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Frugal Utilization of Flue-Cured Virginia *Nicotiana tabacum* Leaf Wastes as a Vicissitudinous Substrate for Optimized Synthesis of Pyridine-3-Carboxylic Acid

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Abstract: Agrotransformation of tobacco leaves into cigarettes and cigars spawns upto 75% wastes which is an environmental and public nuisance owing to its noxious 0.6-3% (w/w) 3-(1-methyl-2-pyrrolindyl) pyridine (MPP) content. Considerately, this volumetric agrowaste is a prodigal loss during tobacco processing. Consequently, the utilization of these frugal wastes as a substrate for pyridine-3-carboxylic acid (PCA) synthesis is a green strategy to obliterate the ecological backlashes of tobacco waste. This concerted study reported the feasibility of utilizing Flue-Cured Virginia (FCV) tobacco waste as a starting substrate for synthesis of pyridine-3-carboxylic acid through MPP as a synthetic intermediate. The intermediate was extracted from powdered FCV wastes using petroleum ether and subsequently oxidized to PCA using 69% concentrated Nitric acid of volumes: 120, 115, 110,105, 100, 95, 90 and 85ml at 87±2°C. The results of the bench scale experiments indicated that the yield of PCA increases with increase in the volume of hot nitric acid; a maximum yield of 25ml was obtained with 100ml of hot nitric acid. The lowest yield of 17ml was from 85ml of hot nitric acid. MPP had a statistical mean boiling point of 249.3±2.082°C, mean density of 1.024±0.006g/cm^3 whereas PCA had a mean boiling point of 262±3°C, mean density of 1.505843±0.05503g/cm^3, mean pH of 3.3±0.19 and a computed mean solubility of 1.5±0.017g/L. The study has shown that FCV tobacco leaf wastes is a green environmental substrate for organic synthesis of pyridine-3-carboxylic acid.

Keywords: Arua, Tobacco, Flue-Cured Virginia, Leaf Wastes, Strategy

1. Introduction

Tobacco have a long smoking history traced back to a couple of centuries ago. First found by Christopher Columbus on his 1492 landing in the Americas, yearthousand art carvings in Central America indicate tobacco smoking dates as early as 1000 B. C. Tobacco is a non-aboriginal crop which was introduced in Uganda during the colonial days. Uganda cultivates two leaf varieties: FCV in West Nile, middle North and North Kigezi, and Burley tobacco in Bunyoro particularly Mubende [1]. Tobacco output accentuated following British American Tobacco (BAT) monopolization of Uganda as the prime source of its tobacco leaf, furnishing 20 nations with approximated exports of US $50 million in 2010 and half a metric ton mean FCV tobacco output per farmer in Arua [2].

The exodus of BAT from Uganda due to stringent international bills and thus the Uganda Tobacco Control Bill promulgated in the Ugandan parliament on its reading of the 28th day of July 2015 led to a decline in tobacco farming. The bill stipulated smoke free public places, tobacco health risks pictorial warnings, prohibited tobacco industry interference, forbade cigarette sales to and by Ugandans below 21 years as well as banned smoking 50 meters near public gatherings [3].

Meridian Tobacco Company is a mega dollar establishment in Arua that carried on with growing and processing of FCV tobacco. Its full processing activities commenced in 2014, on an area of 32,000m^2 and with 30,000 metric ton annual production. Right from harvest, wastes constituted by rejected tobacco foliage, dust, blade bits, petioles and stems accumulates. Nicotine (MPP), PCA (nicotinic acid), nicotinamide, rutin, pectin and other acids can be synthesized from the waste. PCA is an essential nutrient (known as Vitamin B_3) with nutritional sources from milk, green vegetables, coffee, yeast, meat, poultry, red fish, legumes, seeds and tea [4]. Medically, it treat AIDS [5, 6], cancer [7-10], hyperlipidemia, diabetes [11-13] and cardiovascular diseases associated with high cholesterol levels [14]. Solubilized MPP is a bioinsecticide and a fumigant for thrips, aphids and leaf hoppers [15] whereas PCA is a vitamin with coenzyme derivatives playing pivotal roles in anabolic and catabolic processes.

Pyridine-3-carboxylic acid is synthesized by oxidation of the methyl group of 3-picoline to the carboxyl derivative. The substrate with pyridine in a 1:2 ratio are presynthesized by reaction of acetaldehyde, formaldehyde and ammonia. They are first converted to 3-cyanopyridine by ammoxidation, followed by hydrolysis to pyridine-3-carboxylic acid [16] (Figure 1).

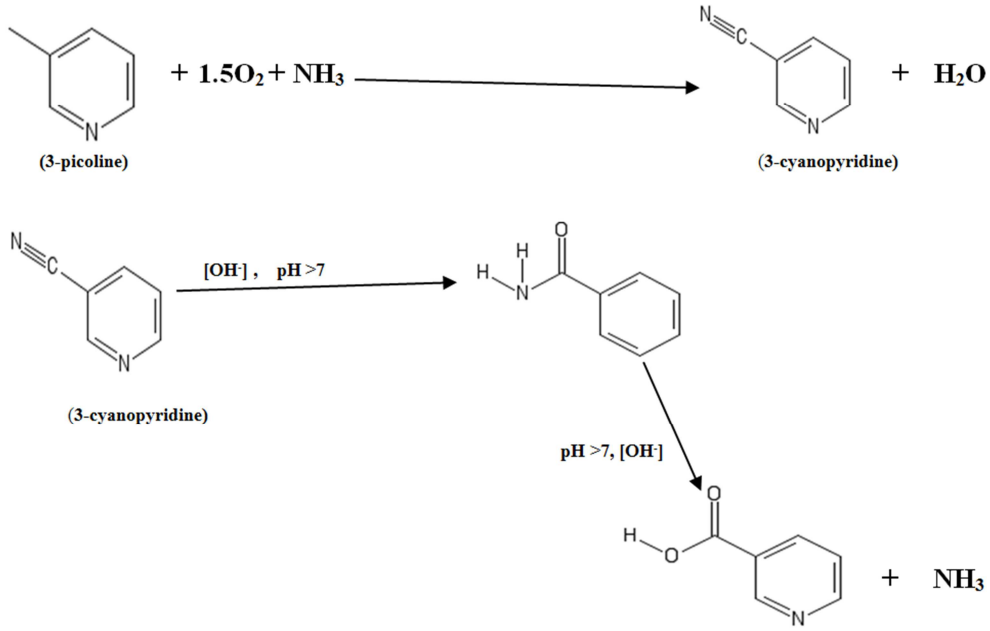

Figure 1. *Gas phase ammoxidation of 3-picoline followed by hydrolysis to pyridine-3-carboxylic acid.*

For a golden jubilee now, gas-phase oxidation of 3-picoline using Vanadium oxide catalysts has been exploited in PCA synthesis. A selective efficient reaction is however nearly impossible as the acid is less stable than the synthetic intermediate and decarboxylates readily, desubliming below 200°C. Picoline is thus selectively oxidized in liquid air with a catalyst combination employed in an acetic acid milieu at elevated temperature and pressure [16].

PCA can also be synthesized from MPP extracted from tobacco waste. Water and methanol were evaluated by Rasjul *et al* [17] for PCA and MPP extraction from cigarette and cigarette smoke. Methanol gave the best yield whereas water required intense heating coupled with constant stirring and evidently larger solvent volume.

Mulyadia *et al* [18] showed that use of a perfect ratio of ether and petroleum ether optimized the extraction time and yield of nicotine from tobacco leaves. They utilized response surface method with central composite design consisting of addition of the solvents. Their findings revealed that addition of ether and petroleum ether influenced the response time of extraction and yield. The obtained optimal solution was 59.46ml of ether and 30.12 ml of petroleum ether. The shortest extraction time was 477.343s and the longest was 887.623s.

Extraction experiments were performed to isolate nicotine in tobacco stems by Purwono and his co-researchers [19]. They employed ethanol, kerosene, hexane and steam. Extraction was perfected with the sample in an extractor connected to the solvent tank with the solvent close to its boiling point. Ethanol gave the best extraction yields with upto 100% of the nicotine in the tobacco stems recovered compared to kerosene, hexane and steam [19].

Green leaf lamina of tobacco was processed for the recovery of proteins, nicotine, solanesol and organic acids by Mahendra and co-authors [20]. Part of the leaf was air cured and other flue-cured. The samples dried rapidly under infrared radiation had 1.27-3.25% nicotine, 0.25-2.14% solanesol on the dry basis and 0.88-3.08% protein.

Biocatalytic conversion of 3-cyanopyridine improves PCA yield. Experimental 3-cyanopyridine conversion to PCA using resting *Rhodococcus rhodochrous* J1 cells having benzonitrilase activity was done by Mathew *et al* [21]. This process prognosticates industrial synthesis with 100% conversion under mild conditions and cheap cell cultivation.

Peter *et al* [22] converted 3-cyanopyridine to PCA using

Nocardia rhodochrous LL100-21. The potential to bioconvert 3-cyanopyridine to PCA plus ammonia was induced in the stationary phase cultures of the bacteria by addition of 2-, 3-, or 4-cyanopyridine or benzonitrile; benzonitrile gave maximal induction. Stoichiometric conversion was achieved up to 0.5M 3-cyanopyridine to PCA. Both 3-cyanopyridine and PCA inhibited further hydrolysis. Bacteria immobilized in calcium alginate beads and used in column bioreactors retained 3-cyanopyridinase activity for over 150 hours with continuous feed of 0.3M 3-cyanopyridine.

A thermostable *nitrilase* harnessed from thermophilic *Bacillus pallidus* Dac521 biocatalyzed the direct hydrolysis of 3-cyanopyridine to PCA without detectable formation of nicotinamide. Total conversion was achieved at 76nmol min/mg/dry cell mass [23] at optimum conditions of 60℃ and pH 8.0 with no detectable mass transfer limitation at maximum cell loading. Kaplan [24] biotransformed 3-cyanopyridine into PCA using fungal nitrilases.

Maira *et al* [25] assessed amidase-catalyzed production of PCA in stirred reactors. Temperature effect, cell load and substrate feeding strategy were assessed with controlled continuous stirred membrane bioreactors. Enzymatic PCA synthesis was most feasible in prototrophs and tryptophan auxotrophs of *Saccharomyces cerevisiae* as they utilized tryptophan for the synthesis in aerobic conditions [26].

This study reported the feasibility of utilizing FCV waste as a green substrate for oxidative synthesis of PCA via MPP as a synthetic intermediate extracted with petroleum ether.

2. Materials and Methods

A twin design approach involving field sampling and laboratory analysis was employed in this study.

2.1. Apparatus and Reagents

Chemicals used in this investigation were of high analytical purity. Volumetric glassware used were sterilized in an autoclave at 121℃ for 15 minutes and oven dried prior to analysis. Mettler PM200 analytical balance (Marshall scientific, USA) was used for weighings. Hanna 211 microprocessor-based bench pH meter (Hanna instruments, Italy) precalibrated using pH 4.01, 7.01, 10 buffers was used for all pH measurements.

2.2. Sample Collection

10kgs of FCV tobacco wastes were collected from the curing site of Meridian Tobacco Company (Arua, West Nile, Uganda). The samples, representative of the bulk waste in the curing unit were transferred into polyethylene packing bags and taken to Department of Biotechnology, Uganda Industrial Research Institute (UIRI), Nakawa, Kampala.

2.3. Extraction of 3-(1-Methyl-2-Pyrrolidinyl) Pyridine

200g of FCV tobacco waste was ground using an electric blender. An aliquot (10g) of fine powder was weighed into a 250ml beaker. 100ml of Sodium hydroxide solution was added and stirred for 30mins using a magnetic stirrer. The mixture was filtered using Buchner glass wool and the residue transferred to a separate beaker. 30ml of distilled water was added to the residue and stirred for 30 mins. It was filtered, and the filtrate collected was re-filtered to remove all the impurities. 25ml of petroleum ether was added to the filtrate, stirred using a magnetic stirrer for 5 minutes and then transferred into a separating funnel. The two layers in the separating funnel were separated and the top layer which is the MPP-ether layer was transferred into a 250 ml conical flask. The conical flask containing the mixture was placed on the water bath maintained at 50-60℃ to evaporate the ether. The concentrated sample in the flask (MPP) was subsequently utilized in synthesis of Pyridine-3-carboxylic acid.

2.4. Synthesis of Pyridine-3-Carboxylic Acid

A measured 200ml of 69% Nitric acid was measured into a reaction flask and heated to 95℃ on a hot plate. 120ml of 69% Nitric acid was measured into a flask followed by 20ml of MPP. To the mixture in the conical flask, 21ml of water was added. The cold mixture was gradually poured into the hot concentrated nitric acid over a period of 2 hours forming pyridine-3-carboxylic nitrate and a temperature of 87±2℃ was maintained while stirring slowly. A slow stream of air was blown over the surface of the heated mixture to concentrate until the crystals of PCA nitrate begun to separate out. The crystal residue was diluted with 18ml of cold water and re-heated to 60℃ to give a clear solution. The experiment was repeated for volumes of hot concentrated nitric acid from 115ml to 85ml at reducing intervals of 5ml.

2.5. Physical Properties of the Synthetic Intermediate and Pyridine-3-Carboxylic Acid

2.5.1. Density

A dry density cup was weighed when empty and its mass, *a* g recorded. The density cup was then filled with the sample of organic liquid and carefully covered without tilting the cup. The overflowing liquid was removed carefully with a cloth. The density cup with the organic liquid was then weighed and its weight *b*g was recorded. The density, ρ in g/cm^3 was computed from (1):

$$\rho = (b\text{-}a) \quad (1)$$

2.5.2. Boiling Point

The sample of the solution was introduced into a micro test tube using a Pasteur pipette and a piece of melting point capillary tubing sealed at one end was dropped in with the open end down. The micro test tube assembly was then attached to a (300℃) thermometer with a rubber band. The whole unit was then placed in a Thiele tube. The lower part of the side arm of the Thiele tube was carefully heated with a small flame from the Bunsen burner moving the flame back and forth along the arm. During the heating, there was an initial stream of bubbles as air was expelled and then, a little later, a rapid and continuous stream of bubbles emerged from the inverted capillary tube. At this point, the heating was

stopped. Soon the stream of bubbles slowed down and stopped. The liquid sample was drawn up into the capillary tube and its temperature was recorded.

2.5.3. pH

An aliquot (5ml) of pyridine-3-carboxylic acid was poured into a clean beaker. The pH electrode of the pH meter was dipped into the solution and stirred using the magnetic stirrer. The pH reading was recorded when the pH reading was stable.

2.5.4. Solubility

2g of crushed PCA crystals was weighed and transferred into a test tube. An aliquot (6 drops) of water was added and stirred until a homogenous solution was formed. A little amount of water was added to ensure complete dissolution in water. The volume of the resultant solution was measured using the measuring cylinder.

3. Results and Discussion

Statistical analysis of the results of the analyses done in triplicate was done using Microsoft excel 2016.

3.1. Properties of the Synthetic Intermediate

The synthetic intermediate (MPP) was a white solid. The key evaluated properties are given in Table 1.

Table 1. *Boiling point and density of 3-(1-methyl-2-pyrrolidinyl) pyridine.*

Sample	Boiling point °C	Density (g/cm^3)
1	248.0	1.0121
2	254.0	1.0250
3	250.0	1.0341

MPP had a statistical mean boiling point of 249.3±2.082℃ (Table 1). The American Standards for Testing Materials (ASTM) standard boiling point for MPP is 247℃. Statistical analysis of results gave a mean density of 1.024± 0.006g/cm^3 (Table 1). ASTM standard density is 1.02g/cm^3. This proved the extracts from FCV tobacco wastes have properties close to MPP from other sources. The slight deviations from the standards may be ascribed to experimental conditions used.

3.2. Properties of Pyridine-3-Carboxylic Acid

With varying volumes of hot conc. nitric acid, the respective volumes of PCA formed were measured and tabulated (Table 2).

Table 2. *Physical properties of pyridine-3-carboxylic acid.*

Sample	B. Point (°C)	Density (g/cm^3)	pH	Solubility (g/L)
1	258	1.4235	3.4	1.52
2	252	1.5643	2.6	1.50
3	265	1.7234	3.6	1.56
4	275	1.4325	4.0	1.46
5	260	1.4452	4.0	1.48
6	266	1.3247	2.7	1.52
7	264	1.6523	3.5	1.44

Figure 2. *Pyridine-3-carboxylic acid crystals on the walls of reaction flask.*

It was observed that the PCA crystals were white (Figure 2), odorless and formed a clear solution on dissolution in water. The resultant solution gave no color change with 2,4-dinitrochlorobenzene and sodium hydroxide at 60-70℃. Available literature confirms that PCA and its immediate derivatives are pyridine ring compounds and shows no color change with 2,4-dinitrochlorobenzene. This confirms that the compound synthesized in this study is pyridine-3-carboxylic acid. The results proved that PCA extracted from FCV tobacco wastes exhibit similar characteristics as that reported in literature [15]. Though there where deviations from the standards, this could be due to conditions not achieved in the laboratory and some errors such as due to parallax.

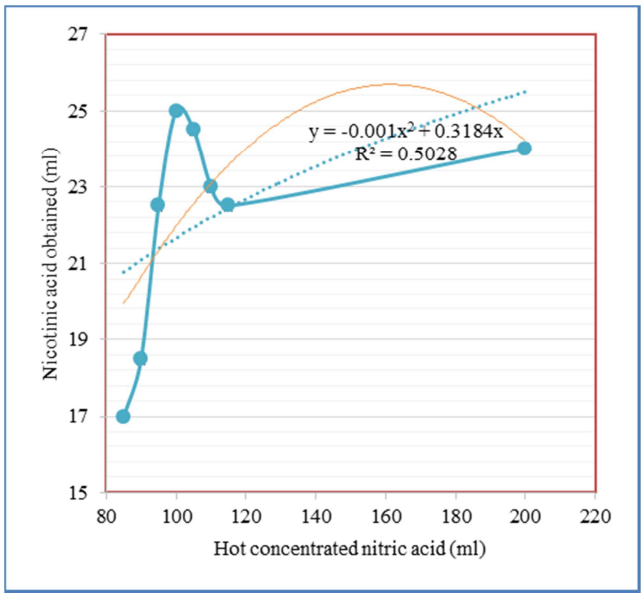

Figure 3. *Variation of pyridine-3-carboxylic acid yield with volume of hot concentrated nitric acid used.*

The statistical mean boiling point was 262±3℃ whereas ASTM boiling point of pyridine-3-carboxylic acid is 260℃.

The mean density of pyridine-3-carboxylic acid was 1.505843±0.05503g/cm^3 (Table 2). The ASTM density is

1.5g/cm^3. It is worth noting that the boiling point of pyridine-3-carboxylic acid obtained in this study is comparable with that reported in literatures [15]. Higher boiling point of pyridine-3-carboxylic acid is attributed to strong intermolecular force of attraction between molecules within the acid solution. The cyclic nature of the acid and higher number of carbon atoms within the chain increases the force of attraction. It thus requires more energy to overcome these forces of attraction. In solution, the positive end of one molecule is attracted by the negative end of another molecule. That means the acid molecules are affected by opposite charge effect hence greater polarity and higher boiling point. There is always vapor in equilibrium with a heated liquid. This gives rise to the initial stream of bubbles.

Statistical mean pH of pyridine-3-carboxylic acid was 3.3±0.19 (Table 2) though the ASTM pH range is between 3.0-4.5. The acid samples had a statistically computed mean solubility of 1.5±0.017g/L (Table 2). The standard ASTM solubility is 1.5g/L. The solubility of the acid is attributed to polar and electronegative elements: oxygen and nitrogen. The polarity of the acid depends on polar C-N, C-OH and C-OH groups. Its low solubility is due to the effect of size of the carbon in its chain on polarity hence cannot easily form hydrogen bonds.

4. Conclusion

This research has shown that Flue Cured Virginia tobacco leaf waste can be utilized as a starting substrate for the optimized oxidative synthesis of pyridine-3-carboxylic acid.

The yield of pyridine-3-carboxylic acid increases with increase in the volume of hot nitric acid. A maximum yield of 25ml can be obtained with 100ml of hot nitric acid. The lowest yield of 17ml can be obtained from 85ml of hot 69% nitric acid.

Meridian Tobacco Company should undertake the initiative of using tobacco waste from its establishment for production of pyridine-3-carboxylic acid to reduce on the environmental degradation caused by the waste.

Further research should be done with microorganisms and compared to ensure maximum extraction of nicotine from the waste.

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