve vitamins, effervescent, pH corrector, blood preservatives, or in the form of iron citrate as a source of iron for the body. These are produced in form of tablets. It is also used in ointments and cosmetics preparations. In the chemical industry, the remaining 10% is employed as a foaming agent for the softening and treatment of textile. In metallurgy, certain metal are utilized in the form of citrate. Citric acid is also used in detergent industry as a phosphate substitute because of less eutrophic effect, and in cement where it is added to slow down the hardening of cement (Max *et al*., 2010; Tong *et al.*, 2020).

Although many strains of bacteria produce citric acid, only few mutants of *A. niger* and *A. wentii*, which are closely related species, are used for industrial production. All enzymes are expressed during the idiophase, except α-ketoglutarate dehydrogenase. The activity of citrate synthetase increases by 10 times, whereas those of the aconitase and isocitrate dehydrogenase are reduced slightly compared to the trophophase. During the trophophase, glucose is mainly used for biomass production and oxidized to carbon dioxide via respiration, whereas during the idiophase, losses by respiration are minimal and the substrate is almost entirely converted to organic acids (Max, 2010; Tong *et al.*, 2020).

The carbon sources commonly used in industrial fermentation are sugar solutions ranging from 15 to 25%. Since the microorganisms used have amylases and invertases, which make them capable of hydrolyzing polysaccharides and other disaccharides and polysaccharides like maltose, sucrose, lactose, and starch. Simultaneous saccharification and fermentation processes can be carried out (Behera, 2020) using as substrates potato starch, sugarcane and sugar beet molasses, among other residues. If glucosidic-hydrolysates are used as culture medium, a pretreatment with precipitating agent or ion exchange resins is necessary to remove cations and then promote the metabolic dysfunction responsible for citric acid accumulation. Alternatively, molasses are often treated with calcium hexacyanoferrate to precipitate heavy metals. Appropriate strains must be selected according to carbon source. To evaluate the optimal cultivation conditions for molasses, no general method of analysis has been developed; hence, selective method for preliminary test of fermentation must be carried out before pilot scale production using the preferred strain. Companies using molasses often optimize fermentation conditions in 30 m^3 pilot plant before moving to production (Khattab *et al.*, 2017).

Yield can be maximized by using Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ze^{2+} and Mo^{2+} ion in concentrations in ppm. Furthermore, the initiation of overflow metabolism is related to limitation of these trace metals as listed above (Paulisen *et al.* 2012); above this limitation the process is affected negatively. For example, iron, one of the cofactors of aconitase, plays a crucial role, favoring biomass growth at concentrations higher than 2 ppm, or citric acid overproduction at concentration of only 0.05-0.5 ppm depending on the substrate used. Interestingly, Cu reverses the effect of Fe. However, sensitivity of microorganisms decreases with increasing temperature (Mahendraprabhu & Elumalai, 2016). In industrial setting, pH is another crucial parameter, which is set at around 5 at the beginning of the trophophase, drop to

3 within the first 48 h of trophophase as result of the nitrogen metabolism, and is then kept at this value during the idiophase to inhibit the formation of oxalic and gluconic acids (Max *et al*., 2010; Kareem *et al*., 2013; Romsdahl *et al.*, 2019).

Conclusion

With increasing demand for citric acid, there is need to explore new area of potentials to meet the growing demand of this commodity as an organic chemical. The use of cashew apple and many other agro-wastes are still green area with high potentials which are yet to be thoroughly exploited. Strain improvement and optimization of process will compliment cheap agro-waste raw material to reduce cost and improve yield of citric acid. Many substrates that are by-products of agro-industrial processing have been reported as useful for citric acid production, but limited account is available on cashew apple which this review has given an insight into.

Author Contribution

A.O. Adeoye: Formal analysis, Investigation, Methodology, validation, writing of the manuscript. G.M. Adegbola: Investigation, Methodology, validation. A. Lateef: Conceptualization Writing- review and editing.

Conflict of Interest Declaration

The authors declare that there is no conflict of interest in the work and its publication.

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