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Inhibition of urea Hydrolysis of human urine using lactic acid from selected fruit and vegetable waste fermentation

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ABSTRACT

In Kenya, enormous amounts of fruit and vegetable waste are improperly disposed of, contributing to environmental pollution and odour. Meanwhile, urine contains nitrogen that can be used as a fertiliser, but its utility is impeded by urea hydrolysis, which causes a rise in pH, nitrogen loss, and ammonia formation. This study investigated the potential of lactic acid fermentation utilising fruit and vegetable waste to prevent urea hydrolysis, enabling nitrogen recovery. Anaerobic fermentation of selected fruit and vegetable waste was carried out in an incubator at a regulated optimum temperature of 37°C for 72 hours. The lactic acid formed was then utilised to treat urine samples for 4, 7, and 10 days. To assess urea hydrolysis inhibition, total nitrogen content was measured using the Kjeldahl method, and pH monitored with a pH meter. The results showed a considerable decrease in the stabilised urine's pH, ranging from 6.1 to between 3.6 and 3.9. The pH for the untreated urine rose to between 7.5 and 8.5 across the days. Statistical analysis using the one-way ANOVA indicated significant difference in the pH across the days (P = 0.047). The highest total nitrogen concentration for the stabilised urine was 2450 mg/L, after seven days of treatment, demonstrating urine stability and nitrogen preservation. The total nitrogen concentration for untreated urine was approximately 607 mg/L across the days, indicating clear nitrogen loss from the original 2643 mg/L obtained in fresh urine. One-way ANOVA test demonstrated a statistically significant fluctuation in TKN concentrations over treatment durations (P = 0.021). The findings showed that lactic acid significantly suppressed urease activity, making it a cheap, ecologically friendly alternative for urine stabilisation. The results showed the effectiveness of lactic acid obtained from fruit and vegetable waste in inhibiting urea hydrolysis in urine, hence, enhancing the recovery of nitrogen nutrient.

Introduction

On-site sanitation systems supply 65% - 100% sanitation services in Sub-Saharan Africa. Of these, Urine-Diverting Dry Toilets (UDDTs) are relatively low-cost, minimise urea hydrolysis and allow for reuse of nutrients from excreta, hence low nutrient loss to the environment (*Riungu et al*., 2019). Collection and treatment of excreta play a crucial role in attaining Sustainable Development Goals (SDGs) regarding clean water, sanitation, sustainable cities and communities (*Xu et al*., 2022).

Human excreta constitute both urine and faeces. Most nutrients are expelled through urine. Human urine is an ultra potent blend of chemicals, including

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metabolic fragments of food, beverages and chemicals. Research by Llambrich et al., (2022) showed 400 volatile compounds in the urine, and more than 15 classes of chemicals, such as alcohols, carbonyls, hydrocarbons, carboxylic acids, among others. Ray (2020) reported that urine contains essential trace elements (such as B, Cu, Cl, Fe, Ni, Zn, Mn and Mo) and major nutrients (like Mg, Ca, and S), as well as three key macronutrients; nitrogen (N), phosphorus (P) and potassium (K). Nitrogen is a prominent macronutrient for crops production, and is a key nutrient that can be recovered from the urine (*Volpin et al*., 2019).

Focusing on nutrient retention and preservation in urine provides a sustainable solution to reduce the tension between the increased demand for food and fertiliser and nutrient deficiency (*Badeti et al*., 2022). As stated by the World Health Organisation (WHO), for urine to be safely used in agriculture, the urine should be stored in containers at a temperature of 22oC and the whole process should take no less than six months (World Health Organisation, 2018). However, storage alone is ineffective for excreta in source-separating toilets due to high nutrient loss as a result of hydrolysis. Stabilising urine as a nitrogen source allows excreta to be reused in a circular economy (*Adejumo et al*., 2019).

Because of its high biological activity, urine requires bacterial growth control measures to stabilise it and enhance nutrient recovery. Stabilisation is largely concerned with preventing urea hydrolysis in urine (*Yang et al*., 2021). Urea hydrolyses rapidly in the presence of the enzyme urease through four steps below: (*Yang et al*., 2021)

Stepwise reactions:

$(NH_2)_2CO + H_2O \rightarrow NH_3 + NH_2COOH$ (2.1)

Urea

$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3$ (2.2)

Carbamic acid

$H_2CO_3 \rightarrow H^+ + HCO_3^-$ (2.3)

$NH_3 + H_2O \rightarrow NH_4 + +OH^-$ (2.4)

Overall reaction:

$(NH_2)_2CO + 3H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^-$ (2.5)

Urea

Fresh urine usually has a pH ranging from 4.8 to 7.5 (*Kim et al*., 2020). However, urea hydrolysis leads to the production of ammonia and enhancement of the ratio of bicarbonate, causing increase in the pH ranges from 6 up to 9-9.3. The concentration of bicarbonate, and ammonia in urine rises, thus increasing the buffering capacity of urine. Since nitrogen is in the form of more highly volatile ammonia, it easily evaporates in air, hence losing nutritive value required for plant growth. Nitrogen in urine can be retained by developing an inhibiting mechanism for hydrolysis of urea (*Nagy et al*., 2019).

Depending on the state of existence, the nitrogen in urine can be recycled as either urea or ammonia (Ray, 2020). Recently, researchers have been studying and developing innovative and cost-effective technologies to boost nutrient recovery from urine and reduce ammonia volatilisation (*Zerihun et al*., 2021). Ion exchange, struvite precipitation, nitrification, acidification and ammonia air stripping are some of the common and studied processes for controlling loss of nitrogen from urine (*Mohamed et al*., 2024). However, the majority of these technologies are costly. The most prominent treatments for stabilising liquid streams are biological processes like partial nitrification, as well as chemical processes like acidification and alkalinisation (*Harder et al*., 2019). Techniques involving pH control by either acidification or alkalisation and the use of urease inhibitors can be used to stabilise urine by inhibiting urea hydrolysis (*Yang et a*l., 2021). Acidification (pH < 5) inactivates the urease-producing bacteria (*Moharramzadeh et al*., 2022).

According to Zerihun et al., (2021), keeping the pH below 4 by adding sulphuric and acetic acids (2.9 g/L) constrained urea breakdown, rendering bacteria inactive. However, most strong commercial acids are expensive. A simple and low-cost system for acidifying urine and reducing urea decomposition could be obtained using organic acids made from locally available waste materials to replace the strong acids. This would allow it to maintain its nitrogen component for agricultural uses (*Oishi et al*., 2023).

Organic acids obtained through biological techniques can provide a viable, low-energy solution with minimal operation and maintenance costs, resulting in a less expensive way of nutrient retention and urine stabilisation. Organic acids are common products of food fermentation and biotechnology.

Fermentation procedures have been used to produce acetic and lactic acids (*Sun et al*., 2020). Using organic acids to stabilise urine helps reduce the pH in urine, lowering the environment for hydrolysis by the urease enzyme. Most organic acids also have microbial activities that help kill microorganisms and pathogens that may contaminate the urine. An effective organic acid that can be utilised for stabilisation of urine is lactic acid (LA) obtained from fruit and vegetable waste fermentation (*Zerihun et al*., 2021).

Lactic acid ($CH_3CH(OH)COOH$), with a molar mass of 90.08 g/mol, is a chiral molecule having two optical isomers: Levo (L-lactic Acid) and Dextro (D-lactic acid) isomers (Ojo & De Smidt, 2023). It dissociates in water with a pKa of 3.86. It can be made chemically or by microbial fermentation of carbon sources. Fermentation looks more attractive than chemical techniques due to the growing eco-friendly awareness, safer, cleaner as well as sustainable utilisation of inexpensive agricultural residues in bioprocess, thus becoming an alternate way to replace expensive raw materials (*Hassan et al*., 2019). Agro-waste is produced during industrial processing and household usage are underutilized and contribute to environmental pollution. These wastes are extremely susceptible to microbial deterioration due to high moisture and total soluble sugar concentrations, posing a significant environmental risk. Fruit and vegetable waste have simple sugars, which can potentially be used as a medium for the manufacture of organic acids(*Al-Dhabi et al*., 2020). Because they have high mineral and vitamin content and a neutral pH, vegetables serve as a natural source for lactic acid bacteria (LAB) fermentation (*Lorn et al*., 2021). Fruit and vegetable wastes are also locally available and ferment quickly, making them suitable for optimal lactic acid production. In research by Zerihun et al., (2021), lactic acid was synthesised using cabbage waste, potato peels, and teff flour extracts for use in pathogen inactivation in faecal sludge. Rotting tropical fruits have also been used for isolating LAB and using it to yield lactic acid (*Ngouénam et al*., 2021).

Utilising local agro wastes to obtain lactic acid reduces cost and promotes sustainability as it enables waste disposal management and creates circular solutions that enable locals to reuse waste. Although previous studies towards optimisation of lactic acid production from organic wastes have been made, little research has been done towards its direct use in urea hydrolysis inhibition in urine (*Al-Dhabi et al*., 2020).

This study evaluated the fermentative utilisation of four fruit peel wastes (mango, orange, banana, and pineapple) mixed with cabbage waste as substrates and carbon sources for lactic acid production for use in urea hydrolysis inhibition, hence nitrogen retention. The high cellulose, lignin, and hemicellulose content in these fruits made them suitable substrates for producing fermentable sugar for lactic acid production (*Aili Hamzah et al*., 2021). Other organic acids can also be created by the anaerobic fermentation of these fruit and vegetable wastes (*Kuley et al*., 2020). To ensure that lactic acid is dominant over other acids, the set conditions included temperature regulation at 37oC , using anaerobic conditions, using carbohydrate-rich substrates and ensuring a specified fermentation time(72 hours) (*Garcia et* al., 2020). Using lactic acid in urea hydrolysis inhibition is sustainable and environmentally friendly for urine treatment, predominantly in areas where centralised wastewater treatment is not feasible, and nutrient recovery from human excreta is desirable (*Arekemase et al*., 2020).

Materials and Methods

Sample Collection

Two kilograms of fruit and vegetable waste (bananas, pineapples, mangoes, oranges and cabbages) were collected from local sources in different clean bags. Three litres of fresh urine provided by volunteers was collected from Urine Diverting Dry Toilets (UDDTs) within Meru University of Science and Technology. Urine was collected in clean bottles and tightly capped. The pH of urine was determined and the samples refrigerated in a refrigerator at -4oC immediately after collection until the time of analysis.

Anaerobic Fermentation of Fruit and Vegetable Waste

The fruit and vegetable waste were washed with distilled water and blended using an electric blender in a 1:1 substrate to water ratio. They were then fermented anaerobically in an anaerobic incubator (Bio base incubator model BJPX-2102C) for 72 hours (3 days) at an optimum temperature of 37oC. After three days, the substrate was filtered using a sieve and centrifuged using the HERMLE Z326K centrifuge to filter the mixture further. The presence of lactic acid (LA) was confirmed using LCMS/MS (Shimadzu LCMS/MS-8030 Triple Quadruple). The lactic acid

obtained was used for the hydrolysis inhibition of urease.

Urine Treatment

The lactic acid obtained above was added to the three urine samples in a ratio of 1:2 (25 mL lactic acid extract and 50 mL urine) and sealed in an air-tight container. The sample mixtures were then stored for 4, 7 and 10 days, respectively. Similarly, 50 mL of urine was measured and set up as a control without the addition of lactic acid. The pH and nitrogen concentration of urine samples were monitored in triplicate before and after the stabilisation.

pH Analysis

The urine pH before and after treatment was determined by a digital benchtop pH meter (86501 AZ EB) after calibration with pH buffers 4.01, 7 and 10. Each time after dipping the probe in a sample, it was rinsed with distilled water, and the pH values were recorded on the monitor. A one-way analysis of variance (ANOVA) test was carried out to assess the effect of urine stabilisation on pH across the days.

Nitrogen Analysis

The samples' total nitrogen content (TKN) was assessed by Kjeldahl method using a Kjeldahl digester and a distillation system (Gerhardt, Vapodest 20s) *(Hicks et al*., 2022).

Exactly 2 mL of separate treated and untreated urine samples were put in the Kjeldahl tubes, and 0.5 g of a catalyst mixture of CuSO4, TiO2 and K2SO4 (ratio 1:1:9) was added, then 20 mL of concentrated sulphuric acid (H2SO4) was added and the solution digested for 2 hours at 400oC. 20 mL sulphuric acid and 0.5 g of catalyst was used as a blank solution. Sulphuric acid oxidised the organic matter in the urine, liberating nitrogen in the form of ammonium sulphate (NH4)2SO4. The residue was then allowed to cool for 10 minutes. It was then distilled with about 0.1 N NaOH in the distillation step. The Kjeldahl flask was linked to a water condenser and heated to boil off ammonia (NH3) from the digest. The ammonia was adsorbed in 50 mL of 4% boric acid (H3BO3). After adding two drops of Tashiro's indicator (mixture of methyl red (0.03%) and methylene blue (0.1%)), the mixture was titrated against 0.1 N HCl until the colour turned pink. A magnetic stirrer mixed the solution during the titration process. Results from the titrations were recorded for nitrogen quantification.

The nitrogen concentrations were calculated using the formulae

$$Nitrogen\ (mg/L) = \frac{(Uf - Ub) \times N \times 14.007 \times 1000)}{(Sample\ volume\ (2\ mL)}$$

Where Uf = volume of titrant, Ub = volume of blank (20 mL H2SO4), and N = normality of HCl (0.1 N).

Given the variables are the titrant volumes, the formula is simplified to:

$$Total\ Nitrogen\ (mg/L) = 700\ (Uf\text{-}Ub)$$

The volume of the blank solutions was deducted from the sample volumes to account for any potential background interferences and errors. Statistical analysis was performed using Microsoft Excel's Data Analysis Tool Pak, using one-way ANOVA to analyse the effects of lactic acid treatment and time on TKN concentrations, with significance set at $P < 0.05$.

Results

Qualitative Analysis of Lactic Acid

The lactic acid extract obtained from the fermentation was identified using LCMS/MS. Chromatograms showed peaks that corresponded to pure lactic acid retention time of 3.682 minutes (**Figure 1** below). The consistent retention time for the samples and standard reinforced the identification of LA, showing it as a major fermentation product. The peak heights for the sample were quite pronounced.

pH and Total Kjeldahl Nitrogen (TKN)

The pH and total Kjeldahl Nitrogen (TKN) of the urine samples were determined before and after the lactic acid treatment. The pH and nitrogen of the untreated urine (control sample) were also analysed. The results for pH and TKN analyses and their relative standard deviations (RSD) are summarised in **Tables 1 and 2** below. The results were used to assess the efficacy of lactic acid in hydrolysis inhibition of urea.

From **Table 1 below**, the initial pH for all urine samples was 6.1, which was slightly acidic. The pH for the stabilised setups dropped greatly, with the final pH ranging from 3.6 – 3.9. The highest pH decrease (6.1 - 3.6) was in the sample treated for 10 days. The pH of the untreated urine rose consistently from 6.1 to

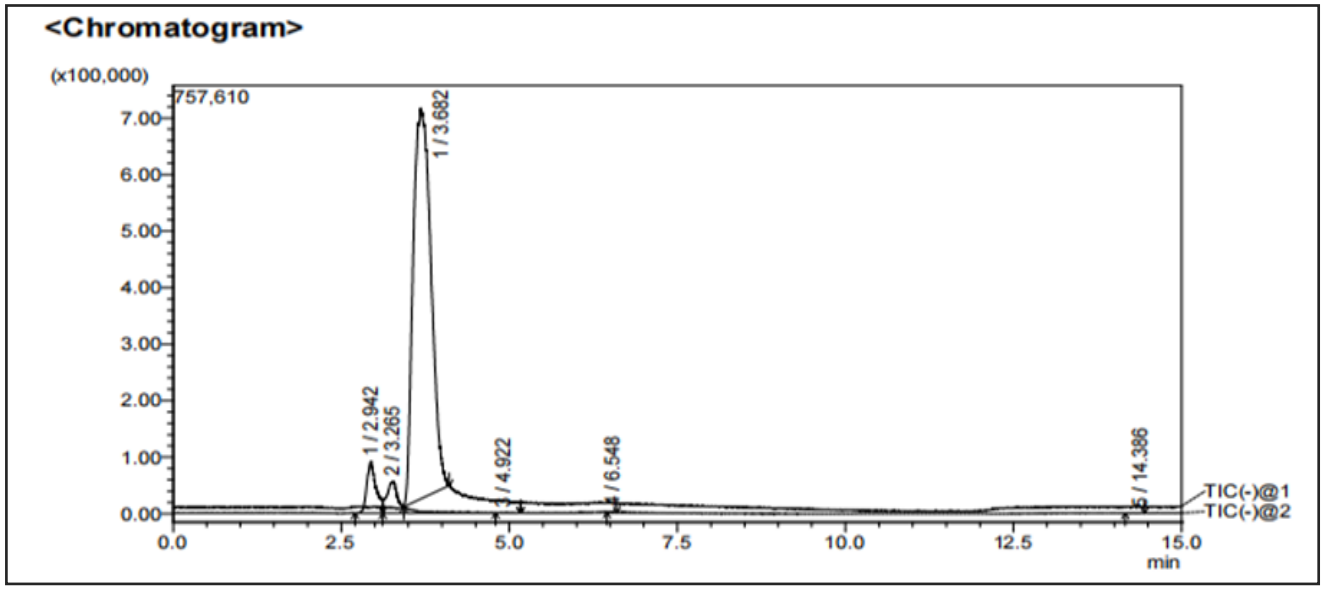

Figure 1: *Chromatogram showing lactic acid at 3.682 Minutes*
The peaks at 2.265–2.842 minutes represented acetic acid. The other minor peaks at 4.922, 6.548, and 14.396 minutes were likely due to the presence of other fermentation products or coeluting substances.

Treatment Duration	pH	
	Control Sample	Treated Sample
Initial	6.1 ± 0.0	6.10 ± 0.0
4 Days	7.5 ± 2.0	3.9 ± 1.8
7 Days	8.0 ± 0.0	3.7 ± 1.8
10 Days	8.6 ± 0.0	3.6 ± 3.9

Table 1: *pH after 4, 7 and 10 days (n = 3)*

Treatment Duration	TKN (mg/L)	
	Control Sample	Treated Sample
Initial	2643± 0.0	2643± 0.0
4 Days	350 ± 0.0	1960 ± 3.6
7 Days	840 ± 8.3	2450 ± 7.6
10 Days	630 ± 11.1	2100 ± 8.8

Table 2: *TKN analysis after 4, 7 and 10 days (n = 3)*

between 7.5 and 8.6 across the treatment duration. The statistical analysis indicated statistical difference in the pH across the days (P=0.047).

The concentrations of nitrogen ranged from 1960 to 2450 mg/L on average for the stabilised samples, as seen in Table 2. Samples treated after 7 days contained the highest nitrogen at 2,450 mg/L. treatment. The concentration for the untreated urine ranged between 350 and 850 mg/L across days, indicating a huge drop from the initial concentration of 2643 mg/L. The one-way ANOVA test showed that the interaction between treatment duration and TKN concentration was also statistically significant (P = 0.021).

Discussion

The fermentation of the fruit and vegetable waste substrates to obtain lactic acid was carried out in anaerobic settings because lactic acid bacteria grow in anaerobic, carbohydrate-rich environments with low pH. These conditions increase the efficiency of lactic acid generation, prevent contamination, and are essential for the metabolic pathways involved (*Rombouts et al.,* 2020). The LCMS/MS chromatograms showed peaks that corresponded to different components of the sample, with retention times indicating the elution time of each component. Lactic acid was identified by the peak with a retention time of approximately 3.7 minutes. The peak height of the samples was quite pronounced, with a high intensity of 7.0 x 105, indicating a high concentration of lactic acid at this retention time. In chromatography, the peak height is proportional to the concentration of the analyte (Zhang, 2024). The sharpness of the peaks indicated that the lactic acid in the samples was relatively pure, with minimal contamination from other components. The peaks at 2.265 – 2.842 minutes were identified as acetic acid. Other minor peaks were also noted at 4.922, 6.548, and 14.396 minutes, representing other fermentation products and coeluting compounds.

The pH measurements were taken to assess the effectiveness of lactic acid in the hydrolysis inhibition of urea. Significant pH drop showed that inhibition had occurred since hydrolysis would normally result in a pH rise of between 9 and 10. The pH for all the sample setups was reduced greatly after treatments with LA. The reported pH after urine treatment ranged from 3.6 - 3.9, compared to pH ranges 4.12 and 4.26 found by Zerihun et al., (2021) in a related study. The lowest pH value of 3.6 was recorded after 10 days of treatment, while the highest was at four days (3.9). The structural aspect of lactic acid is responsible for its high acidity level due to the intramolecular hydrogen bonding between the α-hydroxyl and the carboxylate group. This explains why lactic acid caused a significant drop in the urine pH. The low pH was unfavourable for the hydrolysis action of urease (*Yadav et al*., 2022). The pH of the untreated urine (control) was between 7.5 and 8.6. This was an increase from 6.1, indicating limited microbial activity, which resulted in ammonia accumulation. This showed further the stabilising action of lactic acid. The one way ANOVA statistical analysis indicated statistical difference in the pH across the days (P=0.047). This further reinforced the stabilisation effect of lactic acid on urine in hydrolysis inhibition, which affected the pH.

The results showed a varied difference in the TKN content of the stabilised and untreated urine samples. The highest nitrogen content was 2450 mg/L for 7 days treatment compared with the initial amount of 2643 mg/L. As a result, 92.70% of nitrogen was retained after the lactic acid treatment, showing that hydrolysis inhibition was relatively successful. The theoretical nitrogen for human urine is estimated to reach concentrations of up to averagely 3,700 - 3,830 g N per person per year according to Swedish data (*Nagy et al*., 2019). The difference in nitrogen concentration of the fresh urine from the theoretical can be attributed to possible losses during the collection of samples. The nitrogen in the untreated urine reduced greatly across the days to an average of 607 mg/L. The reduction can be attributed to urea hydrolysis having occurred, leading to a significant loss of nitrogen. Microsoft Excel analysis using one way ANOVA compared the Total Kjeldahl Nitrogen (TKN) concentrations across the treatment duration. The results showed significant difference in TKN concentrations across the treatment days (P = 0.021). The statistical findings emphasised further the effectiveness of lactic acid in stabilising urine and retaining nitrogen.

Conclusion and Recommendations

This study assessed the extent of lactic acid from fruit and vegetable waste fermentation in urea hydrolysis inhibition. The pH of urine was reduced significantly after stabilisation with lactic acid, indicating successful inhibition of urea hydrolysis. Nitrogen retention for the lactic acid-stabilised urine was substantially higher compared to the untreated urine. Lactic acid treatment at 7 days showed the highest degree of urine stabilisation. These findings indicate that lactic acid is suitable for the inhibition of urea hydrolysis by urease enzyme. Utilising fruit and vegetable waste for lactic acid production is a sustainable and environmentally friendly approach to conserve nitrogen in human urine with potential for application in agriculture.

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