Tanzania Journal of Engineering and Technology 2022, **41(4)**:43-57
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Websites: https://www.ajol.info/index.php/tjet; https://tjet.udsm.ac.tz

ISSN 1821-536X (**print**); ISSN 2619-8789 (**electronic**)
DOI: https://doi.org/10.52339/tjet.v41i4.873

Full Length Research Paper

Determination of Effectiveness of Combined Biological and Physicochemical Treatment of Vinasse

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ABSTRACT

Combined biological and Physicochemical treatment method was used to treat vinasse using microorganisms (*Aspergillus niger (fungi)* and *Bacillus subtilis (bacteria))*, coagulants (aluminium sulphate ($Al_2(SO_4)_3$) and iron III chlorides ($FeCl_3$)) and followed by filtration. Three factors; coagulant type, type of microorganism and coagulant concentration was analysed using full factorial design (2^n) with replication, on their percentage removal of Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Total Suspended Solids (TSS) and turbidity. Pareto chart and regression analysis with a 95% confidence interval (5% significant level) were used to analyse the results using MINITAB 22 (current version). Initially, the vinasse had (COD), (BOD), (TSS), and turbidity of 10240 mg/ℓ, 5340 mg/ℓ, 400 mg/ℓ, and 1980 NTU, respectively, and a pH of 5.68. COD, BOD, TSS, and turbidity were reduced by 99.5%, 99.7%, 99.7%, and 93.7%, respectively, after the treatment, while pH was raised to 7.5, which is neutral. Using *Bacillus subtilis* and aluminium sulphate, the combined biological and physicochemical process produced good results for the treatment of vinasse.

ARTICLE INFO

Submitted: **May 30, 2022**

Revised: **November 22, 2022**

Accepted: **December 5, 2022**

Published: December **30, 2022**

Keywords: *Combined Biological and Physicochemical Treatment, flocculants, Vinasse, Pareto chart, Bacillus subtilis*

INTRODUCTION

Vinasse is the final by-product of the biomass distillation from sugar crops (beet and sugarcane), starch crops (corn, wheat, rice, and cassava), or cellulosic material (harvesting crop leftovers, sugarcane bagasse, and wood) used primarily for the manufacturing of ethanol (Hor *et al.*, 2022; Christofoletti *et al.*, 2013). Vinasse is released in large quantities during the industrial manufacturing of ethanol from molasses. For every 1 litre of ethanol produced in the industry, 9 to 14 litres of vinasse is produced (Marafon *et al.*, 2020; España-gamboa *et al.*, 2016). Potable alcohol distillery at the Kilombero sugar mill site has the annual production capacity to produce around 12 million litres of ethanol (Daily News reporter, 2021). This is approximately 138 million litres of vinasse produced. This is a significant amount of industrial wastewater to be discharged into the environment. Discharging such large number of pollutants has a negative impact on the environment (Christofoletti *et al.*, 2013).

Because of their high organic content, dissolved solids, and many other compounds that are toxic or could be contaminants

under certain environmental conditions, direct disposal of stillage on land or in groundwater (rivers, streams, or lakes), or even for direct irrigation of crops, pollutes the environment. Highly coloured effluent can impede sun light penetration into shallow waters, which is necessary by aquatic plants for photosynthesis to sustain oxygen levels. These coloured effluents can cause aquatic plants to die and rot, increasing oxygen demand and causing eutrophication (Kaishev *et al.*, 2022). The low pH, electric conductivity, and chemical elements present in sugarcane vinasse may cause changes in the chemical and physical–chemical properties of soils, rivers, and lakes with frequent discharges over a long period of time, and also have adverse effects on agricultural soils and biota in general (Parsaee *et al.*, 2019; Christofoletti *et al*., 2013). Disposal of this by-product can cause serious environmental damages, such as leaching of metals present in the soil to groundwater, reduction of soil pH, environmental acidification and high CH_4 emission potential (Carpanez *et al.*, 2022). The physical components of vinasse are shown in Table 1.

Treatment of the vinasse before discharge to the environment is very crucial for aquatic and terrestrial life, and alcohol distilleries are rated as one of the world's 17 most polluting industries in the world (Mikucka & Zielińska, 2020). Disposal of this by-product can result in serious environmental damages, such as metals in the soil leaching into groundwater, soil pH lowering, ecosystem acidification, and a high CH_4 emission potential (Eduardo *et al.*, 2019).

Table 1: Physical components of vinasse from different works of literature

Parameter	Value
pH	3.7 – 5.5
BOD5 at 20°C (mg/ℓ)	6800-31250
COD (mg/ℓ)	11076-132250
Turbidity (NTU)	850-941
Total Suspended Solids (TSS), mg/ℓ	7338-20273

Source: Eduardo *et al.* (2019), Karimi *et al.* (2019) , España-gamboa *et al.* (2016), España-Gamboa *et al.* (2012), Vilar *et al.* (2018) and Hakika *et al.* (2019)

Vinasse Treatment Methods

Biological treatment, physicochemical treatment, and combined biological and physicochemical treatment are all used to treat vinasse. The biological treatment process might be aerobic or anaerobic. Adsorption, coagulation/precipitation, oxidation, and membrane filtering are examples of physicochemical approaches.

Biological treatment

Biological treatments (aerobic and anaerobic) have been recognized as effective methods of treatment for highly polluted industrial effluents. Wastewater/effluents from agro-industrial industries, including distilleries, are often treated using both anaerobic and aerobic systems. Anaerobic treatment is the most attractive primary treatment which removes over 80% of BOD while also recovering energy in the form of biogas (Yellezuome *et al.*, 2022).

Anaerobic Treatment

The application of thermophilic anaerobic digestion is a logical choice for the treatment of sugarcane vinasse (industrial wastewater from ethanol production) because this process enables the recovery of energy as hydrogen and methane without requiring energy for heat or interfering in its quality as a bio-fertilizer (Montiel-Rosales *et al.*, 2022; Júnior *et al.*, 2016). In comparison to the single-phase process, Fuess *et al.* (2021) and Yeoh (1997) investigated the thermophilic two-phase anaerobic treatment system of a cane-molasses ethanol distillery using bioreactors for acidogenic and methanogenic phases. The results showed that even with larger substrate loading, the two-phase approach removed over 85 percent of BOD5 and 65 percent of COD; respectively. The

acidogenic phase provided satisfactory conversion of initial COD to volatile fatty acids (VFAs), with a degree of acidification of 15.6 percent.

Junior *et al.* (2020) used the fungus *Pleurotus sajor-caju* to treat sugar-alcohol vinasse, resulting in the production of a laccase enzyme and the elimination of colour and turbidity. After 15 days of cultivation in the medium with *Pleurotus sajor-caju* fungus under anaerobic conditions, the highest values for colour removal and turbidity were 92 percent and 99.2 percent, respectively. This proves that anaerobic bacteria are very effective in colour remove in wastewater comparing to COD and BOD removal.

Aerobic Treatment

España-gamboa *et al.* (2016) treated ethanol vinasse by removing colour and phenol in an air-pulsed bioreactor using *Trametes Versicolor* fungus. Batch operation of the bioreactor removed 71% of total phenol, 18% of colour and 40% of COD. The maximum laccase activity achieved was 428 UI/L. Moreover, in continuous mode, the bioreactor removed 80% of total phenol, 17% of colour and 60% of COD. Laccase activity ranged from 956 to 1630 UI/L. The results indicated that continuous operation of an air-pulsed bioreactor under the conditions proposed favoured biodegradation of vinasse.

Menezes *et al.*, (2020) used air-lift bioreactors to study the decomposition of sugarcane vinasse. Chemical oxygen demand (COD) and biological oxygen demand (BOD) reductions ranged from 53% to 58% and 71% to 58%, respectively. Bezuneh (2016), examined the role of microorganisms, including bacteria and fungi, in distillery wastewater treatment. As demonstrated in Table 2, a wide range of bacterial cultures were investigated and their efficiency reported.

Table 2: Bacterial cultures employed for treatment of distillery effluent

Microorganism	Treatment Process	COD removal Efficiency (%)	Time (days)
Pseudomonas sp	A pure culture of the isolate was incubated at room temperature in a diluted spent wash (10%)	63	3
B(Bacillus). cereus	Immobilization of bacteria on sodium alginate and anaerobic degradation was carried in a batch reactor.	81	2
X. fragariae		76	
B. megaterium		76	
B. circulans	Molasses spent wash was Bioremediated and incubated for 15 days in a supplemented medium.	80.8	15
B. megaterium		80.9	
B. frmus		78.9	
Mixed culture of: B. thuringiensis, B. brevis, Bacillus sp. (MTCC6506)	Mixed bacterial culture was used to decolourize Sucrose-Glutamate-Acid (SGA).	63.39	1
Lactobacillus L-2	Bioremediation of bacteria was used to digest spent wash anaerobically.	57	7
A bacterial consortium of: P. aerugenosa A01, S. maltophia, P.microbilis	The degrading and decolorizing of anaerobically treated distillery wastewater were treated by using an isolated bacterial consortium	51	3

Acetogenic bacteria of strain No. BP103	Decolourization of molasses wastewater was carried in a replacement culture system.	70.9	7

Source: Bezuneh, (2016)

The fungi also have been investigated by Bezuneh (2016) for their ability to degrade COD, BOD and decolorizing distillery wastewater as well as the treatment condition and the results are portrayed in Table 3.

Chuppa-tostain *et al.* (2020) evaluated the Filamentous Fungi and Yeasts aerobically for the biodegradation of sugarcane distillery wastewater and the results are shown in Table 4.

Table 3: Fungal cultures employed for treatment of distillery effluent

Microorganism	Treatment Process	COD removal Efficiency (%)	Time (days)
C. cladosporioides	A pure culture of the isolate was transferred in a medium with 10% diluted spent wash and incubated at room temperature	62.5	10
Penicillium sp. *P. decumbens*	Aerobic degradation of beet molasses alcoholic fermentation wastewater diluted to 50%	57	5
A. fumigatus *A. terreus*	Bioremediation of molasses spent wash was carried in medium supplemented with glucose, yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$ and kept for incubation for 15 days	84 84	15
Trametes sp. I-62	20% (v/v) of distillery effluent was added to the culture medium and incubated for 7 days at 28°C under sterile conditions.	61.7	7
P. chrysosporium	The fungus was immobilized on different support materials, such as polyurethane foam (PUF) and scouring web (SW), in rotating biological contactor (RBC)	48	17
F. flavus	The isolated fungi were immobilized on a polyurethane foam cube and decolourized 10% diluted molasses wastewater	50	5
T. pubescens MB 89	The isolated fungus was used in flask cultures and a bubble lift bioreactor to treat 10% diluted wastewater.	79	15

Source: Bezuneh (2016)

Table 4: Strains used in the effects of aerobic treatment of vinasse on physicochemical parameters

Strains (Genera/Specie)	Reduction of COD (%)	Effect on OD_{475nm} (%)	Reduction of Minerals Content (%)	Final pH
Arthroderma otae	59.22	98.26	36.99	6.91
Aspergillus alutaceus	69.23	58.12	73.49	7.91
Aspergillus flavus	70.86	80.00	20.88	8.72
Aspergillus itaconicus	73.23	64.64	40.94	7.64

Aspergillus niger	70.11	73.04	77.57	8.31
Aspergillus oryzae	65.98	77.97	66.62	8.86
Aspergillus parasiticus	74.6	57.54	53.66	8.46
Aspergillus terreus var africanus	76.53	110.14	66,00	9,00
Aspergillus terreus var terreus	73.5	61.16	72.4	9.05
Candida albicans	56.56	118.01	29.3	8.69
Candida dubliniensis	45.98	152.53	32.18	8.45
Candida glabatra	57.34	130.64	26.62	8.12
Candida tropicalis	50.41	138.72	20.75	8.42
Clavispora lusitanea	54.72	116.84	28.35	7.39
Colletotricum graminicola	57.75	92.17	39.23	7.94
Cryptococcus albidus	44.89	135.35	30.29	8.13
Fennellia flavipes	58.65	75.65	61.46	8.74
Strains (Genera/Specie)	**Reduction of COD (%)**	**Effect on OD_{475nm} (%)**	**Reduction of Minerals Content (%)**	**Final pH**
Flavodon flavus	28.99	77.10	37.48	6.17
Fusarium sporotrichioides	55.13	140.00	43.81	8.25
Galactomyces geotrichum	56.95	104.64	46.39	8.26
Gibberella fujikuroi	37.89	106.96	36.58	6.76
Gibberella zeae	55.8	109.28	33.52	8.04
Issatchenkia orientalis	48.13	139.73	49.56	8.08
Komagatella pastoris	69.7	107.41	56.84	7.99
Penicillium rugulosum	62.48	86.38	56.28	8.72
Penicillium verrucosum	62.09	168.99	49.05	9.03
Phanerochaete chrysosporium	23.51	74.20	70.49	7.01
Pichia angusta	49.52	114.14	20.78	6.53
Pichia guilliermondii	54.78	138.38	28.21	7.54
Pichia jadinii	40.91	141.08	46.33	8.21
Pseudozyma antarctica	51.33	136.03	22.11	8.9
Rhizopus microsporus var oligosporus	67.32	95.94	52.04	8.85
Saccharomyces cerevisiae	55.84	190.91	41.17	8.88
Thanatephorus cucumeris	65.28	105.22	44.63	6.66
Trametes hirsute	74.01	57.54	62.86	7.8
Trametes versicolor	73.64	67.54	39.05	7.79

Source: Chuppa-tostain *et al.* (2020)

Physicochemical Treatment

Karchiyappan *et al.* (2019) treated sugarcane vinasse liquid using electrocoagulation/flocculation followed by ultrafiltration in a monopolar electromagnetic reactor with two aluminium (purity of Al 95 to 97%) plates, namely anode and cathode (dimension 400x400 mm), as electrodes. The tubular ceramic membranes namely Al_2O_3/ZrO_2 formed by isostatic pressing used in ultrafiltration of the effluent after being electro coagulated. Electrocoagulation/flocculation, ultrafiltration were used as pre-treatment and post-treatment respectively. The procedure demonstrated improved colour (91%), turbidity (88%), and COD removal efficiency (85%).

Prajapati & Chaudhari (2015) studied the coagulation/flocculation of molasses-based alcohol distilleries (vinasse) utilizing inorganic coagulants such as aluminium derivatives, iron derivatives and lime, as well as the effect of effluent pH on coagulation. With 60 mM/dm^3 $AlCl_3$, $FeCl_3$, and 30 mM/dm^3 polyaluminium chloride, respectively, at their optimum initial pH of 5.5, 3 and 5.5, the study found that the pH variation on the distillery effluent was significant, and the coagulation/flocculation yielded about 55, 60, and 72% COD reductions and about 83, 86, and 92% colour reductions. Furthermore, alum was found to be superior to ferric sulphate, ferric chloride, and aluminium chloride, as well as being significantly less expensive.

Hakika *et al.* (2019) used the Fenton Reaction (an advanced oxidation technique) to lower COD in sugarcane vinasse. The hydroxyl radical (•OH) was created by catalysing the interaction between Fe^{2+}/Fe^{3+} with hydrogen peroxide. The COD value reduced to 48.10%, and its biodegradability increased nearly double at a pH of 3.8, according to the results of the experiment. However, if utilized as a pre-treatment and then followed by a biological treatment, whether anaerobic or aerobic, the treatment is effective.

Combined biological and physicochemical treatment

Vilar *et al.* (2018) treated vinasse using *Pleurotus Sajor-caju* fungus in a combined biological–Electrochemical oxidation treatment and yielded 97% efficient colour removal, 99% turbidity removal, 50.6% chemical oxygen demand (COD) and total organic carbon (TOC) removal was 57.3%.

Campos *et al.* (2014) treated the vinasse by series of fermentation with high flow system (activated sludge batch) followed by filtration and successfully reduced BOD (96.7% of pollution reduction), suspended solids (99.9%), pH, copper (88%), iron (92.9%), and manganese (88%). However, Ojha *et al.* (2015) used complex combined treatment plant and reduced 99.07% in BOD_5 and 98.61% reduction in COD. The observed differences were contributed to the difference in the methods and equipment used as well as microorganisms used to treat the vinasse.

Campos *et al.* (2014) treated the vinasse from molasses ethanol distilleries by fermentation with high flow system (activated sludge batch), followed by filtration by filters containing layers of gravel, crushed stone, coarse sand and fine sand in descending sequence, followed by chemical flocculation (using calcium oxide, aluminium sulphate and ferric chloride) then followed by fermentation of low flow system (activated sludge batch) then neutralization to minimize the corrosive power of liquid waste treated using orthopolyphosphate then finally disinfection whereby sodium chloride was added. The active sludge was added at a ratio of 1:10 of active sludge to the vinasse and the flow rate of feeding the vinasse was maintained at 4.4 ℓ/min with batch mode. The filtration was done using the layers of gravel (0.19 g) in a descending particle size flowed by crush stone (0.21 g), coarse sand (0.19 g)

and fine sand (0.18 g). Then the flocculation was done using calcium oxide (30 g/ℓ), aluminium sulphate (5 g/ℓ) and ferric chloride (0.3 g/ℓ) and after the vinasse was allowed to flocculate for 6 hours. However, the fermentation with a low flow system was done at a rate of 0.4 ℓ/min and the Orthopolyphosphate (5 mg/ℓ) was added to reach a pH value of 7.0 for the neutralization and minimizing the corrosive of the vinasse and the final treatment was adding sodium hypochlorite (4%) in which it functioned as a disinfectant.

Comparison between treatment methods

Table 5 shows different effluent treatment methods and their efficiency on COD, BOD, colour, phenol, turbidity and suspended solids.

Table 5: Characteristics of vinasse after treatment by using different methods

	Biological (Aerobic)	Biological (Anaerobic)	Physicochemical	Combined biological and Physicochemical
COD	28.99[1] – 85[6]	65[2]	48.1[9] – 85[7]	50.6[10] – 98.61[12]
BOD_5	58[5] – 95[6]	85[2]	–	96.7[11]-99.07[12]
Colour	17[4] – 81[6]	92[3]	35[8] – 91[7]	97[10]
Phenol	71[4]-85[6]	–	–	–
Turbidity	–	99.2[3]	88[7]	99[10]
Suspended Solids	–	–		99.9[12]

Source: [1]Chuppa-tostain *et al.* (2020), [2]Yeoh, (1997), [3]Junior *et al.*, (2020), [4]España - Gamboa *et al.* (2016), [5]Menezes *et al.* (2020), [6]Bezuneh, (2016), [7]Karchiyappan *et al.* (2019), [8]Prajapati & Chaudhari, (2015), [9]Hakika *et al.* (2019), [10]Vilar *et al.* (2018), [11]Campos *et al.* (2014) & [12]Ojha *et al.* (2015)

As demonstrated in Table 5, the combined biological and physical method is the most effective way for removing biological and physicochemical components. The biological and physicochemical methods have widely been studied and experimented, while few studies have been done on combined biological and physicochemical methods. The objective of this study was to analyse the effectiveness of the combined biological and physicochemical treatment for treatment of vinasse from ethanol production plant by using locally available microorganisms.

MethodS

Raw materials collection and storage

Vinasse samples were collected from Kilombero Sugar Company, located at Morogoro region in Tanzania, and characterized and analysed at the University of Dar es Salaam's Chemical and Processing Engineering (CPE) laboratory, as well as the Molecular Biology and Biotechnology (MBB) Laboratory. At the laboratory samples were stored at 4ºC using a refrigerator to avoid microbial activities.

Characterization

The vinasse was characterized before and after treatment by measuring BOD, COD, Colour, pH range, total suspended solids, turbidity, percentage of ethanol and phenol content. The standard method used for the five-day BOD Test was EMDC1 1173: Part 3 5-day, Chemical Oxygen Demand (COD) Closed Reflux Titrimetric Method (EMDC1 1173: Part 4 Dichromate), Turbidity Test (APHA STD: 2130 Nephelometric Method) and Total Suspended Solids Test Method (EMDC1

1173: Part 1 Gravimetric Method), while the pH Test value was measured using HI 2211 pH/QRP Meter (EMDC1 1173: Part 2-Electrometric).

Selection, Isolation and Inoculation of the Microorganisms

Selection and isolation of Microorganisms

The microorganism was selected based on its availability, performance, and operating condition. The microorganisms used in this study were *Aspergillus niger* (fungi) and *Bacillus subtilis* (bacteria). The species were isolated from natural environment, the *Aspergillus niger* from coffee pulp and *Bacillus subtilis* from soil. The two species were used to analyse their performance on COD and colour removal.

Microorganisms (Bacteria and Fungi) Inoculation

The bacteria and fungus were inoculated in the culture media at room temperature (25ºC) using PDA and SDA for fungi and NA for bacteria. *Aspergillus niger* was grown in 5 glass Petri dishes using Potato Dextrose Agar (PDA), whereas *Bacillus subtilis* was grown in Nutrients Agar (NA).

Treatment methods

The proposed treatment method was a combined biological and physicochemical treatment. The physicochemical treatment was coagulation and sand filtration. The treatment sequence is shown in Figure 1.

The study adopted the RNN-LSTM design, following five stages: data collection, preprocessing of data, building a network, training a network, and testing a network, as shown in Figure 1.

Coagulation

Due to their availability and performance in coagulation, two types of coagulants were used in this study to study their performance: aluminium sulphate $Al_2(SO_4)$ and iron III chloride ($FeCl_3$). Coagulant dosages were 0.1 g/ℓ and 0.2 g/ℓ, respectively. The vinasse was kept for 6 h to allow coagulants to settle before being filtered to separate the coagulant and vinasse solution.

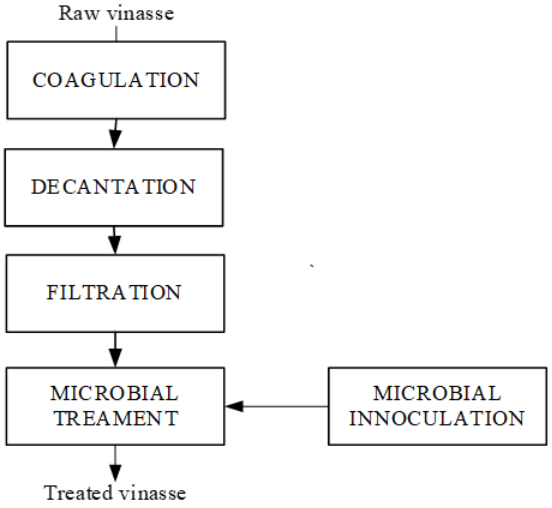


Figure 1: Treatment methods developed for vinasse.

Filtration

Filter paper (Whatman, Grade 1) was used to filter the vinasse sample for 2 hours, after which the filtrate was biologically treated with the selected microorganisms. The selected microorganisms were added to a 100 mL of vinasse sample that has already been coagulated for BOD and COD digestion for 24 hours.

Design of experiment (DoE)

Minitab 22 software was used to design the experiment. Full factorial design, 2^n with two replications was used to analyse effects of coagulant type, microorganism type, and coagulant concentration on COD, BOD, TSS and turbidity removal. For the coagulant type (F_1), $Al_2(SO4)_3$ was set to low (A) and $FeCl_3$ to high (B). Microorganisms: *Bacillus subtilis* (low) was designated as C, while *Aspergillus niger* (high) was designated as D for the type of microbe used (F_2). Coagulant Concentration (F_3): was set as low as 0.1 g/l and as high as 0.2 g/l. The three factors were designed into low (-) and high level (+) and their values and representations are shown in Table 6. A total of 16

experiments were done with 8 runs and two replicates.

Table 6: Values of the factors to be conducted in the experiment

		Coagulant concentration (g/ℓ)			
Microorganisms		A		B	
	C	0.1	0.2	0.1	0.2
	D	0.1	0.2	0.1	0.2

Hence; number of runs = $2\times2^3=16$. $\text{number of runs} = 2x2^3 = 2\ x\ 8 = 16$The experimental design had eight (8) runs with two replications that made a total of 16 runs as shown in Table 7.

Table 7: Factorial design of the experiment

Runs	F_1	F_2	F_3	Yield
1,9	+	+	+	Y_1
2,10	+	+	-	Y_2
3,11	+	-	+	Y_3
4,12	+	-	-	Y_4
5,13	-	+	+	Y_5
6,14	-	+	-	Y_6
7,15	-	-	+	Y_7
8,16	-	-	-	Y_8

Yields are the results found in the experiment as percentage removal of COD, BOD, TSS and turbidity as calculated using Equation (4).

Data analysis

The data were analysed using the Pareto chart to standardize the effect and regression analysis to determine whether the effect was negative or positive and by how much using MINITAB 17 with a 95% confidence interval to check for significance (5% significant level). The results were also compared to the TBS standard (TZS 860: 2015 Municipal and Industrial Wastewater) and removal efficiency was estimated using Equation (1). The $Y_{\%rem}$ is percentage removal; V_{bt} is value before treatment; and V_{at} is value after treatment.

$$Y_{\%rem} = \frac{V_{bt} - V_{at}}{V_{bt}} \times 100\% \quad (1)$$

RESULTS AND DISCUSSION

Vinasse Characterization

The results of COD, BOD, TSS, pH, and turbidity characterization in raw vinasse are shown in Table 8. The given values are the averages of the three replicated measurements.

Table 8: Physical characteristics of the raw Vinasse

Parameter	Measured Value
pH at 26.0℃	5.68 ± 0.006
COD (mg/ℓ)	10240 ± 10
BOD_5 at 20℃ (mg/ℓ)	5340 ± 10
TSS (mg/ℓ)	400
Turbidity (NTU)	1980 ± 50

Source: Data collection

The raw vinasse was found to be extremely turbid (1980 NTU) when compared to other researcher's findings of 941 NTU (Vilar *et al.*, 2018) . Very small inorganic and organic matter, as well as dissolved coloured organic compounds, can be attributed to this variation. Both BOD and COD values are significantly higher compared to TBS standards. This is due to the presence of large amount of carbohydrate in the vinasse. This has an impact on aquatic life because bacteria and other microorganisms decompose the present organic matter using the available oxygen; resulting in less oxygen to the aquatic life for the wastewater.

Microbial Inoculation

Figure 2(i) shows the inoculation of *Bacillus subtilis* and its kinetic growth is shown in

Figure 3(i) and Figure 2(ii) shows the inoculation of *Aspergillus niger* with kinetics growth shown in Figure 3(ii). Figure 3 shows *Bacillus subtilis* growth in three phases: lag (0–4 hours), log (4–12 hours), and stationary (from 12 hours till they are run out of food and start to die where death phase start). *Bacillus subtilis* grew to full size in under 24 hours. *Bacillus subtilis* was harvested during the early log phase of cell growth, which is the optimal time for removing BOD and COD and treating wastewater since the bacteria are at their peak rate of growth. The growth of *Bacillus subtilis* colonies can be seen in Figure 2(i). After 10 days of growth, *Aspergillus niger's* kinetics enters a stationary phase, as seen in Figure 4. The lag phase lasts from 0 to 1 day, the log phase lasts from 1 to 8 days, and the stationary phase lasts from 8 days until they run out of food and die, at which point the death phase begins. *Aspergillus niger* took three days to fully mature and be ready for treatment, but *Bacillus subtilis* only took one day (24 hours) to fully mature and be ready for treatment.

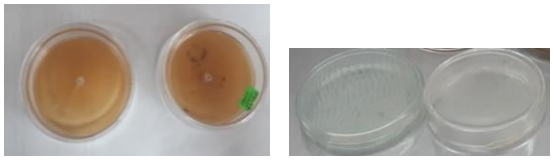
Vinasse Treatment

Analysis of combined Physicochemical and Biological Treatment

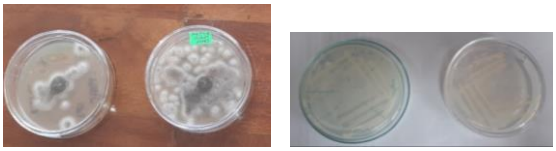
Figures 5, 6, 7 and 8 show the effect of F1, F2 and F3 on pH, Turbidity, TSS, BOD and COD removal from industrial wastewater (effluent).

pH Analysis

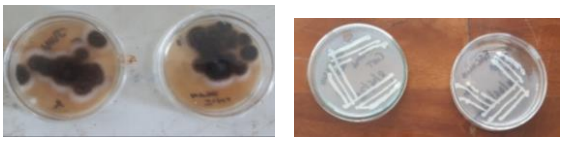
After combining physicochemical and biological treatment, the pH of the effluent ranged from 6.17 to 8.31, with the best pH of 7.52 obtained utilizing *Bacillus subtilis* and aluminum sulphate.



a) After 0 hours of inoculation



b) After 24 hours of inoculation



c) After 48 hours of inoculation

Figure 2(i) Inoculation of *Aspergillus niger* in PDA (a, b and c).

Figure 2(ii) Inoculation of *Bacillus subtilis* in NA (a, b and c).

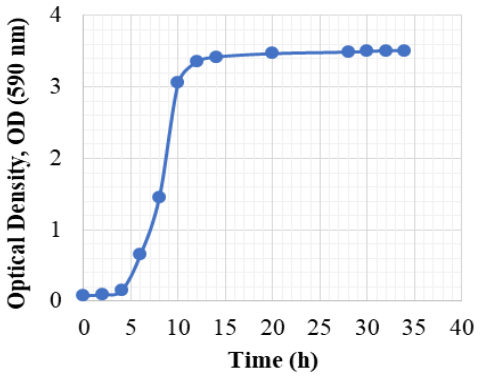


Figure 1(i): Kinetic growth of *Bacillus subtilis.*

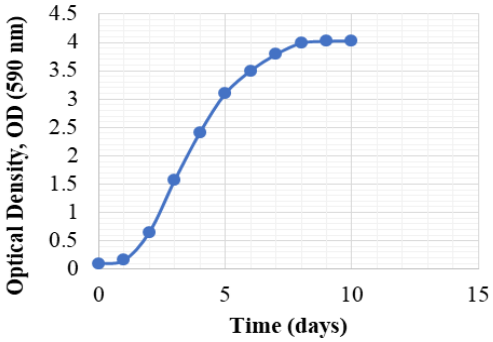


Figure 3(ii): Kinetic growth of *Aspergillus niger.*

Turbidity Analysis

The maximum turbidity removal was 165 NTU (equals to 96.72% removal) after microbial treatment. As the number of *Aspergillus niger* and *Bacillus subtilis* cells increased, the turbidity increased, but it was still within the TBS Standard limit (TZS 860: 2015).

TSS Analysis

The final TSS value ranged from 1.2 to 3.1 mg/ℓ (equals to 99.23 percent removal), and all values were within the TBS Standard for Industrial Wastewater (TZS 860: 2015) of 200 mg/ℓ, but these values increased from the values obtained after physical treatment, which ranged from 0.01 to 0.85 mg/ℓ (equals to 99.79 percent) due to growing suspended particles of *Aspergillus niger* and *Bacillus subtilis* into the treated waste water.

BOD_5 Analysis

Figure 4 shows the standardized effect of BOD_5 in treated vinasse. As can be seen in Figure 4 varying the factors is significant except the interaction between F1 and F3 as well as the interaction between F1, F2 and F3 which are insignificant. This is further elaborated by the regression analysis shown in Table 9 and the regression Equation (2).

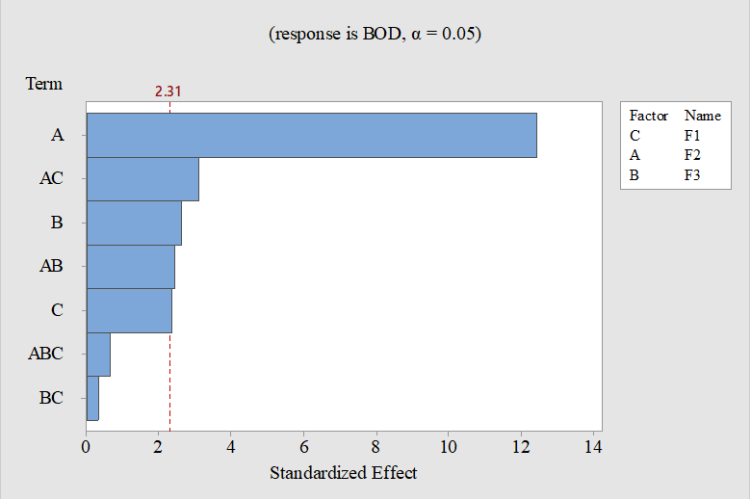


Figure 4: Pareto Chart of standardized effect for BOD_5.

Table 9 shows that increasing the value of each of the three factors (type of coagulant (F_1), type of microorganisms (F_2), and coagulant concentration (F_3) individually decreases the mean value of 98.8318 by 0.0011, 1.1177, and 0.1917, respectively, implying a decrease in the effectiveness of BOD removal. Increasing the kind of coagulant involves using more iron III chloride, which reduces the efficiency of BOD removal; thus, aluminium sulphate is the optimum coagulant for removing COD from vinasse. In cases where *Bacillus subtilis* is more successful than *Aspergillus niger*, raising F2 suggests more *Aspergillus niger* is used. Increasing F3 means increasing the coagulant's concentration hence less removal of BOD.

Table 9: Coded Coefficient for the regression analysis of BOD_5

Term	Effect	Coeff	SE Coeff	P-Value	VIF
Constant		98.8318	0.0546	0.000	
F1	-0.022	-0.0011	0.0546	0.003	1.00
F2	-2.2354	-1.1177	0.0546	0.000	1.00
F3	-0.3833	-0.1917	0.0546	0.004	1.00
F1*F2	-0.4653	-0.2327	0.0546	0.009	1.00

F1*F3	-0.0012	-0.0006	0.0546	0.864	1.00
F2*F3	-0.2689	-0.1345	0.0546	0.012	1.00
F1*F2*F3	-0.0002	-0.0001	0.0546	0.636	1.00

Source: Data collection

$$COD = 99.8318 - 0.0011F1 - 1.1177F2 - 0.1917F3 - 0.232F1*F3 - 0.1345F2*F3 \quad (2)$$

After biological treatment, BOD removal ranged from 22 mg/ℓ (equals to 99.7% removal) with *Bacillus subtilis*, which was within TBS standard of 30. While BOD after treatment with *Aspergillus niger* was 123 mg/ℓ, which was not within TBS standard with. The physicochemical treatment, on the other hand, proved ineffective in reducing BOD alone, since all values exceeded the TBS limit for BOD in industrial wastewater.

COD Analysis

Figure 5 shows the Pareto chart of the standardised effect for COD. The COD was significant at a 5% confidence level with various microbial (F_2) and coagulant (F_3) concentrations, as well as their interaction (F_2*F_3). Yeoh (1997) obtained the same result using a thermophilic two-phase anaerobic reactor. The COD results were attributed to the bacteria feeding on the nutrition in the vinasse, resulting in a reduction in COD. The concentration of the coagulant, as well as the interaction of different bacteria and varying the coagulant concentration, all affected the COD value. However, the type of coagulant used had no effect on the removal of COD in the vinasse; both had the same effect on COD removal and switching from one to the other had no effect on the result, and either could achieve the same results. Table 10 and regression Equation 3 demonstrate the factors' increasing and decreasing effects in the regression analysis.

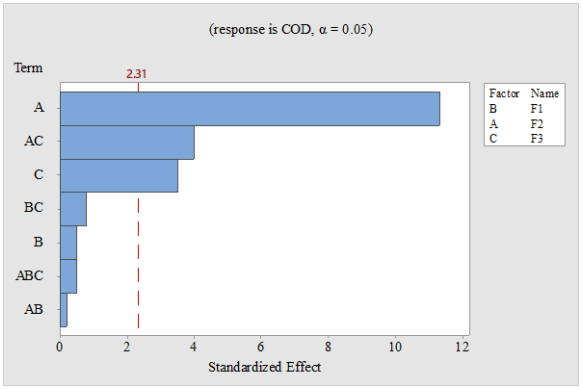


Figure 5: Pareto Chart of standardized effect for COD.

Table 10: Coded Coefficient for the regression analysis of COD

Term	Effect	Coeff	SE Coeff	P-Value	VIF
Constant		99.4484	0.0504	0.000	
F1	0.0147	0.00735	0.0504	0.613	1
F2	-3.2354	-1.6177	0.0504	0.001	1
F3	-0.4523	-0.2262	0.0504	0.004	1
F1*F2	0.0281	0.01405	0.0504	0.654	1
F1*F3	0.0469	0.02345	0.0504	0.787	1
F2*F3	-0.0719	-0.036	0.0504	0.012	1
F1*F2*F3	0.0156	0.0078	0.0504	0.636	1

Source: Data collection

$$COD = 99.8318 - 1.677F2 - 0.2262F3 - 0.0360F2*F3 \quad (3)$$

General results after treatment

Table 11 shows the summary of the results, which are the characteristics of the vinasse before treatment and after treatment.

Table 11: Comparison of vinasse characteristics before and after treatment

Parameter	Characteristics before treatment	Characteristics after treatment with *Bacillus subtilis*	Characteristics after treatment with *Aspergillus niger*	Tanzania Bureau of Standard for waste water
pH at 26.0℃	5.68 ± 0.006	6.28-8.31	6.17-8.12	6.5 − 8.5
COD (mg/ℓ)	10240 ± 10	49-61	118-256	60
BOD_5 at 20℃ (mg/ℓ)	5340 ± 10	22-31	52-123	30
TSS (mg/ℓ)	400	1.2 - 3.1	1.4-3.0	100
Turbidity (NTU)	1980 ± 50	85-88	65-123	300

CONCLUSION AND RECOMMENDATION

Using *Bacillus subtilis* and aluminium sulphate, the combined biological and physicochemical process produced good results for the treatment of vinasse. COD, BOD, TSS, and turbidity were reduced by 99.5%, 99.7%, 99.7%, and 93.7%, respectively, after the treatment, while pH was raised to 7.5, which is neutral. *The Bacillus subtilis* was found to be more effective than A*spergillus niger* on COD removal. The COD removal was 99.52% and 97.5% with *Bacillus subtilis* and *Aspergillus niger*, respectively. The best coagulant was found to be aluminium sulphate at 0.1 g/l, with *Bacillus subtilis*.

Further studies for comparing effectiveness of the analysed microorganisms (*Bacillus subtilis* and *Aspergillus niger*) with others is recommended. With the fact that the waste effluent itself might have different properties, the effectiveness of the treatment using other sample sources for comparison is also recommended. Composition analysis of the treated vinasse is important to confirm its suitability to be reused for irrigation.

ACKNOWLEDGEMENT

Authors are grateful to Mrs. Winnie Kimaro from Molecular Biology and Biotechnology department, University of Dar es Salaam for her support during data collection.

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