PHYSICOCHEMICAL ANALYSIS, TOXICITY ASSESSMENT AND NUTRITION SOURCE POTENTIAL OF SUGARCANE DISTILLERY SPENT WASH

H.D.KURUPPUARACHCHI

(168881T)

Degree of Master of Science

Department of Civil Engineering

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Dissertation submitted in partial fulfillment of the requirements for the Degree of Master of Science in Environmental Management

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DECLARATION

"I declare that this is my own work, and this dissertation does not incorporate without acknowledgement any material previously submitted for a Degree or Diploma in any other University or institute of higher learning, and to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where the acknowledgement is made in the text.

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…… ……

Mr. K. M. S. Ruvinda Date:

Name of the supervisor: Prof. Jagath Manatunge

…… ……

Prof. Jagath Manatunge Date:

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ABSTRACT

Sugarcane molasses-based ethanol industries in Sri Lanka generate large volumes of high strength spent wash, causing severe environmental issues. The potential toxicity of spent wash on biological systems and the possibility of using it as a nutrition source in agriculture has been given less attention in Sri Lanka. The present study was conducted to assess the physicochemical characteristics of the raw spent wash and possible cytogenotoxic effects of diluted spent wash using a plant-based bioassay with *Allium cepa* (common onion). Further, to evaluate the potential of raw spent wash as a liquid nutrition source to improve the growth of commonly grown vegetable variety, tomato (*Solanum Lycopersicum*).

Selected physicochemical parameters of raw spent wash collected from the distillery industry of Lanka Sugar Company (Pvt) Ltd, Pelwatta, were evaluated using APHA (2017) standard procedures. Toxicity assessment was carried out after exposure of *Allium cepa* bulbs to diluted spent wash (1:8) along with aged tap water (negative control) following standard protocols. The tomato crop experiment was conducted as an open field experiment using agricultural guidelines provided by the Department of Agriculture with certified organic fertilizer as positive control and tap water as a negative control. Growth morphometric attributes of the plants and fruits were monitored for 60 days. The data were statistically analyzed using univariate statistical methods.

The physicochemical analysis revealed that the spent wash is highly acidic with high EC (21.93 ± 0.09 mS/cm), COD (92,101 ± 0.33 mg/L), BOD (26,116 ± 2.33 mg/L)TSS (4,076 ± 0.55 mg/L), TDS (68,656 ± 0.13 mg/L), Nitrate (255 ± 0.04 mg/L) and phosphate (38 ± 0.07 mg/L), and contained heavy metals viz. Cd, Cu, Ni, Zn, As and Mn and K in trace amounts. Significantly decreased root growth was found in *Allium* roots exposed to diluted raw spent wash with the highest root growth delay (92%) after two days of exposure compared to the negative control ($p < 0.05$). The mitotic index did not show any difference in all exposure conditions. Significantly increased nuclear abnormalities, including micronuclei, nuclear buds, binuclei and condensed nuclei, were observed in root tip meristematic cells of diluted spent wash compared to the negative control ($p < 0.05$). Among growth-related morphometric attributes of tomato plants treated with different spent wash doses (0.5, 1.5, 2.5, 5.0, 7.5 and 10 mL), shoot lengths, the number of leaves and number of buds and flowers were found to have less significant variations. In contrast, other treatment categories recorded significantly reduced fresh fruit weight compared with the positive control ($p < 0.05$). Measured fresh fruit weight was more favourable toward high-end doses. However, this should be confirmed by repeated scientific studies. Results of the experiments reflect that the raw spent wash may have a cytogenotoxic effect, and the spent wash may use as a nutrition supplement by mixing with other organic ingredients. Further experiments for different crops, soil testing, and frequent biological effects monitoring are recommended to verify the spent wash's nutritional use and toxic effects.

Keywords: Sugarcane molasses, Spent wash, Bioassay, Cytogenotoxic, Growth morphometric attributes

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LIST OF ABBREVIATIONS

DOA	-	Department of Agriculture
SLSI	-	Sri Lanka Standard Institution
CEA	-	Central Environmental Authority
ANOVA	-	Analysis of Variance
ATW	-	Aged Tap Water
PC	-	Positive Control
MI	-	Mitotic Index
DAT	-	Days after transplanting
BOD	-	Biochemical Oxygen Demand
COD	-	Chemical Oxygen Demand
TSS	-	Total Suspended Solids
TDS	-	Total Dissolved Solids
EC	-	Electrical Conductivity
BMDE	-	Bio Methanated Distillery Effluent
DSW	-	Distiller Spent Wash
TMD	-	Tonnes of Molasses per Day
TCD	-	Tonnes of Cane per Day

LIST OF APPENDICES

1. INTRODUCTION

1.1. Sugarcane based ethanol industry

Food and Agricultural products have an increasing demand due to the growing world population at regional and global levels to meet the societal needs. Sugar is an essential commodity in the world agricultural market and one of the heavily traded agricultural commodities. Sugar is produced by either cane or beet and some countries (India, Brazil, Thailand, China, the US, Mexico, Russia, Pakistan, France, and Australia) lead the global sugar production (FAO 2021). Sugarcane is major sugar crop which accounts for nearly 80% of global sugar production (Koizumi 2003) and represents 21% (1.9 billion tonnes) of worldwide global crop production during the 2000 – 2019 period (FAO 2021). Sugar crops provide many other alternative productions such as livestock feed, fiber and energy, particularly biofuels (sugar-based ethanol) and co-generation of electricity (cane bagasse). Sugarcane is also considered as one of the most considerable and efficient sources of biomass since bioethanol emerged as a renewable biofuel source. Most of the world's sugarcane utilizes for the production of ethanol therefore world sugar and ethanol markets have a mutual interaction. Less than half of the sugarcane is consumed for the production of sugar (35% – 47.2%) and rest other goes for the production of ethanol at present (Impacts of trade liberalization on the world sugar market, n. d.).

Due to wide range of industrial applications such as chemicals, pharmaceuticals, cosmetics, beverages, biofuel, food and perfumery industry, etc., ethanol (C_2H_5OH) distilleries are growing rapidly worldwide. The art of distillation and production ethanol has been recorded by naturally fermenting vegetative materials among ancient Egyptians and Chinese. Fermentation is a complex biological process and the oldest biotechnology used for the production of ethanol in the distillery (Fito *et al*. 2019[a]).

1.2. Ethanol production process in distilleries

Around 80% of the sugarcane-based industries are integrated the sugar and ethanol production. Alcohol production in distilleries is based on sugarcane molasses in the industries of Asia and South America (Fito *et al*. 2019[a]). The alcohol manufacturing process in distilleries consists of four main steps viz. feed preparation, fermentation, and distillation and packaging (**Figure 1.1)**. Cane Molasses, a by-product generates during the production flow of sugar is then diluted to obtain favorable sucrose level. Using sulfuric acid, pH is adjusted to below 5 before fermentation and supplements (assimilable nitrogen source like ammonium sulfate or urea, phosphate if necessary) are added. Composition of the cane molasses varies according to the agroclimatic regions (Fito *et al.* 2019[b]). The feed is then inoculated with about 10% by volume of yeast inoculum. Fermentation step continues by an exothermic reaction and controlled conditions which take place in either batch or continuous reactors. During this process, cellulosic materials in cane molasses first delignified and hemi-cellulose and cellulose are subsequently acid hydrolyzed into simple sugars. These sugars are fermented to yield ethanol (6%–8%) and carbon dioxide (Satyawali and Balakrishnan 2008).

According to Satyawali and Balakrishnan (2007), distillation is a two-stage process and is typically carried out in a series of bubble cap fractionating columns (**Figure 1.1)**. The first stage consists of the distillation column and is followed by rectification columns. The cell free fermentation broth is preheated to about 90 $^{\circ}$C by heat exchange with the effluent and sent to the degasifying section of the distillation column. Here, the liquor is heated by live steam and fractionated to give about 50% alcohol.

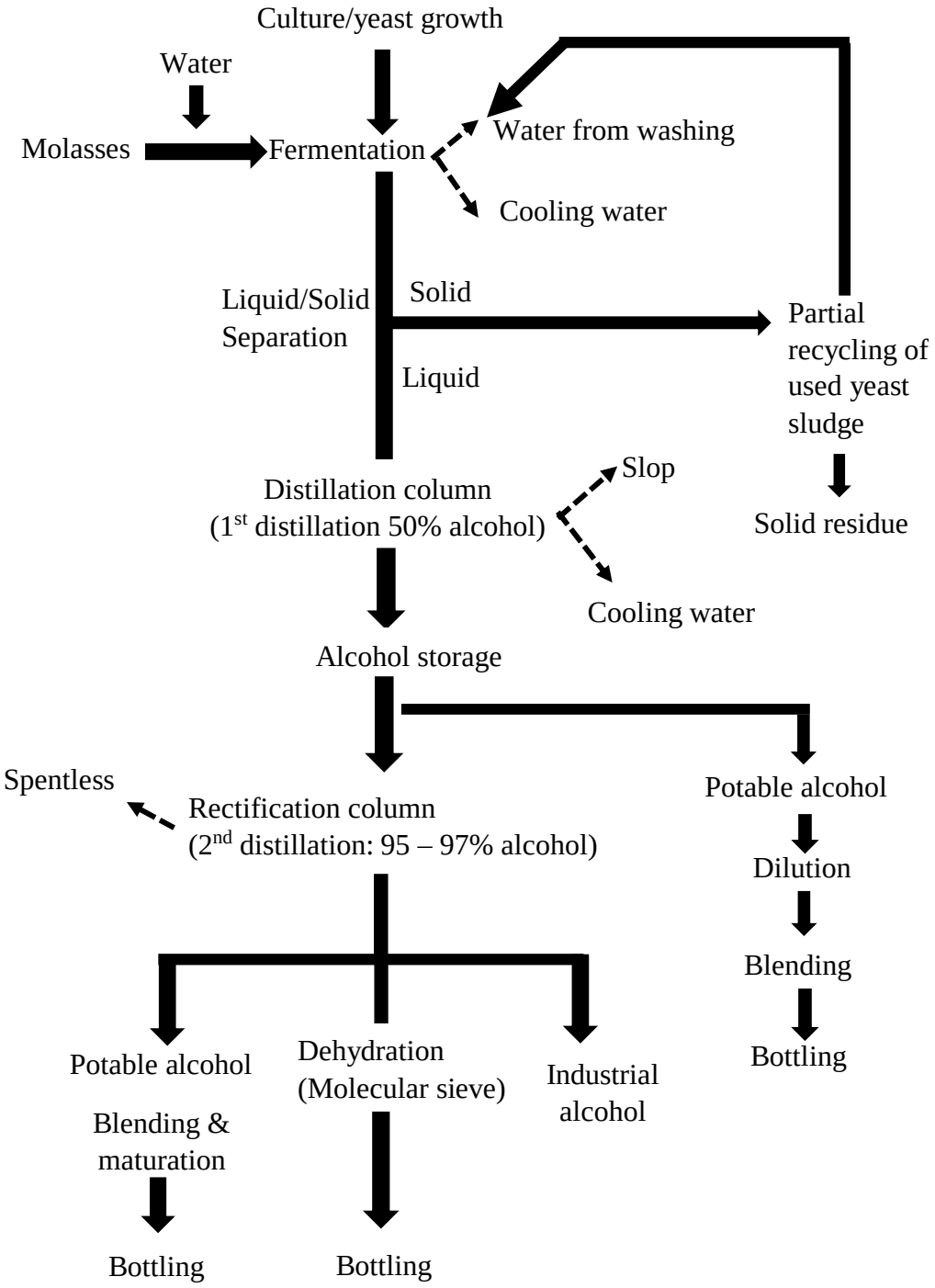

Figure 1.1 Process of sugarcane-based ethanol distilleries.

(Source: Satyawali and Balakrishnan 2008; Kharayat 2012)

1.3. Characteristics of spent wash generated in ethanol production

Sugarcane molasses-based distillery industries consume highest amount of raw water (25 – 175 L/L of alcohol) for process consumption such as yeast propagation, molasses preparation and non-process applications, like cooling water, steam generation, etc. The major source of wastewater is considered as fermented sludge and spent less and spent wash. The fermented sludge generates after filtration of the fermented wash through fermentation process. Spent less generates after rectifying column and usually being recycled usually. Spent wash is the major wastewater produces from the ethanol distillery, which also known as distiller spent wash water (DSW), slop, vinasses and stillage (Umair Hussan *et al*. 2021). According to the estimations, 88% of the molasses components end up as waste (Satyawali and Balakrishnan 2008). The distillery spent wash is considered as one of the most polluted wastewaters due majorities of the raw materials are converted into waste and generation of high strength liquid waste.

The spent wash has similarities with sugar industry wastewater in composition, whereas the distillery spent wash is a high strength wastewater. General physicochemical properties of the sugarcane-based distillery crude (raw) spent wash extracted from different studies which were conducted over the world are indicated in **Table 1.1**. The spent wash is a dark brown coloured and high strength effluent generates in huge volumes after the distillation step. Production of 1 L of alcohol releases an average of 10 – 15 L of wastewater (Satyawali and Balakrishnan 2008).

Various studies have reported that the distillery effluent consists of many types of organic and inorganic pollutants such as melanoidin, polysaccharides, reduced sugar, proteins, waxes, N, K, Ca, SO_4^{2-}, PO_4^{3-} etc. The effluent is generally characterized by unpleasant odor, low pH, highly acidic in nature, high temperature, dark brown colour, ash content and high percentage of dissolved organic and inorganic matter with high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values and COD/BOD ratio as well as high potassium, phosphorus and sulfate content (Mahimairaja and Bolan 2004). Cane molasses spent wash also contains low

molecular weight compounds such as lactic acid, glycerol, ethanol and acetic acid. Characteristic dark brown colour of the spent wash is an impact of the presence of the major pigment melanoidin and other colorants such as caramels, phenolics and furfurals. Melanoidin are recalcitrant, antioxidant, acidic, nitrogen containing dark brown polymeric compound formed as one of the final products of the non-enzymatic (Maillard) reaction between sugar and amino compounds at high temperature. Generally, molasses-based ethanol distillery spent wash is the major source of melanoidin wastewater source (Mikucka and Zielińska 2020).

Table 1.1 Physicochemical properties of the crude spent wash generated in ethanol production.

Physicochemical property		Metal	(µg/mL)
Colour	Brownish black	P	77.6 ± 2.51
Odour	Pungent	S	1,609 ± 37.3
pH	4.0–5.0	Fe	68.5 ± 3.04
EC (dS/m)	12.5	Mn	3.0 ± 0.17
COD (mg/L)	102,500	Zn	4.01 ± 0.52
DO (mg/L)	Nil	Cu	2.66 ± 0.14
BOD (mg/L)	50,500	Cd	0.025 ± 0.01
SS (mg/L)	20,000	Cr	0.172 ± 0.01
TDS (mg/L)	8,000	Ni	0.863 ± 0.05
Total N (mg/L)	1,000	Pb	1.24 ± 0.12
PO_4^{3-} P (mg/L)	45		
Exchangeable K (mg/L)	10,500		
Exchangeable Na (mg/L)	250		

(Source: Kaushik *et al.* 2005; Jain and Srivastava 2012; Garcia *et al.* 2017; Fito *et al*. 2019[b]).

1.4. Environmental impacts of discharging untreated spent wash

Untreated industrial effluent discharges may cause adverse impacts to the natural eco systems which has been known for a long time. Specially, discharge of melanoidins containing spent wash with untreated pollutant loads can increase the turbidity and block the sunlight penetration in water bodies (Mikucka and Zielińska 2020). Reduced photosynthetic activity in the water bodies with the drastically reduce oxygen concentrations (oxygen depletion) can be detrimental to the aquatic life. This can also result in loss of productivity of natural waters and deterioration of water quality to such an extent that the water becomes unusable (Baskar *et al*. 2013). Furthermore, highly acidic nature in the effluent may stimulate the dissolution of metals in water and impair the biological activities of organism such as create infertility conditions in their life cycles.

Insufficiently treated spent wash is rich in nutrients such as phosphates, nitrates and sulfates therefore eutrophication possibility of water bodies has also been identified. Unpleasant odour of the spent wash can distract the natural predators may collapse the food chains and food webs which are essential to sustain the balance of the ecosystems (Fito *et al*. 2019[a]). Obnoxious odour of H_2S from sulfates, indole and skatole from the yeast cells can cause irritations and destruction to aquatic life a well (Baskar *et al*. 2013). Ground water contaminations may result due to leaching of distillery spent wash (Fito *et al*. 2019[a]). Several cases have been reported in villages of India regarding the colour change in ground water due to the distillery effluent leaching (Baskar *et al*. 2013).

Direct dumping of distillery spent wash can cause a risk of soil salinization and contamination by zinc and manganese. Inappropriate land discharge has been reported to inhibit seed germination, reduce soil alkalinity, cause soil manganese deficiency, and damage agricultural crops. Generally, untreated distillery spent wash considered as an eco-toxic effluent, unsafe to use and harmful to agricultural and aquatic ecosystems Therefore, discharge of untreated or partially treated distillery spent wash poses serious threat to the eco systems and its organisms (Fito *et al*. 2019[b]).

1.5. Spent wash treatment and management

Treatment of spent wash has been identified as crucial before being discharged into the environment due to the presence of various types of pollutants in high strength (Tewari *et al*. 2007). The impacts due to direct discharge of untreated or partially treated spent wash have been reported in various studies worldwide (Chandra *et al*. 2008). The treatment of industrial effluent is a mandatory aspect in terms of many countries' legal background to fulfill the regulatory norms imposed by responsible institutions. Treatment efficiency, treatment cost, local geography, climate, land use, regulatory constraints, and public attitude of the treatment may influence the selection of treatment option (Kharayat 2012). Current treatment options for distillery spent wash consist of physicochemical, biological and alternative methods.

1.5.1. Physicochemical treatment and management of spent wash

Physical treatment methods such as screening, flow equalization mixing, flotation, and sedimentation are used at the initial stage of effluent treatment (Fito *et al*. 2019[b]). Adsorption is a one of the most widely used physical method where activated carbon is widely employed for removal of colour and specific organic pollutants. Physical treatment is used to settle suspended/settable solids from wastewater via sedimentation by using the force of gravity (Chowdhary *et al*. 2018). In chemical wastewater treatment, oxidizing agents such as chlorine (Cl_2), oxygen (O_2), ozone (O_3), and permanganate (MnO_4^-) are added to oxidize the wastewater components into carbon dioxide (CO_2), water, inorganic matter, and other harmless products. Coagulation and flocculation processes are used to remove suspended, inert, or undesirable colloidal materials in industrial wastewater (Kharayat 2012). Forty percent of removal of wastewater pollutants has been achieved by treatment of distillery wastewater using iron sulfate ($Fe_2 (SO_4)_3$) as a coagulant (Pikaev *et al*. 2001). Biological treatment system can be divided into primary, secondary, and tertiary steps to achieve the desired level of pollutant.

The primary treatment traps solids, oil, and fats, while secondary treatment involves the removal of organic matter and nutrients, and tertiary treatments are generally referred as the polishing stage (Chowdhary *et al*. 2018).

1.5.2. Biological treatment and management of spent wash

Normally, Biological wastewater treatment involves microorganisms to utilize wastewater pollutants for growth and convert the organic substrate in the wastewater into simpler substances such as CO_2 and water. Waste waters with high organic contents are generally considered to be suitable for anaerobic treatment hence molasses spent wash makes more attractive towards anaerobic treatment in comparison to direct aerobic treatment. Various anaerobic reactor configurations are being used currently (Satyawali and Balakrishnan 2008). Hence, anaerobic lagoons are the simplest choice for anaerobic treatment of molasses-based wastewater treatment (Tewari *et al*. 2007). Methane, CO_2 and water are generated in the anaerobic treatment process through interactions among physiologically diverse groups of microorganisms in different process steps. Generated biogas can be utilized for steam generation in the boilers thereby meeting the energy demands of the unit (Tewari *et al*. 2007). Low nutrient requirements and stabilized sludge production are other associated benefits of the anaerobic treatment.

An aerobic treatment required after post-anaerobic treatment stage since the biomethanated effluent still has high organic loading and dark brown color. Most commonly used aerobic wastewater treatment is the activated sludge process where in aerobic bacterial and fungal groups involved in reactions. Research efforts are targeted at improvements in the different reactor configuration and performance. Solar drying of biomethanated spent wash is another option but the large land area requirement limits this practice. Generally, aerobic treatment required high energy consumption making the process less feasible (Satyawali and Balakrishnan 2008).

1.5.3. Alternative treatment and management of spent wash

Major sugar and ethanol producers in the world (Brazil and India) practice fertigation, biocompost and concentration by evaporation (incineration) as common methods to manage spent wash generated from the sugarcane-based ethanol industries. In fertigation, soil is used as a medium for treatment and disposal of industrial wastewater. Agricultural irrigation (Fertigation) can help in overcoming water shortage in non-irrigated areas where the availability of freshwater very limited. As the wastewater is rich in nutrients, this practice can reduce fertilizer consumption and irrigation water cost. Similarly, disposal of distillery spent wash on land can cause hazardous and toxic effects to seed germination and growth of plants. Soil quality can be impacted though direct application by altering the physical properties such as permeability, aeration, soil structure and composition (Fito *et al*. 2019[a]).

Evaporation is another alternative where the wastewater can be burned in special boilers and the condensate removed by evaporation can be treated and reused by the factory to decrease water use in the facility. However, this process has several constraints such as fast incrustation of evaporators, high energy demanding process, and spontaneous crystallization as the concentration of solids increases (Fito *et al*. 2019[a]).

In biocomposting, press mud and distillery spent wash are mixed to preparation of an organic manure. Biocomposting solves problems of waste disposal and pollution and replenishes the soils and reduces fertilizer cost. Raw distillery spent wash contains high BOD and COD hence the spent wash has to be subjected to a primary anaerobic digestion (bio-methanation) prior to use for biocomposting process. Difficulty to manage the huge volume of distillery spent wash through very slow biocomposting process and requirement of large treatment space can be considered as constraints of this process (Baskar *et al*. 2013).

Post-biomethanated effluent, which is a rich growth medium has been used for pisciculture activities near Chennai city in southern India. Constructed wetlands

which are artificially built engineered systems that use natural functions of vegetation, soil, and as a polishing method after biological treatment prior the land discharge for irrigation purposes. Some aquatic weed plants have proven to be effective in further reduction of COD, BOD and other nutrients in bio-methanated spent wash (Fito *et al*. 2019a). Fungal and bacterial treatments are reported to be successful in decolorization of the melanoidin pigment and further reduction in BOD and COD in anaerobically and aerobically treated spent wash. Some fungi have been more effective in decolorizing raw molasses spent wash than the anaerobically and aerobically treated spent wash. The prokaryotic, oxygen-evolving photoautotrophs (cyanobacteria) have been reported to be useful in degradation and metabolization of the melanoidin in distillery effluent (Kharayat 2012).

Alcohol distilleries over the world use different treatment methods and management options to treat spent wash as described in section 1.5. However, these treatment methods alone could not guarantee the removal of various compounds which are identified as cytogenotoxic, and potentially mutacarcinogenic (Chandra *et al*. 2008).

1.6. Use of higher plants-based bioassays for assessment of cytogenotoxic potential of spent wash

Complex mixtures of industrial effluents may contain possible genotoxic and mutagenic chemicals as well as other chemicals which can cause toxic effects on biological systems (Radić *et al*. 2009). It is obvious that the industries are legally bound to discharge their effluents into the water bodies after a proper treatment. However, these treated wastewaters still may contain chemicals that can cause genotoxic/mutagenic or phytotoxic effects (Barberio 2013). Bioassays are an elegant fit to use in concurrence with physicochemical analysis and useful to fulfill the gap of measuring biological parameters of waste waters (Gana *et al*. 2008).

Spent wash is widely being used as a fertilizer in fertigation of the own sugarcane cultivations yet application of raw spent wash may cause problems in soil such as unbalance of nutrients, leaching of metals, salinization etc. Higher plants are widely used as a sensitive and simple model to assess soil contaminations as plants are direct

receptors of pollutants. Higher plants are also considered as excellent genetic models for detecting environmental mutagens and environmental monitoring (Pedro-Echer *et al*. 2016). Also, higher plants and associated cytogenetic tests (chromosome aberrations in mitotic cells) could be used as early responders of adverse long-term effects and predict the levels of future harm upon exposure to environmental stresses such as discharge of waste waters (Hemavathi *et al*. 2015). Among most widely used higher plant-based bioassays, *Allium cepa* (common onion) is one of the best test-systems which has been proven as an effective, economical and sensitive test so far for variety of environmental contaminants. The test shows a good correlation with mammal test-systems reported as similar to 82% of the results obtained with rodents (Pedro-Echer *et al*. 2016).

1.7. Sugarcane-based ethanol industry in Sri Lanka

Sri Lankan sugarcane industry operates as a complex of plantation-sugar factory and distillery located within the same premises. Currently there are three functioning sugar producing industries namely, Pelwatta, Sevanagala, and Hingurana with processing capacities of 3300 Tonnes of cane per day (TCD), 1,250 TCD, and 2,000 TCD respectively and each sugar factory is accompanied a distillery plant (Kulasekara *et al*. 2021). Cane molasses-based ethanol production has a high potential than using any other feed stock. Pelwatta and Sevanagala distilleries together produce around 12 million liters of alcohol per annum. Under Sri Lankan context, 1,000 kg of cane produces 140–160 L of spent wash and 30–35 kg of filter-mud averagely (Sustainable Biofuel for Sri Lanka, 2012).

Pelwatta Distilleries Limited is the largest and leading alcohol producer in Sri Lanka uses “molasses”, a by-product generates from the sugar manufacturing as a raw material to produce alcohol. The distillery industry has a capacity of 100 tons of molasses per day (TMD). Main product of Pelwatta Distillery is potable alcohol (25 m^3 /day) and by products includes compost and methylated spirit (denatured alcohol). The industry utilizes 100 Metric tons of molasses and urea (600 kg), sulfuric acid (600 kg), di ammonium phosphate (12 kg), hydrochloric acid (70 kg)

and magnesium sulphate (3 kg) as other raw chemicals for the daily ethanol production (Production Manager, personal communication, Pelwatta Distillery Limited).

Distillery industry requires around 600 m^3 of water for the daily production process and generates around 300 m^3 of wastewater/ spent wash per day. Currently, wastewater generates from the production process are being disposed into a concreted lagoon and use to irrigate the sugar cane cultivations maintained within the company premises. Since nearly half of the input water turns into wastewater, adequate treatment of wastewater is required to avoid the discharge of untreated waste waters into the environment. Disposal of large volumes of high strength molasses-based wastewater can cause serious environmental problems. Treatment of spent wash is a mandatory requirement to comply with the national regulations and standards before being discharged into water bodies or use for irrigation and agricultural purposes (**Appendix 01**).

Spent wash generates after the analyzer column, is a thick aqueous liquid contains fair amounts of major plant nutrients such as N, P, K Ca, Mg and appreciable amounts of micronutrients (Baskar *et al*. 2003). Use of spent wash in irrigating crops (fertigation) has proven advantages in terms of improving soil quality and crop productivity since it can supply substantial amounts of nutrients and solve the problems of unnecessary discharges environmental pollution. Countries lead the ethanol production such as China, Brazil have identified spent wash as a viable mineral source for various crops (Prado *et al*. 2013).

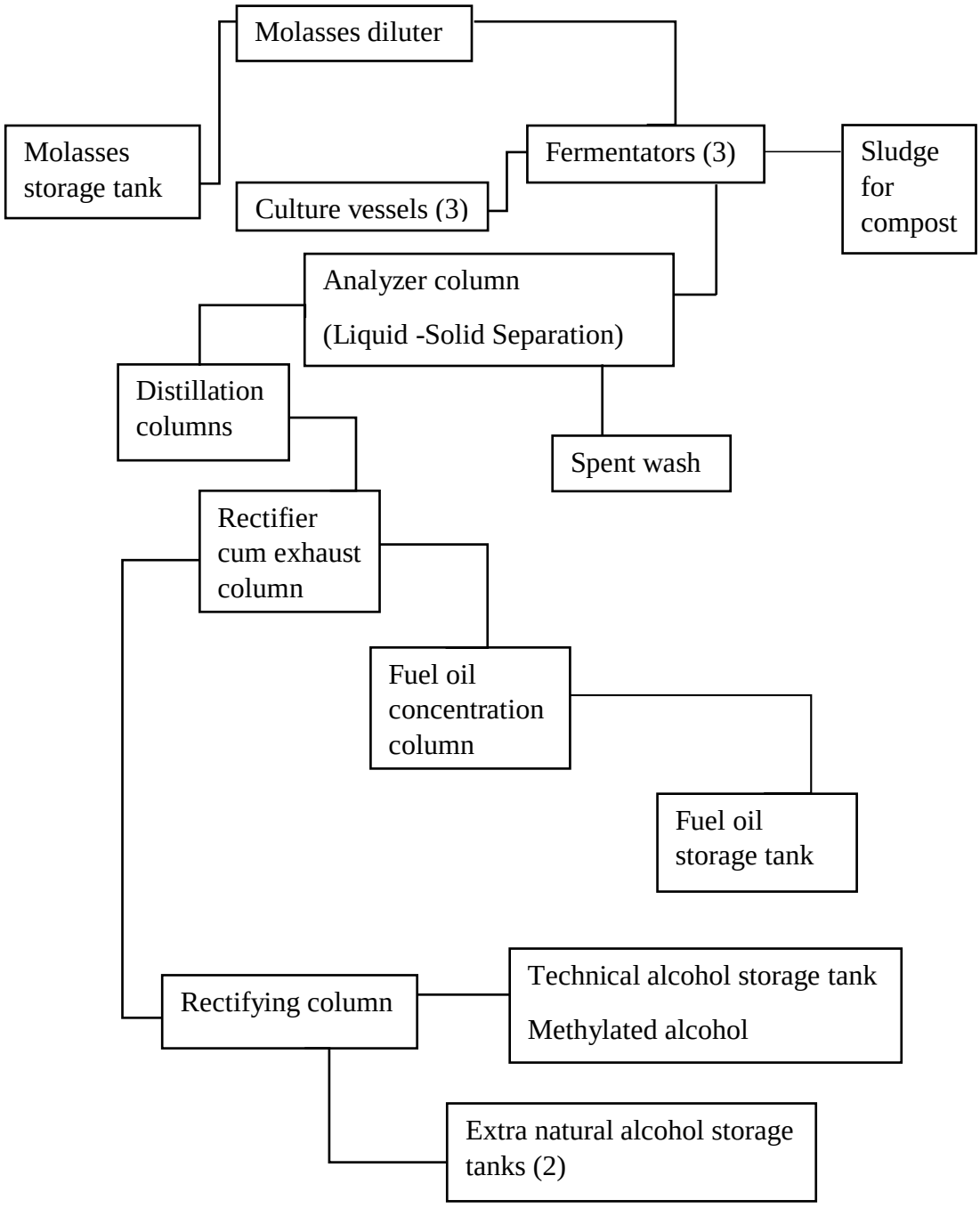

Figure 1.2 Simplified production process flow of ethanol at Pelwatta Distillery.

1.8. Rationale, Hypothesis and Objectives of the present study

As discussed in the previous sections, compared to the studies which have been conducted related to industrial effluents over the world, less attempts have been made in Sri Lanka to study the physicochemical characterization and feasibility of sustainable utilization of industrial effluents (Kannangara and Pathiratne, 2015). Out of main three sugar production industries, Pelwatta Distilleries Limited is the largest and leading molasses-based alcohol producer in Sri Lanka generates around 300 $m^3$of wastewater/ spent wash per day (Production Manager, personal communication, Pelwatta Distillery Limited). Disposal of large volumes of high strength molasses-based wastewater can cause serious environmental problems (Chowdhary *et al*. 2018, Mikucka and Zielińska 2020). However, physicochemical monitoring, potential environmental impacts or potential utilization of sugarcane spent wash as a nutrition source for vegetable crops have given less attention so far in Sri Lanka (Kulasekara *et al*. 2021).

Present study was designed to assess the potential cytogenotoxicity of spent wash and potential usage of spent wash as a nutrition source for the crops. Analyzing the physicochemical characteristics of the spent wash should be prioritized before any other experiment or management option (Diangan Jr. *et al*. 2008). Hence, some selected physicochemical parameters of the spent wash were measured using standard analytical protocols. Due to significant advantages such as ease of handling and storage, simplicity, less expensiveness and rapid and effective biological sensitivity, plant bioassays were selected for assess the cytogenotoxic effect of sugarcane distillery spent wash (Fiskesjo 1985; Siddiqui *et al*. 2011). The cytogenotoxic effect of sugarcane distillery spent wash was assessed using *Allium cepa* as test as a practically feasible tool for measuring the biological impacts of industrial effluents.

Use of spent wash in irrigating crops (fertigation) have proven advantages in terms of improving soil quality and crop productivity since it can supply substantial amounts of plant nutrients and solve the problems of unnecessary discharges environmental pollution (Baskar *et al*. 2003). The second experiment was conducted to test the

feasibility of using raw sugarcane spent wash as liquid nutrition source for common vegetable variety grows in up-country of Sri Lanka. Several growth-related morphometric measurements of transplanted tomato (*Solanum lycopersicon*) plants were selected to test the impacts of raw sugarcane spent wash.

Scientific Hypotheses

The spent wash generated by Pelwatta Distilleries Limited does not exceed the physicochemical standard for irrigation purpose water introduced by Central Environmental Authority (CEA) and water does not contaminate with toxic substances which can cause cytogenotoxicity on biota.

Hence, the spent wash generated by Pelwatta Distilleries Limited can be used as a liquid nutrition source for selected agricultural crop which can be measured as selected agronomical morphometric measurements.

Objectives

- To determine physicochemical characteristics of the raw sugarcane distillery spent wash using standard chemical analytical methods.

- To assess the cytogenotoxicity of the sugarcane distillery, spent wash using standard plant bioassay with Common onion (*Allium cepa*).

- To assess the growth-related morphometric attributes of tomato (*Solanum lycopersicon*) plants treated with different dosages of raw sugarcane distillery spent wash as a liquid nutritional source.

2. LITERATURE REVIEW

2.1. *Allium cepa* standard plant bioassay as a tool for evaluating potential cytogenotoxicity of industrial effluents

Higher plants and their seedlings have been used intensively to study the cytogenotoxic effects of heterogeneous mixtures of industrial effluents introduced into the aquatic ecosystems (Barberio 2013). They have been proven as outstanding genetic models to detect environmental mutagens and frequently used in monitoring studies (Leme and Marine-Morales 2009). Unlike the animal models, advantages of using plants are easy to store and handle, relatively simple to perform, inexpensive, biologically sensitive and rapid methods (Fiskesjo 1985; Siddiqui *et al*. 2011). Plant root system is the prominent part of the biological testing because the root tips directly exposed to the chemicals in soil and water. Observation of the root system can be therefore considered as the most sensitive method for environmental monitoring. Both the growth restriction at macroscopic level and the cells/tissue studies at microscopic level will give more information about the harmful effects of environmental contaminants (Fiskesjo 1988).

The most frequently used assay among the plant categories is the *Allium* (Common onion) genotoxicity test. This test is based on the mitotic and cytokinetic abnormalities and chromosomal aberrations occur in the meristematic zone (cell division zone) in *Allium cepa* or *Allium* ascalonicum root system (Kristen 1997). *A. cepa* is an efficient bio indicator due to the cellular proliferation kinetics, the rapid growth of the roots, large number of cells in division, high tolerance to different cultivation conditions, year-round availability, easy management and reduced number of large chromosomes (2n = 16) (Carita and Marin-Morales 2008; Barberio 2013).

The *Allium* test has a distinctive place because it can be adopted for screening soil and water pollutants and industrial waste chemicals as well as pesticides (Kristen 1997). Another importance in this test is that no need of condensation or purification of the sample (Vujosevic *et al*. 2008).

Roots of *Allium* can be allowed to grow direct contact with the complex industrial effluent samples and enable to predict the damage to DNA of eukaryotes (Tedesco and Laughinghouse 2012). The USEPA has used *Allium cepa* test to assess the toxic and genotoxic effects of industrial effluents (Junior *et al*. 2007).

Several studies were carried out in Sri Lanka to assess the cytotoxicity and genotoxicity of wastewater discharged by industries into the surface waters. Potential cytogenotoxic effects of industrial wastewater and water receiving canal, Dandugan Oya in the Western Province had been studied using the *Allium cepa* bioassay in combination with the physico-chemical analysis by Kannangara and Pathiratne (2015). Another study that had been carried out to assess the cytogenotoxicity of nine treated industrial effluents which are discharged into the Kelani River basin suggested *Allium cepa* bioassay as a practically feasible monitoring tool that can be used in concurrence with the physicochemical analysis to identify the genotoxic contaminants which have not been removed by the treatment processes (Pathiratne *et al*. 2015). Similar study that was carried out by Hemachandra and Pathiratne (2015) revealed the importance of using *Allium cepa* bioassay to identify the potential genotoxic effects of heavy metals in industrial effluents. Root growth inhibition, depression of mitotic index and nuclear abnormalities and chromosomal aberrations had been used as toxicity endpoints in these studies.

2.2. Toxicity assessments of distillery effluent using *Allium cepa* and other plant bioassays

Many studies have been conducted with different types of industrial effluents but limited number of experimental studies were carried out to assess the toxicity of sugar cane-based distillery spent wash using *Allium cepa* bio assay. A study has been conducted to assess the mutagenic and genotoxic activity of spent wash collected from a fuel alcohol plant, located in the municipality of Frontino, Northwestern Colombia (Oñate *et al*. 2015). The spent wash sample was subjected to biological oxidation and Fenton reaction consecutively.

Portions of untreated spent wash biologically treated spent wash and fenton treated spent wash samples were diluted to different concentrations (1%, 0.75%, 0.50% and 0.25%) and onion bulbs were exposed to different concentrations. After 48 hours, root tips were cut and preserved for the microscopic observe of mitotic index, occurrence of micronuclei and chromosome alterations in root meristem cells. Study has revealed that purification treatments significantly reduced the frequency of chromosomal aberrations. (Oñate *et al*. 2015).

Different concentrations (v/v%) of effluent viz. 2.5%, 5%, 7.5% and 10% were used to treat *Allium sativum* (Garlic) bulbs at room temperature to detect cytotoxic efficacy of distillery effluent on somatic cells (Hemavathi *et al*. 2015). Slides with preserved root tips were prepared for the microscopic studies of meristematic cells. With the increased effluent concentration, percentage mitotic index was found to be decreased significantly. However mitotic index in 2.5% concentration was found to be higher than control. As the effluent concentration increased, chromosomal abnormalities were found to be increased when compared to control (Hemavathi *et al*. 2015).

In some cytotoxic and genotoxic potential assessment studies, seeds of *Allium cepa* has been used. The study conducted in Brazil with two different sugarcane spent wash samples which were obtained from two different harvests (Samples I and II). Seeds of *Allium cepa* were exposed to raw spent wash and different diluted concentrations such as control soil + raw spent wash; 50% diluted spent wash + control soil (V 50%); 25% diluted spent wash + control soil (V 25%); 12.5% diluted spent wash + control soil (V 12.5%) in petri dishes. Low pH and high concentration of potassium showed when the spent wash samples were chemically characterized. Results revealed that the two raw spent wash samples tested are toxic as there was no seed germination observed. In microscopic studies, cytotoxic potential was observed of root tip meristem cells of the seeds tested with SV and V (50%). All groups in samples I and II have shown statistically significant chromosomal alterations when compared with negative control (Pedro-Escher *et al*. 2016).

Most of the groups exposed to different concentrations of raw spent wash has shown an increase in frequency of micronuclei in meristematic cells. However, this study did not reveal a significant reduction in the Mitotic Index (MI- frequency of cellular division) in the root tip meristem cells exposed to sugarcane spent wash since a correlation could not be given between the reductions of MI with the presence of cytotoxic pollutants in the environment. This study concluded that the genetic material of the test-system could be damaged when exposed to sugarcane spent wash (Pedro-Escher *et al*. 2016).

Garcia *et al*. (2017) assessed the genotoxicity of sugarcane and orange spent wash. Sugarcane spent wash was found to be widely used for irrigation of crops with water (fertirrigation) and has a great fertilizer potential. Hence evaluating the ecotoxicological effects on plants is similarly important. *Allium cepa* was used as the test organism in this study. Petri dishes were lined with filter papers to place 100 *A. cepa* seeds and exposed to 4 mL of each concentration (2.5% and 5%) of sugarcane and orange spent wash with triplicates. Negative and positive controls were treated in the same manner. Radicles were cut and fixed in Carnoy 3:1 (ethanol: acetic acid, v/v) after 5 days of exposure period. After this process, the radicles were transferred to a new fixative and stored at 4 °C until analysis.

Slides were prepared to observe characteristics of meristematic cells and F1 region cells through light microscope following standard procedures. Frequency of micronuclei (FMN) and the occurrence of chromosomal aberrations (CA) at different stages of cell division (prophase, metaphase, anaphase and telophase), such as chromosome stickiness, chromosome bridges, C-metaphase, chromosomal delays and nuclear buds were observed and recorded and compared with the negative and positive controls. According to the results, these two effluents were genotoxic and mutagenic at both dilutions. Study also revealed that presence of metals and low pH are the main parameters responsible for the toxicity. As genotoxic effects in *A. cepa* were recorded when exposed to the sugarcane and orange spent wash, conducting further studies were recommended to ensure the safeguard when applying spent wash as a fertilizer into the environment.

Kumar *et al*., (2021) conducted a study recently on post methanated distillery effluent (PMDE) has revealed that effluent contained high BOD, COD, TDS along with heavy metals like Fe (572.64 mg/L), Mn (4.269 mg/L), Cd (1.631 mg/L), Zn (2.547 mg /L), Pb (1.262 mg/L), (Cr 1.257 mg/L), and Ni (0.781 mg/L) exceeded the permissible limits for discharge of effluent. Evaluation of cytotoxic and genotoxic effects of PMDE using *Allium* bioassay reflected that the mitotic index (MI) was inhibited and chromosomal abnormalities in root tip cells especially in higher concentrations (Kumar *et al*. 2021).

2.3. Application of sugarcane distillery spent wash as a liquid fertilizer for various crops

There has been a substantial increase in the use of wastewater for crop irrigation, especially in arid and seasonally arid areas of both industrialized and developing countries. There is a growing recognition by water resource planners and researchers about the potential of using wastewaters for agricultural purposes as they are rich source of nutrients and provides all the moisture necessary for crop growth which significantly increase crop yield. Apart from that, increasing scarcity of alternative waters for irrigation, increasing urban demand for potable water supplies, high cost of artificial fertilizers and high-cost treatment technologies can be considered as other for the growing interest in agriculture use (Hussain *et al*. 2002; Kumar and Chopra 2012).

Many attempts have been made to quantify the effects of treated and untreated wastewater on number of quality and yield parameters under various agronomic situations. Most of these studies suggested that treated wastewater can be used for producing crops with higher yields and better quality than the crop with untreated waste (Hussain *et al*. 2002). After primary treatment, distillery spent wash is called distillery effluent. The distillery effluent (primary treated) is a dark brown liquid with an unpleasant smell of burnt or caramelized sugar. The composition of this effluent is found very similar to farmyard manure (Baskar *et al*. 2013).

Baskar *et al*. (2003) reported that the sugarcane distillery spent wash is non-toxic, biodegradable and contains large quantities of soluble organic matter and plant nutrients absorbed from the soil, as the origin of the spent wash is purely plant based. The untreated spent wash and treated spent wash contains nutrients and salts such as N, P, K, S and other micronutrients which are essential nutrients for plant growth. Therefore, fertilizer potential of this spent wash in agriculture can be assessed by controlled land application following proper methods (pre-plant application or with proper dilution).

Utilization of distillery waste waters in agriculture would save costs on chemical fertilizers and reduce the environmental pollution (Mohna *et al*. 2009). As the distillery spent wash is highly acidic in nature and contains favorable amounts of Ca and Mg, it could be utilized as an organic amendment for the reclamation of sodic soils. Soil sodality is the replacement of the Na^+ ion from soil exchange sites by cations like H^+, Ca^{2+} and Mg^{2+} ions (Saliha and Manivannan 2008). The distillery spent wash is an acidic liquid (pH 3.8 to 4.0) and it contains fairly high amounts of N, P, K, Ca, Mg and S with appreciable amounts of micronutrients which the sugarcane crop has absorbed from the soil. BOD (45,000 – 55,000 mg/L) and COD (90,000 – 110,000 mg/L) of the liquid are generally high.

Therefore, undiluted spent wash / effluent (primary treated spent wash) could not be directly applied to the growing crops. The crop should be given sufficient time to naturally oxidize the organic matter in spent wash by applying spent wash before planting the crop. Also, in some studies it is recommended to apply spent wash after proper dilution with water and applied for the growing crops. Some researches revealed that the application of undiluted spent wash followed by irrigation is far more effective rather than the dilution of spent wash at the time of its application in the reclamation of sodic soils (Baskar *et al*. 2013).

In pre-plant application, distillery spent wash is applied in fallow lands during summer, facilitating oxidation of organic matter and reduction of BOD and COD levels to solve soil fertility problems and increase the crop yields.

In countries leading sugarcane industry such as Brazil and India, spent wash is used to irrigate sugarcane to provide nutrients to crop whether it is treated or not. Many field studies have conducted using bi-annual crops such as rice, sugarcane, and maize which are cultivated in huge land extents. Application of untreated DSW on land would require a large land area to facilitate natural oxidation of high organic matter content. Unless lagooning or anaerobic digestion prior to irrigation would reduce this requirement (Baskar *et al*. 2013; Diangan Jr. *et al*. 2008).

A field study was carried out by Srivastava *et al*. 2012 to monitor the effect of application of molasses-based distillery effluent on yields of sugarcane and soil properties. Application of undiluted distillery effluent and diluted distillery effluent as pre-sown irrigation under good soil drainage conditions significantly increased the cumulative yields of sugarcane over no application of effluent on sugarcane (control). No significant effect was noted on soil pH, electrical conductance and exchangeable "Na" upon applying different treatments. In comparison, significantly higher build-up of organic C in surface soil was noted in one specific diluted treatment. The study concluded that the molasses-based distillery effluent had favourable effect on cumulative cane yields and could deduct the fertilizer consumption of the farmers.

Apart from the field studies, many other studies have been conducted in lab scale or pot cultures as controlled experiments. Jain and Srivastava (2012) conducted a pot culture experiment using sugarcane (*Saccharum* spp. hybrid cultivar CoLk 8102) to assess the growth and biochemical attributes by application of raw and digested spent wash (Jain and Srivastava 2012). High concentrations of essential nutrients (P, S, Fe, Mn, Zn, Cu) and heavy metals (Cd, Cr, Ni and Pb) were found to be present in raw spent wash (RSW–BOD 40,000 mg/L, pH 4.0) when compared to the digested spent wash (DSW–BOD: 4,000 mg/L, pH 9.0). Sugarcane which was grown soil pot culture were treated with different rates of RSW (5, 10, 20 and 100 mL/kg soil) along with digested spent wash 100 mL/kg soil).

Results recorded after 30 days planting revealed that the sugarcane pots treated at low rate of crude spent wash (5 mL/kg soil) had shown improvement in bud

sprouting (10.5%), settling height (40%), root number (9.4%), root length (13.2%), over control while higher doses (20 and 100 mL/kg soil) of both RSW and DSW decreased the above growth parameters markedly. This study has concluded that application of spent wash will improve crop productivity and mitigate environmental pollution problems (Jain and Srivastava 2012).

In another study, effect of bacteria treated and untreated post-methanated distillery effluent (PMDE) on seed germination, seedling growth and amylase activity in black gram (*Phaseolus mungo* L) were analyzed by conducting a petri dish experiment. Study revealed that untreated PMDE had high BOD, COD values along with high metals content was highly toxic in nature while bacterial treated effluent had reduced values by 64.58% and 74.20%, respectively. The study had been conducted in a dilution series and results revealed that 40% and 60% PMDE has deleterious effects on seedling growth parameter and seeds treated with 80% and 100% PMDE showed no root development. 20% bacteria treated PMDE was identified as the optimum level for plant growth. Further, in most concentrations of untreated PMDE, treated *Phaseolus* seeds showed reduced amylase activity/ no amylase activity while seeds treated with bacterial degraded PMDE showed amylase activity (Bharagava and Chandra 2010).

An experiment was conducted using a randomized plot design in the field to evaluate the growth parameters of the seeds of wheat (*Triticum aestivum*) and pea (*Pisum sativum*) when exposed to the treated sugarcane distillery effluent and diluted distillery effluent (0%, 25%, 50%, 75% and 100%) (Pandey *et al*. 2005). Seeds were sown in the plots after proper tillage and were irrigated with 5 liters of different concentrations of the distillery effluents at the interval of five days or as per its requirements. After sisty five days, plants were uprooted to evaluate percentage germination, root length, and shoot length, number of secondary roots, girth of plants and leaf area. Based on the results, the study suggested that 25% to 50% concentration of the effluent showed positive response on the growth parameter probably due to effect of effluent as liquid fertilizer. As the concentration raises gradually, retardation of growth was observed possibly due to excess nutrients

(nitrogen, phosphate, potassium, sulphate, calcium, and chloride) have affected the water absorption and other metabolic process in plants (Pandey *et al*. 2005).

The post biomethanated spent wash/primary treated spent wash (PTSW) with different dilutions has been used to irrigate root vegetables, leaf vegetables and creepers to assess the impact on yields. The seeds of selected leaf vegetables were Amaranth (*Amaranthus gangeticus*), Coriander (*Coriandum sativum*), Fenugreek (*Trigonella foenum graceum*), Shepu (*Peucedanum graveolens*) and Spinach (*Spinacia oleracea*). The field experiment was carried out in a prepared block to sow the seeds and irrigated with raw water, 33% and 50% diluted PTSW at the dosage of twice in a week and rest of the period with raw water. At the time of maturity, leaves vegetable plants were harvested and analyzed for vitamins, minerals and trace elements present in the plants. Results revealed that the nutrient uptake of plants irrigated with 33% and 50% distillery spent wash were at a good condition compared to raw water control. Overall results concluded that 33% dilution is good for the cultivation of leaves vegetable plants (Basavaraju and Chandraju 2008).

In a similar type of experiment, top vegetables (creepers) such as Bottle Gourd (*Lagearia Vulgaris*), Ash Gourd (*Benincasa hispida*), Pumpkin (*Cucrbita mzxima*), Snake Gourd (*Trichosanthes anguina*), Ridge Gourd (*Luffa acutangula*) and Bitter Gourd (*Momordica Charavatia)* were irrigated with the same dilutions of PTSW (33% and 50%). The results of the experiment revealed that, yields were very high in plants irrigated with 33% diluted spent and moderate in plants irrigated with 50%, while very poor in raw water. Post-harvest soil tested for nutrients was found to be rich in plant nutrients (nitogen, phospurus and pottasium) and no adverse effect on other parameters (Chidankumar *et al*. 2009). Another was conducted using root vegetables such as potato (*Solanum tuberosum*), sweet potato (*Ipomoea batatas*), radish (*Raphanus sativus*), carrot (*Daucus carota*), beet root (*Beta vulgaris*) and onion (*Allium cepa*) irrigated with the same dilutions of PTSW (33% and 50%). The results of the experiment revealed that, yields were very high in plants irrigated with 33% diluted spent and moderate in plants irrigated with 50%, while very poor in raw water (Chidankumar and Chandraju 2009). Above three studies overall conclude

that, the diluted spent wash can be conveniently used for the cultivation of leaf vegetables, top vegetables (Creepers) and root vegetables without applying chemical fertilizers externally.

A laboratory experiment was conducted by Raman *et al*. (2002) to study the effects on selected vegetables viz. Tomato, Chili, Bottle gourd, Cucumber and Onion in pot exposures at different dilutions (0%, 5%, 10%, 15%, 20%, 25%, 50%, 75% and 100%) of raw spent wash. Twenty-five seeds of each species were placed in sterilized glass petri dishes lined with two filter paper discs and moistened with 5 mL of distilled water for control and same quantity of various dilutions of the distillery effluent (5%, 10%, 15%, 20%, 25%, 50%, 75% and 100%) for the others with four replications per each treatment. The germinated seeds were counted and removed on each day after count until there was no further germination (Raman *et al*. 2002).

When comparing the results of the study, tomato has shown inhibitory effects on seed germination even at lowest concentration (5%). The concentration 25% was identified as the critical value for the other crops (except for tomato) for seed germination. At highest concentrations (75% and 100%), all the crops' species had shown complete failure of germination where the speed of germination, peak value and germination values also followed a similar trend. According to this study, the crops can be arranged in an order (cucumber > chili > onion > bottle gourd > tomato) based on the tolerance for distillery effluent. Study concluded that distillery effluent could be used for pre-sowing irrigation under great concern hence the effect of the distillery effluent is crop-specific (Ramana *et al*. 2002).

Utilization of distillery effluent along with water for the crops was experimented by Kumar and Chopra (2012). A field study had been carried out in randomized block design using Fenugreek (*Trigonella foenugraecu*). Poly bags were filled with air-dried and sieved soil (5 kg) and mixed with equal quantity of farm yard manure. Soils were fertigated with 500 mL of diluted distillery effluent viz. 5%, 10%, 25%, 50%, 75%, and 100% along with bore well water and allowed for mineralization for 2 weeks.

When the Fenugreek seed (ten per each concentration with four replicates per each) were sown, each crop plant received a fertigate dose of 500 mL from each dilution and bore well water twice a week and no drainage was allowed. Physicochemical parameters of the soil were analyzed before sowing and after harvesting the crop following standard methods. Study has been conducted for ninety (90) days and different agronomical parameters were recorded in germination stage, vegetative stage, flowering stage and maturity (harvesting) stage. Seed emergence, seed germination, shoot length root length, number of leaves, number of flowers, number of pods, and crop yield, dry weight, chlorophyll content were recorded and leaf area index (LAI), harvest index were calculated. Several soil parameters as moisture content, EC, pH, Cl^{-}, total organic carbon (TOC), HCO_3^-, CO_3^- , Na^+, K^+, Ca^{2+}, $Mg^{2+,}$ Fe^{2+}, TKN, Nitrate, Phosphate and Sulphate showed a significant effect on post-harvest soil. Hence, irrigation improved the soil nutrient status. Nutrient accumulation was better in all effluent concentrations (Kumar and Chopra 2012).

The recorded agronomical parameters showed a gradual increase at higher effluent dilutions, i.e., from 5% to 50% while a gradual decrease at lower effluent dilutions, i.e., from 75% to 100% as compared to control at various stages. The effluent 50% dilution was identified as the concentration which showed a maximum growth performance of the plants. Overall performances of the crop indicated that low nutrients accumulate at low concentrations induced the growth performance and high nutrient accumulation at high concentrations inhibit growth performance. Therefore, distillery effluent can be used as an agro-based biofertilizer after appropriate dilution to obtain a maximum yield (Kumar and Chopra 2012).

2.4. Application of sugarcane distillery spent wash as a liquid fertilizer for various crops- country overview

Several investigations have been conducted by Sugarcane Research Institute of Sri Lanka to find out the feasibility of organic fertilizers produced from sugarcane by-products such as sugarcane press mud and sugarcane spent wash. These studies mainly focused on evaluating performance of sugarcane plant (*Saccharum* spp.) when exposed to different types of fertilizer mixtures made from sugarcane by-products.

Kulasekara *et al*. (2021) conducted a preliminary investigation to find out the feasibility of organic fertilizers prepared from by-products of sugarcane industry using nutritional characteristics and growth characteristics favorable for sugarcane plant. The study consists of two consecutive experiments. First experiment was to preparation of the organic fertilizer mixtures (OFM) using sugarcane trash, filter mud and spent wash as input substances. Four treatment combinations were prepared using different ratios of the above said input substances. Decomposing piles were prepared in three replicates per each treatment combination and undisturbed topsoil was added in the top layer of each soil as a microbial inoculant. At each time of turning the piles, predetermined amount of spent wash had been sprinkled over the piles. After two months of decomposing, piles were sieved and separated organic fertilizer mixtures as per the pre introduced treatment combinations and the chemical properties were analyzed.

In the second experiment, *Saccharum* spp. grown in pots under net house condition were treated with five treatments, three types of organic fertilizer mixtures (OFMs) in 20 ton/ha level along with half of the recommended chemical fertilizers (CF), recommended CF and zero fertilizer (control) for three months.

Results of the first experiment indicated that use of filter-mud and spent wash is more effective in organic fertilizer preparation and rich in plant nutrients. According to the results of the pot culture experiment, plants treated with OFM + half of recommended chemical fertilizer have shown similar growth characteristics to the

plants treated with recommended chemical fertilizers only. Soil analysis revealed that both OFM + chemical fertilizers treated soil and chemical fertilizer treated soil alone are similarly rich in major plant nutrients. Therefore, this investigation revealed that the fertilizers prepared with the combination of filter mud and spent wash could reduce the amount of application of chemical fertilizers at a remarkable level.

3. METHODOLOGY

3.1. Spent wash sampling

Distillery industry of Lanka Sugar Company (Pvt) Ltd, Pelwatta is located in Monaragala District of Uva Province, Sri Lanka (6.727484° N, 81.202725° E). Large volume of molasses based raw spent wash discharged after the fermentation process in the industry does not undergo any preliminary or physicochemical or biological treatment currently. The spent wash is temporarily stored in two concreted lagoons and used for the irrigation purposes of sugar cultivation crops of the company. Spent wash samples were collected from the lagoon depicted in the **Figure 3.1**.

Sampling of spent wash was carried out in July 2021. Wastewater samples were

Figure 3.1 Sugarcane distillery spent wash receiving lagoon at distillery industry of Lanka Sugar Company (Pvt) Ltd. Pelwaththa.

collected from three sub sampling sites in the wastewater lagoon. In-situ physicochemical parameters viz. pH, temperature, salinity, dissolved oxygen, conductivity, total dissolved solids of each sub sample were measured onsite. All physicochemical parameters were measured according to the procedures of APHA standard procedures (APHA 2017). pH was measured using a calibrated pH meter (WTW model No: 315i/SET) by dropping the probe in the sub samples and allowed the reading to be stabilized. Other in situ parameters were measured using calibrated multimeter (YSI/Model: 556 MPS) by dropping the probes in each random sample.

Three random samples were mixed to make a composite sample and filled in glass bottles for further analysis of ex-situ physicochemical parameters viz. Biochemical Oxygen Demand (BOD, APHA 5210 B), Chemical Oxygen Demand (COD, APHA 5220 D), Total Dissolved Solids (TDS, APHA 2540 C), Total Suspended Solids (TSS, APHA 2540 D) Nitrate (APHA, 4500 NO_3^- B), Phosphate as Phosphorous (APHA 4500 P E) in the laboratory as described by APHA (2017). Sample bottles were stored in ice containers and brought into the laboratory of Central Environmental Authority, Uva Provincial Office. Bottles were refrigerated at 4 ^{0}C until analysis. Polypropylene bottle, which was filled with 10% nitric acid to the top were emptied and refilled with the composite wastewater sample to measure the heavy metals in the laboratory.

In addition to the water samples collected for physicochemical analysis, one composite water sample filled into a glass bottle was brought to the laboratory for the *Allium cepa* bioassay. Another spent wash sample was filled in a polythene bag for the open field tomato crop experiment. Spent wash sampling was done again in October for the open field experiment. Samples were refrigerated at 4 ^{0}C throughout the crop experiment period.

3.2. Determination of physicochemical characteristics of the spent wash

3.2.1. Determination of Biochemical Oxygen Demand (BOD)

Five-day BOD test (5210 B) was carried out as described in APHA (2017). Dark bottle (250 mL) was filled with water completely without allowing the air to be trapped inside. Sample bottle filled to determine the initial dissolved oxygen concentrations were fixed immediately using Manganese sulphate solution (1 mL) and alkali-iodide-azide reagent (1 mL) and shaken vigorously (**Appendix 02**). The bottle was kept stand and allowed the precipitate to be settled down. Sample bottle filled to determine the dissolved oxygen after 5-days (DO_5) interval was brought into the laboratory and necessary dilutions (dilution factor, 10,000) were made with the oxygen saturated dechlorinated water for the effluent sample. Diluted samples in the dark bottles (250 mL) were incubated in the dark for five days at 20 $^\circ$C. Another two

dark bottles (250 mL) were filled with dilution water to determine initial DO and DO after 5 days as similar to the water samples.

The precipitate in the initial DO (DO1) bottle were dissolved using concentrated sulfuric acid (1 mL) at the time of processing. Portion (100 mL) of the sample was pipetted out and titrated immediately with sodium thiosulphate titrant (0.025 M) using starch as indicator (**Appendix 02**). Few drops (0.20 mL) of starch were added into the titrand when the solution become pale yellow colour while titrating with sodium thiosulphate. Appeared dark blue solution was then titrated again until it turns into colourless. The burette reading was taken at this end point. Same procedure was carried out for the diluted sample which was collected to determine the DO after 5 days. BOD was calculated using the following formula.

BOD in water sample = 1,000/ V [$(S_m - S_t) - (D_m - D_t)$] mg/L

Where, V = Volume of the sample in 1 L of the mixture, S_m = DO of the mixture before incubation, St = DO of the mixture after 5 days incubation, D_m = DO of the dilution water before incubation, D_t = DO of the dilution water after 5 days incubation.

3.2.2. Determination of Chemical Oxygen Demand (COD)

Chemical Oxygen Demand in water was determined using closed reflux colorimetric method (5220 D, APHA, 2017). For a portion of (2.5 mL) of the diluted spent wash sample (200 times diluted) in the vessel, digestion solution (1.5 mL, **Appendix 02**) and sulphuric reagent (3.5 mL, **Appendix 02**) was added. Mixture was allowed to digest for 2 hours at 150 $^{\circ}$C Same procedure was followed for the reagent blank (distilled water) and standards (50, 250, 500 ppm) assuring the total volume of each reaction vessel is same. Standards were prepared using Potassium hydrogen phthalate (KHP) stock solution (**Appendix 02**). The COD concentration was determined using the standards calibration curve.

3.2.3. Determination of Nitrate level

Ultraviolet Spectrophotometric Screening Method (4500 – NO_3^- B, APHA, 2017) was used to determine the nitrate concentration in the spent wash sample. A portion of the diluted sample (50 mL) was thoroughly mixed with 1N HCL solution. The absorbance of the sample was measured at 220 nm and in 275 nm wavelengths from the UV-Visible spectrophotometer (CECIL/Model: CE1021). Distilled water was used as the blank solution. Nitrate calibration standards were prepared (0.0, 1.0, 1.5, 2.0 ppm) from stock Nitrate solution (**Appendix 02**). Nitrate standard curve was developed by taking absorbance at 220 nm and subtract that by the double of reading at 275 nm. Absorbance was read against redistilled water set at zero absorbance. The wavelength 220 nm was used to obtain nitrate reading and wavelength of 275 nm was used to determine interference due to dissolved organic matter content.

3.2.4. Determination of Phosphate (as Phosphorus)

The Phosphorus concentration was measured using the Persulfate Digestion Method (4500 –P B.5) followed by the Ascorbic Acid Method (4500-P E) as described by APHA (2017).

A 50 mL sample fixed in-situ was pipetted out to from Erlenmeyer flask after thoroughly mixing and Sulfuric acid (1 mL, **Appendix 02**) and solid Potassium persulfate (0.5 g) were added. Then the solution was boiled gently on a pre-heated hotplate until the final volume of 10 mL was reached. The sample was cooled to the room temperature, diluted to 30 mL with distilled water and Phenolphthalein indicator (0.05 mL) was added. Then each sample was neutralized by adding Sodium hydroxide (1N) and the final volume was then made up to 100 mL with distilled water.

Neutralized sample (50 mL) were pipetted out into a clean dry Erlenmeyer flask and Phenolphthalein indicator (0.05 mL) was added. Then Sulfuric acid (5 N, **Appendix 02**) was added drop wise to until it gets a faint pink colour and then 8 mL of the

combined reagent (**Appendix 02**) were added. Sample was mixed thoroughly and kept for 10 minutes until the blue colour developed. The absorbance was measured using the 325 – 1,000 nm visible Spectrophotometer (CECIL/Model: CE 1011) at 880 nm using the reagent blank as the reference solution.

Standards were prepared (0.25, 0.5, 1.0, 1.5 ppm) using standard phosphate solution which was prepared using stock phosphate solution (**Appendix 02**). Standards were treated in the same manner as the samples. A standard curve was constructed by plotting absorbance against phosphate concentrations of the standards. The total dissolved phosphorous and total phosphorus concentrations were determined using the standard curves (APHA, 2017).

3.2.5. Determination of Total Dissolved Solids (TDS)

Total Dissolved Solids were measured according to the Gravimetric method (2540 C, APHA, 2017). A portion of the well mixed diluted spent wash sample was filtered through a glass fiber filter with a vacuum. Filtrate was transferred to a pre-weighed dish and evaporated in an oven at 180 $^{\circ}$C until a constant weight reached. The weight increase compared to the empty pre-weighed dish represents TDS.

Total Dissolved solids mg/L = (A – B) × 1,000/ Sample Volume (mL)

A = Final weight of the residue + dish
B = Weight of empty dish

3.2.6. Determination of Total Suspended Solids (TSS)

Total suspended solids were determined according to the gravimetric method (2540 D, APHA 2017). A portion of the well mixed diluted spent wash sample was filtered through a standard glass-fiber filter inserted with a 70 mm filter paper. The filter residue was then dried to a constant weight in an oven at 105 $^{\circ}$C and allowed to be cooled in a desiccator. The weight increases of the dried filter paper with residue compared to the weight of empty filter paper represents total suspended solids.

Total suspended solids mg/L = (A – B) × 1,000/ Sample Volume (mL)

A = Final weight of the filter paper with dried residue

B = Weight of the empty filter paper

3.2.7. Determination of metals

Heavy metals and light metals were determined using ICP-OES-iCAP 7000 with asx560 series. Using the "Qtegra" programe, run deionized water as "wash". Standards were prepared (2, 5, 10 and 20 ppm) and analyzed. Using the programme, again run the "wash". Finally, run the samples. Intensities and concentrations of the metals were displayed on the screen.

3.3. *Allium cepa* (common onion) bioassay for cytogenotoxicity assessments

The *A. cepa* bioassay was conducted as described by Pathiratne *et al*. 2015 and Fiskesjo 1985 with some modifications. Commercial variety of common onion was used for the determination of different toxicity endpoints of meristematic cells. Equal sized healthy onion bulbs were chosen, and the outer scale of the bulbs was removed by gently scraping to make the apices of root primordia exposed. Twenty bulbs were exposed (submerged in the test tubes) to different exposure conditions in glass test tubes (20 mL) from each four exposure conditions, (Aged tap water as negative control, 1:8 diluted raw spent wash (12.5%) and potassium dichromate as positive control) were tested (**Figure 3.2**). The bioassay was conducted under tropical

exposure conditions (temperature, (20-29 °C) and the bulbs with exposure media were kept in dark to avoid the direct sunlight.

After 48 hours (two days) of exposure, root lengths of randomly selected ten onion bulbs from each test media were measured in millimeters (Fiskesjo 1985). Root tips (5-6 from each onion bulb) of 1 – 2 mm length were processed for microscopic studies of toxicity end points. Root tips were fixed in ethanol: glacial acetic acid (3:1, v/v) solution for overnight at 4 °C. Rootlet tips were then transferred into 70% alcohol and stored at 4 °C until the time of processing.

When processing the root tips, they were hydrolyzed in Hydrochloric acid (1N) solution for 5 minutes at 60 °C and washed with distilled water. Root tips were then placed in watch glasses containing acetocarmine (**Appendix 02**) for 30 minutes to allow the stain to penetrate to the primordial cells. After staining, root tips were placed on glass slides and a slight pressure was applied on the cover slip to squash the tip cells over the slide. Prepared slides for each exposure medium were observed under the light microscope at 400 × magnification to score of mitotic stages, occurrence of micronuclei, nuclear abnormalities in the interphase cells.

Mitotic index was calculated as the number of dividing meristematic cells in 100 total meristematic cells by counting 1,000 meristematic cells in each slide.

Mitotic Index = (A)/(B) × 100

Where, A = Number of dividing meristematic cells, B = 1,000 total meristematic cells.

Nuclear abnormalities were detected in 1,000 observed interphase cells (Fiskesjo 1988). The frequency of nuclear abnormalities including micronuclei was detected in 1,000 observed interphase cells and recorded. Rest of the other bulbs (remaining 10 bulbs out of 20) were exposed to the media continuously (Fiskesjo 1985). After seven days, root lengths of the bulbs were measured in millimeter to assess the phytotoxicity endpoints.

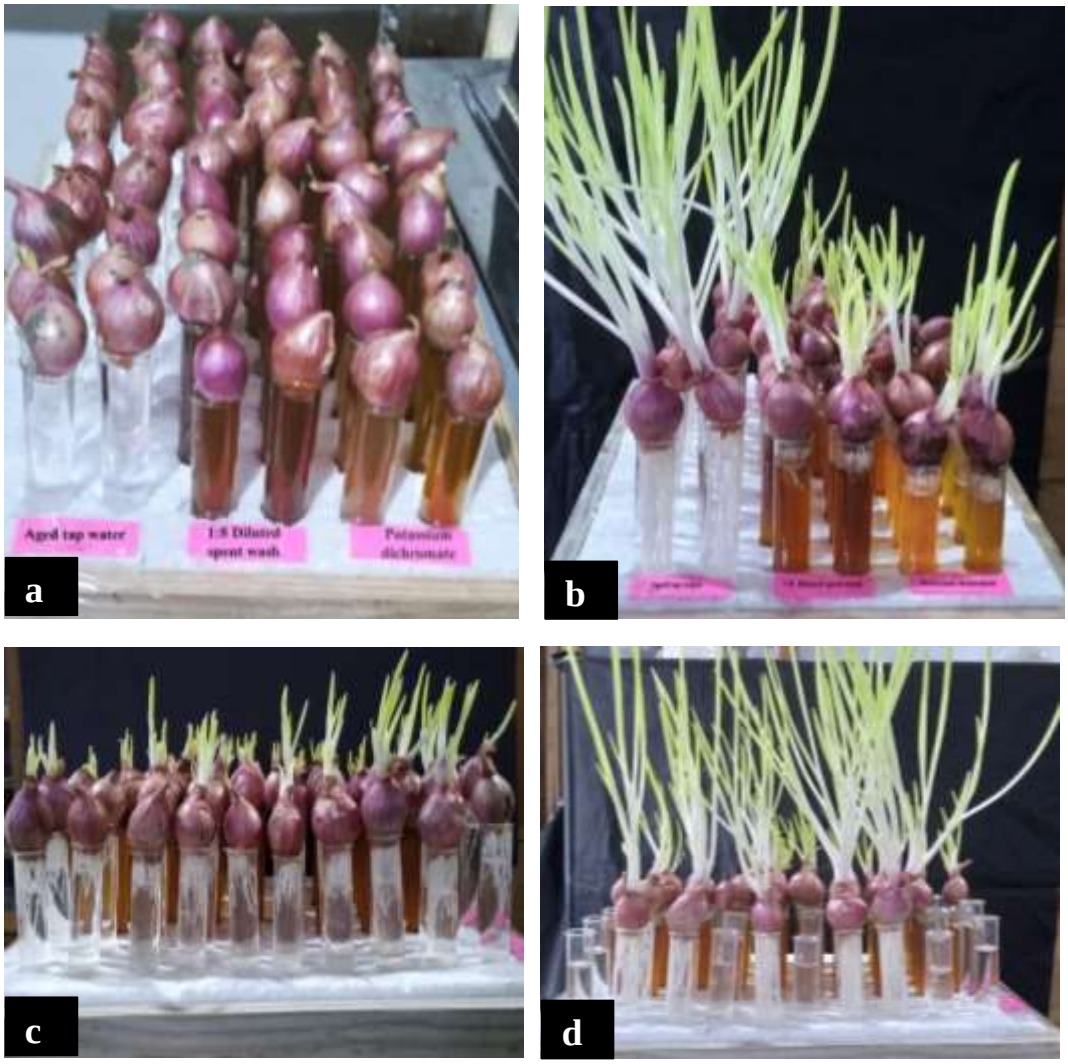

Figure 3.2 Exposure system and root growth of *Allium* bulbs exposed to aged tap water, 1:8 diluted spent wash and potassium dichromate (positive control). **(a)** Initial setup, **(b)** Root and shoot growth after 7 days, **(c)** and **(d)** Comparison of root and shoot growth in aged tap water after 2-days and 7-days exposure period.

3.4. Raw spent wash as a liquid nutritional source for tomato (*Solanum lycopersicon*) crop

3.4.1. Experimental setup

The experimental setup was designed according to the methodology described by Kumar and Chopra, 2012 with some modifications. The location falls under upcountry intermediate zone in Badulla district according to the classifications of agroecological zones by Department of Agriculture (DOA), Sri Lanka with an average annual rainfall of 1,750 – 2,500 mm average temperature of 20 – 28 $^{\circ}$C and at elevation of 1,039 m (Elevation map for Badulla, Sri Lanka, 2020). An open field experiment was conducted in the home garden (GPS Coordinates 6.874077° N, 81.053394° E) during the period of August 2021 to November 2021 which covers both dry and wet periods under the average temperature range of 20 – 28° C and average monthly average rain fall range of 74 – 280 mm.

A local variety of Tomato (*Solanum lycopersicon*) (Family Solanaceae) was selected as agricultural crop for the present study due to popularity and ease of growing in all agroclimatic regions except in upcountry wet zone as a perennial crop (Department of Agriculture, DOA). Nursery of tomato was maintained for one month with a commercially available "Thilina" variety (75% seed germination, 99% purity) according to the guidelines given by the Department of Agriculture (DOA).

Transplanting was done at the end of August 2021 in porous polythene bags with the seedlings of 10 – 15 cm tall with 3 – 5 true leaves. Each poly bag (Diameter: 20 cm, Height: 20 cm) was filled with 5 kg of well-prepared soil mixture, consists of garden soil (collected from a depth of 15 to 20 cm, air dried and sieved) and commercially available cattle manure mixed in 1:1 ratio. Open field experiment was conducted including several treatment categories which were based on different doses of sugarcane distillery raw spent wash used to apply as an external source of nutrition for the tomato crop. Dosages were prepared following the recommendations given by

DOA (Handbook of organic manure, 2021) for the application of "fish tonic" liquid fertilizer (50 mL of fertilizer per 10 L).

Based on the fish tonic fertilizer dose (50 mL of fertilizer per 10 L), range of different volumes of the raw spent wash (100, 75, 50, 25, 15, and 5 mL) were determined. Minimum water requirement per plant (1 L) was estimated by applying tap water to a soil filled porous poly bag until water overflows through pores. Determined volumes of spent wash were adjusted for 1 L by calculating according to the recommended dose (50 mL of fertilizer per 10 L) by DOA as follows.

Spent wash per plant = (Determined volume of spent wash/10,000 mL) × 1,000 mL

Accordingly, six treatment categories viz. S_1 (0.5 mL), S2 (1.5 mL), S3 (2.5 mL), S4 (5 mL), S5 (7.5 mL), and S6 (10 mL) of raw spent wash, tap water (S_0) as negative control and commercially available certified organic fertilizer, BIOTIN-V, Fish Hydrolysate as positive control (S_7) were maintained with four replicate seedlings per each series. Proper distance was maintained between each series and each plant (30 cm). Tomato (*Solanum lycopersicon*) plants in $S_1 – S_6$ series were irrigated with calculated dosages of sugarcane spent wash/1 L of tap water while plants in positive control series (S_7) were irrigated with recommended dose of certified organic fertilizer/1 L of tap water and plants in negative control series (S_0) were irrigated with tap water/1 L twice a week in the afternoon of each day after transplanting the tomato (*Solanum lycopersicon*) seedlings in the polythene bags.

Watering was conducted using pipe bourn born water as per the requirement (1L, similar quantity for each plant) considering day to day changing weather pattern and climatic conditions. Staking was done in the 4^{th} week after transplanting. **Figure 3.3** shows the different growth stages of the tomato (*Solanum lycopersicon*) crop.

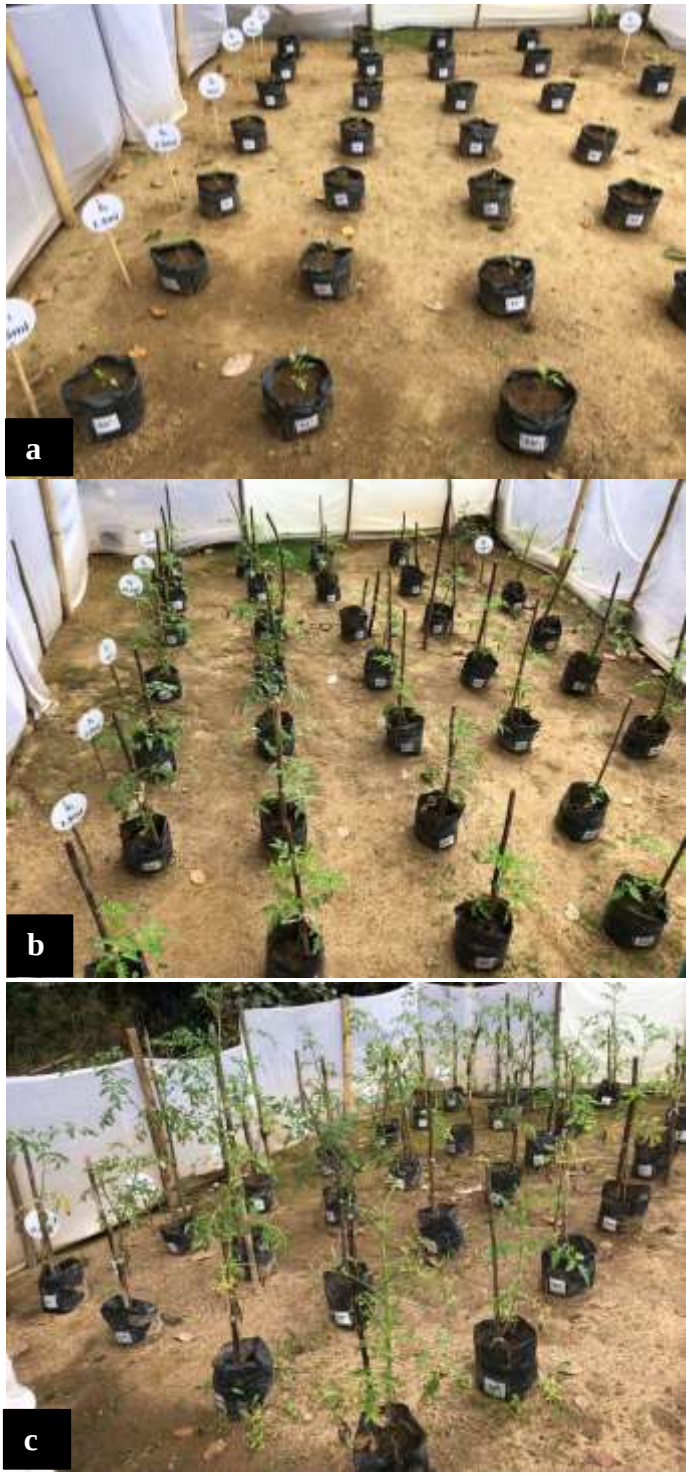

Figure 3.3 Different growth stages of the tomato (*Solanum Lycopersicon*) tomato crop during field experimental period. **(a)** Transplanting stage of plants, **(b)** vegetative stage and **(c)** fruiting stage.

3.4.2. Measuring morphometric attributes of tomato (*Solanum lycopersicon*)

Growth related morphometric characteristics, i.e., the shoots lengths of each plant, number of leaves were measured once in a two week during the study period. Pests were removed manually, and plants were maintained natural photoperiods (12:12 day and night). Flower buds and flower count were recorded 40 days after the transplanting (40 DAT).

The fresh weight of fruits in each treatment category were recorded using an electronic balance (accuracy resolution 0.01 g) 60 days after transplanting (60 DAT) the tomato seedlings. The fruits were harvested at the early stage despite the fruit size. At this stage, surface of the tomato (*Solanum lycopersicon*) is completely green and the shade of green could be varied from light to dark.

3.5. Statistical analysis of data

The mean and the standard error of the mean of the replicate data were obtained using the descriptive statistics in the Minitab 17.0 version for all the physicochemical parameters which were measured in situ (pH, temperature, conductivity, turbidity) and exsitu (COD, TDS, TSS, Nitrate, Phosphate as P). Microsoft office excel (version 2013) was used to depict the data wherever necessary. One-sample T-test was used to compare the means of COD, TDS and EC with the tolerance limits for discharge of industrial effluents for irrigation purposes (**Appendix 01)**. Accepted level of significance was $p < 0.05$.

For biological parameters including two days and seven days root lengths, mitotic index and nuclear abnormalities of *Allium cepa* data were transformed into Log_{10} (X+1) scale to assume normality in their distribution and analyzed using one way ANOVA. If there were significant differences, Tukey's pairwise comparison test was used for comparison of means where appropriate. Accepted level of significance was $p < 0.05$.

The morphometric attributes such as shoot length, number of leaves and number of flowers and fresh fruit weight of tomato were analyzed using one way ANOVA confirming the normal distribution with Anderson Darling test. If there were significant differences, Tukey's pairwise comparison test was used for comparison of means where appropriate. Accepted level of significance was $p < 0.05$.

4. RESULTS AND DISCUSSION

4.1. Physicochemical characterization of the raw distillery spent wash

Tables 4.1 represents on-site, and off-site physicochemical parameters of raw distillery spent wash as mean of three values. When considering the physicochemical characteristics, spent wash has a low pH and high levels of COD, TSS, TDS, and EC. Nitrate and Phosphate as P show low levels compared to the other parameters. According to the **Table 4.1**, COD, TDS, pH and EC levels of the raw spent wash exceed the tolerance limits for industrial waste discharged on land for irrigation purpose. Hence the spent wash may need a proper treatment prior the direct discharge for irrigation purposes. According to the results of One-sample t-test**,** COD, TDS and EC values of the spent wash are significantly higher when compared with the limits for discharge of industrial effluents for irrigation purposes specified by the CEA ($P < 0.05$). The metal concentrations recorded in the raw spent wash are given in **Table 4.2**. Heavy metals in the raw spent wash viz. Cd, Cu, Ni, Zn, As, Pb, Mn and K were detected in low levels. As mentioned in the **Table 4.2**, metal levels in untreated raw spent wash do not exceed the tolerance limits for industrial waste discharged on land for irrigation purpose.

Among the recorded onsite parameters, pH of the spent wash is very low (3.3 ± 008) which indicates the highly acidic nature. The acidic nature of the spent wash is due to the various types of acids used in fermentation step of the distillery industry such as sulfuric and phosphoric (Fito *et al*. 2019[b]). High electrical conductivity (21.93 ± 0.09 mS/cm) of the spent wash indicates the high amounts of soluble salts and ion concentrations. Temperature is a factor that controls the rate of chemical reactions take place in the process flow. In the present study, the temperature was measured randomly in the spent wash of receiving lagoon with the value range of (38.7 ± 0.15 $^{\circ}$C). Generally, spent wash is associated with high temperatures due to the complex reactions occurs in the distillation column (Fito *et al*. 2019[b]).

Table 4.1 Physicochemical properties of the raw distillery spent wash collected from Palwatta Distillery (Pvt) Ltd.

Physicochemical Parameter		Tolerance limits of industrial wastewater for irrigation purpose (CEA)
pH	3.30 ± 0.08 (3.2–3.5)	5.5–9.0
EC (mS/cm)	**21.9 ± 0.09 (21.83–22.12)**	**2.25 mS/cm, max**
Temperature ($^{\circ}$C)	38.7 ± 0.15 (38.5–39.0)	-
Turbidity (NTU)	1,420 ± 0.66 (1420–1422)	-
COD (mg/L)	**92,101 ± 0.33 (92,100–92,101)**	**400 (mg/L, max)**
BOD_5 (mg/L)	**26,116 ± 2.33 (26,112-26,120)**	**250 (mg/L, max)**
TSS (mg/L)	4,076 ± 0.55 (4075–4077)	-
TDS (mg/L)	**68,656 ± 0.13 (68,656–68656)**	**2100 (mg/L, max)**
Nitrate (NO_3^-) (mg/L)	255 ± 0.04 (255.2–255.3)	-
Phosphate as P (mg/L)	38.0 ± 0.07 (37.9–38.2)	-

Results are presented as mean ± SEM (n=3) and ranges and bold values are significantly different from stipulated tolerance limits of water for irrigation purpose (One-sample t- test, $p < 0.05$)

Table 4.2 Metal concentrations (light and heavy) of the distillery spent wash.

Parameter	concentration (mg/L)	LOD (mg/L)	Tolerance limits-CEA (mg/L, max)
Cd	<LOQ (0.005)	0.107	2.0
Cu	<LOQ (0.039)	0.493	1.0
Ni	<LOQ (0.033)	0.088	-
Zn	<LOQ (0.004)	0.123	-
As	<LOQ (0.0067)	6.675	0.2
Pb	<LOQ (ND)	2.466	1.0
Mn	<LOQ (0.015)	0.068	-
K (light metal)	<LOQ (3.39)	16.229	-

Turbidity is a measure of the clarity of water which impacts the light penetration of aquatic eco systems. In the present study, onsite turbidity measurement shows high values (1,420 ± 0.66 NTU) which may be due to the presence of high amounts of dissolved and suspended matter.

Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) represents the amount of organic matter in spent wash. Organic compounds, such as polysaccharides, reduced sugars, lignin, proteins, melanoidin, and waxes are associated with the high COD and BOD values of distillery spent wash (Kharayat 2012).

Current study recorded high COD (92,101 ± 0.33) mg/L and BOD (26,116 ± 2.33) mg/L in raw spent wash. Results represent high concentrations of organic matter in spent wash sample. Another comparative study which has been conducted based on six cane distilleries of India have recorded similar results for COD of untreated distillery spent wash range between 86,000 - 105,000 mg/L and BOD range between 40,000 - 52,000 mg/L (Kumari and Phogat 2010).

Total Suspended Solids (TSS) and Total Dissolved Solids (TDS) are associated with various types of suspended and dissolved organic and inorganic solids. The present study reported high TSS (4,076 ± 0.55 mg/L) and TDS (68,656 ± 0.13 mg/L) concentrations of the distillery sent wash. Presence of inorganic matter such as chlorides, carbonates nitrates sulfates, metal constituents and organic matter may increase the total solids in spent wash (Fito *et al*. 2019[b]). Analysis of physico-chemical properties and potential for the production of commodity chemicals by sugarcane distillery spent wash conducted by Shinde *et al*. 2020 has reported that fermented cell, cellulose microfibers, salts and biomass slime generates after the fermentation of molasses may attribute to the high TSS contents recorded in the spent wash.

In the recent study, low nitrate (NO_3^-, 255 ± 0.04 mg/L), Phosphate as P (38 ± 0.07 mg/L) and Potassium (K^+) (3.39 ± 0.004 mg/L) levels were recorded in the spent wash sample. Low amounts of nitrate and phosphates may due to the amount of urea

[CO $(NH_2)_2$], sulfuric acid (H_2SO_4) and diammonium phosphate [$(NH_4)_2HPO_4$] use for the yeast propagation and pH adjustment in the fermentation process. Trace am amounts of nitrate and phosphates were recorded by the similar studies (Melamane *et al*. 2007).

However, high amounts of total nitrogen (N), total phosphorous (P) and total potassium (K) levels have been recorded by comparable studies, total N (1,660 – 4,200 mg/L), total P (225 – 3,038 mg/L) and total K (9,600 – 17,475 mg/L) by (Umair Hassan *et al*. 2021).

As per **Table 4.2**, trace amounts of heavy metals such as Cd (0.005 mg/L), Cu (0.039 mg/L), Ni (0.033 mg/L), Zn (0.004 mg/L), As (0.0067 mg/L), Mn (0.015 mg/L) were recorded and Pb was not detected. K (3.39 mg/L) was detected. All the metals were below the detection limit. A study conducted by Fito *et al*., 2019[b] in a distillery industry during winter and summer has recorded Cr (0.25 – 0.55 mg/L) Cu (1.4 – 1.1 mg/L), Ni (0.14 – 2.5 mg/L), Zn (1.7 – 2.65 mg/L), Mn (1.6 – 6.45 mg/L) whereas Cd and Pb levels were not detected. Also, comparatively high levels of K (1,189.5 – 2,926.4 mg/L) have been recorded by the same study.

When considering the physicochemical characteristics mentioned above, distillery spent wash sample is highly rich in organic matter with comparatively low amounts of other inorganic constituents. However, cane distillery spent wash considered as a high strength wastewater which contains both organic and inorganic constituents. Characteristics of the spent wash are highly variable depending on the various raw materials used for the alcohol production process (Satyawali and Balakrishnan 2008). However, the raw spent wash generally has a dark brown colour with an unpleasant odour. Colour of the cane molasses-based waste waters is generally due to the pigment called “melanoidin” which is formed as a final product by series of non-enzymatic browning reactions and presence of other colorants such as phenolic (Satyawali and Balakrishnan 2007). The unpleasant odour is due to the formation of chemically volatile fatty acids (Kumari and Phogat 2010).

Discharge of untreated raw spent wash with numerous pollutants and toxicants can cause serious environmental problems. Therefore, characterization of the sugarcane

distillery spent wash is highly important for decision making such as choosing a most appropriate treatment method or any other management option (Melamane *et al*. 2007). Cane distillery spent wash has a plant-based origin and other chemicals are incorporated during the process flow. Therefore, the composition of the raw spent wash may vary with the other chemical inputs used during the process flow of ethanol production (Mahimairaja and Bolan 2004).

In the present study, some physicochemical characteristics of the spent wash, i.e., EC, COD, BOD, TSS, and TDS indicate extreme values when compared with the tolerance limits for discharge of industrial effluents for irrigation purposes (**Table 4.1**). The distillery industry uses the spent wash to irrigate the sugarcane crops of spent wash with these exceeding stipulated tolerance limits values of the above-mentioned characteristics. Therefore, it would be better to treat the spent wash using a suitable treatment method prior discharge into the land or water bodies to minimize the environmental hazards of direct discharge of excessive pollution loads into the environment. However, characterization of the spent wash proves that the spent wash may contain minerals and nutrients where the land application can enhance the crop yield of selected crops (Satyawali and Balakrishnan 2008).

4.2. Assessment of toxicity of sugarcane raw distillery spent wash using *Allium cepa* (common onion) bioassay

4.2.1. Root growth pattern of *Allium cepa* bulbs exposed to raw distillery spent wash

Table 4.3 and **Figure 4.1** indicate the root lengths (Mean ± SEM) of *Allium cepa* bulbs exposed to aged tap water, 1:8 diluted distillery spent wash and potassium dichromate ($K_2Cr_2O_7$) after two days and seven days of exposure periods. Significant differences in the root growth of onion bulbs exposed to aged tap water, 1:8 diluted distillery spent wash and potassium dichromate (ANOVA, Tukey's test, $p < 0.05$) were recorded after two days of exposure. Also, the root emergence of the diluted spent wash was very poor.

Root growth of the rest of the *Allium* bulbs were measured after seven days of exposure. As indicated in the **Table 4.3** and **Figure 4.1**, significant differences (ANOVA, Tukey's test, $p < 0.05$) in the root growth of onion bulbs exposed to 1:8 diluted distillery spent wash and potassium dichromate were recorded when compared with the root growth of aged tap water category. Root growth of bulbs exposed to diluted spent wash and potassium dichromate did not show any significant difference after seven days of exposure. Highest root growth delay was shown by the onion bulbs exposed to diluted spent wash (92%, 83%) when compared with the root growth of onion bulbs exposed to aged tap water.

Table 4.3 Root growth of Allium bulbs exposed to aged tap water (ATW), 1:8 diluted spent wash (12.5%) and potassium dichromate (PC).

Exposure	Root lengths (mm)			
	After 2 days	Root growth delay (%)	After 7 days	Root growth delay (%)
Aged Tap Water (Negative Control)	20.73 ± 1.05 [a] (15.15–24.13)	-	86.13 ± 6.50 [a] (50.12–111.39)	-
1:8 diluted distillery spent wash	1.5 ± 0.62 [b] (0.00–4.00)	92	14.58 ± 0.68 [b] (11.00–17.00)	83
Potassium Dichromate (Positive Control)	13.09 ± 2.39 [c] (0.00–23.50)	36	16.03 ± 7.40 [b] (0.00–59.00)	81

Data are presented as mean ± SEM (n=10). Numbers in parentheses indicate the range. Means indicated by different superscript letters are significantly different from each other for different exposure media. (ANOVA, Tukey's test, $p < 0.05$). Percentage of root growth delay is presented in relation to the mean root growth of onion bulbs exposed to aged tap water, for the statistically significant cases.

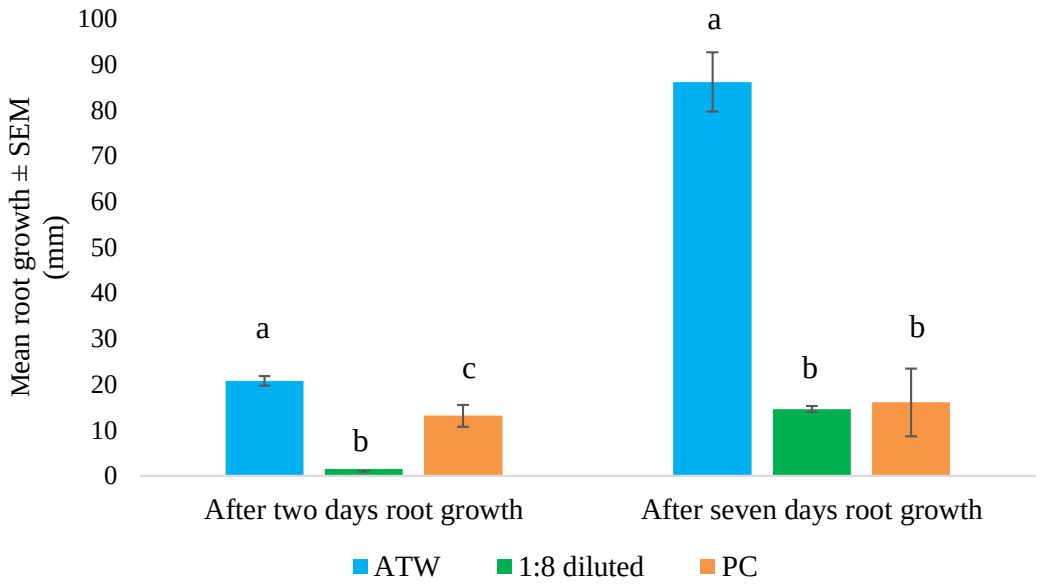

Figure 4.1 Root lengths (mean ± SEM) of Allium bulbs exposed to aged tap water (ATW), 1:8 diluted spent wash and potassium dichromate (PC). Different superscript letters are significantly different from each other (ANOVA, Tukey's test, $p < 0.05$), n=10 for each exposure.

It is extremely important to assess the toxicity of different waste waters to determine the biological effects associated when these waste waters discharge into the environment with or without treating. Higher plants have been identified as most economical, versatile and reliable bioassay among different types of bio monitoring tools by the researchers over the past years. Among the higher plant bioassays, *Allium cepa* bioassay has been proven as effective to detect environmental contaminants discharged by various industries (Hemachandra and Pathiratne 2018). In these studies, both macroscopic and microscopic endpoints have been evaluated including inhibition of root growth, mitotic index, occurrence of micro nuclei and nuclear abnormalities. *Allium cepa* root growth inhibition test is commonly used to assess the general toxicity of the chemicals where root growth inhibition is used as a phytotoxicity endpoint (Pathiratne *et al*. 2015).

In the present study, same toxicity endpoints were used to assess the toxic effects of sugarcane distillery spent wash. However, limited number of studies have been conducted in literature to assess the toxicity of sugarcane distillery spent wash using the *Allium cepa* bioassay.

The significant reduction in root growth and increased root growth delay in onion bulbs exposed to diluted spent wash indicate the phytotoxic effects occurred possibly due to the high acidity of the spent wash indicated by low pH (3.30 ± 0.08) which may interrupt the cell division of the root tip cells.

Kumar *et al*. 2021 have conducted an experiment to evaluate the physiological and cytological effects on root growth of *Allium cepa* exposed to different dilutions of (1%, 3%, 5%, 8% and 10%) of bio methanated distillery effluent (BMDE). Eight percent diluted BMDE has shown 42.8 mm of root growth where as 10% BMDE has not shown any root growth after 96 hours of exposure. The study stated that the root growth inhibition at high concentrations of BMDE may be due to the presence of metal and high salt concentrations compounds.

In a study conducted by Pathiratne *et al*. 2015 to assess the toxicity of effluents discharged from different industrial profiles, onion bulbs exposed to undiluted textile effluents have shown a mean root growth of (1.6 ± 0.1 cm) where bulbs exposed to diluted effluent from a rubber industry has shown a mean growth of (1.6 ± 0.1 cm) after seven days of exposure. Another study which was conducted to assess the toxicities of industrial effluent receiving water body has revealed significant delay in root growth of onion bulbs after exposure to undiluted wastewater indicating phytotoxicity (Kannangara and Pathiratne 2015).

When considering the findings of the present study and above-mentioned past studies, root growth of *Allium* could be used as a reliable phytotoxic endpoint to detect toxic effects of distillery spent wash in future studies.

4.2.2. Mitotic index (MI) of the root tip cells of *Allium cepa* bulbs exposed to sugar distillery spent wash

Table 4.4 and **Figure 4.2** indicate the mitotic index (mean ± SEM) of the *Allium* root tip cells exposed to aged tap water, 1:8 diluted distillery spent wash and potassium dichromate. In this study, no significant differences (ANOVA, Tukey's test, $p > 0.05$) were observed among the mitotic indexes of different exposure categories. Results may indicate that the presence of cytotoxic substances in the diluted spent wash can be considered as minimal or scarce. According to **Table 4.4**, 58% decline in mitotic index of diluted spent wash was reported when compared with the aged tap water control. The decline in the mitotic index indicates the loss of cell division and inhibition of DNA synthesis in the root meristematic region which could be due to the presence of heavy metals in the spent wash. Presence of heavy metals attributes to altering the normal cell division cycle and DNA synthesis (Srivastava and Jain 2010).

Mitotic index (MI) is a microscopic parameter that has been used to assess the cytotoxicity of the industrial effluents. Mitotic index is used to evaluate the changes occur in the cell division of the root tips when they have been exposed to environmental contaminants (Hemavathi *et al*. 2015). Mitosis is a process where a single cell undergoes division to produce to genetically identical two cells. The five stages of the cell division are interphase, prophase, metaphase, anaphase and telophase (Stages of mitosis, n.d). Interphase cells and the cells undergoing mitotic division in *Allium cepa* root tips as seen in the present study are presented in **Figure 4.3 (a – e)**. The mitotic index reflects the proportion of mitosis (prophase, metaphase, anaphase and telophase) in the root tip cells compared to the total number of cells in the meristematic region.

A study has been conducted by Oñate *et al*. 2015 using spent wash samples collected from a bioethanol production plant to assess the genotoxic and mutagenic activity of the diluted spent wash samples and spent wash subjected into different treatments.

Mitotic index and occurrences chromosomal abnormalities in the root tip cells were observed using *Allium* test. Results indicated that there was no significant difference in the mitotic index of the diluted spent wash samples when compared with the negative control. However, chromosomal aberrations have shown significant difference when compared with the control. Hence, similar outcome has been produced by this literature study in terms of mitotic index when compared with the MI of the diluted distillery spent wash of the current study.

Table 4.4 Mitotic index of *Allium* root tip cells exposed to ATW, 1:8 diluted spent wash and PC.

Exposure	Mitotic index (%)	Percentage decrease of mitotic index
Aged tap water (ATW)	27.95 ± 4.98 (10.27– 45.54) [a]	
1:8 diluted spent wash	11.68 ± 4.91 (0 – 7.00) [a]	58
Potassium Dichromate (PC)	27.9 ± 3.92 (10.04 – 42.46) [a]	0.18

Data are presented as mean ± SEM (n=10). Numbers in parentheses indicate the range. Means indicated by different superscript letters are significantly different from each other for different exposure medias (ANOVA, Tukey's test, $p > 0.05$). Percentage decrease of mitotic index is indicated in comparison with aged tap water.

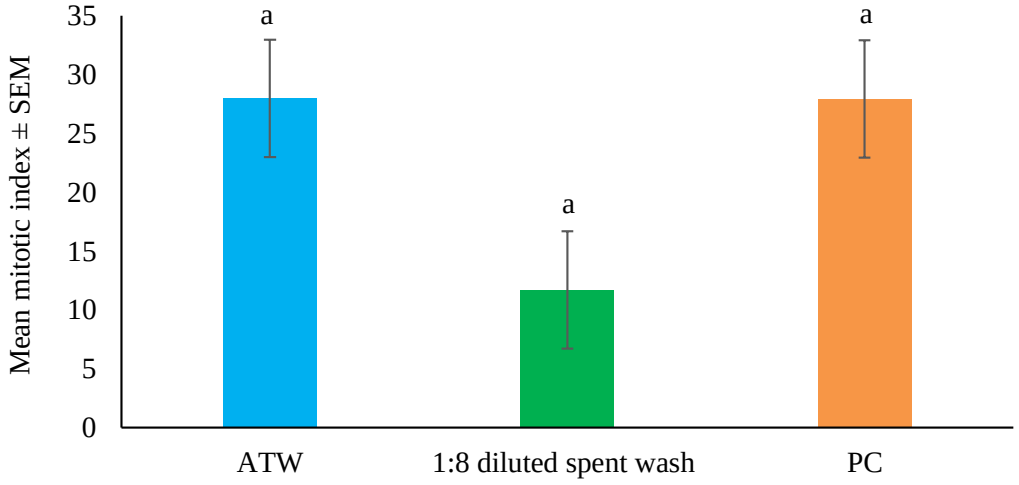

Figure 4.2 Mitotic index (mean ± SEM) of *Allium* bulbs exposed to ATW, 1:8 diluted spent wash (12.5%) and PC. different superscript letters are significantly different from each other (ANOVA, Tukey's test, $p < 0.05$). n = 10 for each exposure condition.

Most studies have been conducted to evaluate the toxicity of industrial effluents receiving water bodies. Some of the industrial effluent receiving water samples indicated root growth retardation and decreased mitotic index (cytotoxicity) also showed a significant genotoxicity potential as well. However, cytotoxicity of some water samples did not directly correlate with the genotoxicity. Water samples that did not induce any cytotoxic effects also did not show any genotoxic effects. The study revealed that the presence of trace metals and other pollutants may be responsible for the decreased mitotic index of some samples. The study also suggested that the strong toxic effects displayed by some water samples may hard to explain by the typical physicochemical analysis (Radić *et al.* 2010).

A study with similar aspect has been conducted by Srivastava and Jain (2010) to assess the effects of distillery spent wash on the cytological changes in sugarcane settlings. The settlings were treated with crude spent wash, digested spent wash and diluted spent wash for a certain time period and the root tip assay for sugarcane root tips has been carried out. Study concluded that rate of cell division measured by mitotic index showed a decrease in the crude spent wash and digested spent wash treatments. However, an appreciable increase in the mitotic index has been recorded in the diluted crude spent wash treatment suggesting that the diluted crude spent wash can induce the early growth characteristics.

In another study, mitotic index was calculated in different dilutions (1%, 3%, 5%, 8% and 10%) of bio methanated distillery effluent (BMDE) revealed 10.78% of MI of *Allium* at 8% dilution and decline of MI for the lower concentrations. The BMDE in this study has similarities in terms of the physicochemical composition with high COD, TSS, TDS and metal contaminants to the current study. However, no root growth was recorded in 10% dilution (Kumar *et al*. 2021) Retarded root growth and MI was resulted in the present study with a 12.5% diluted (1:8) raw spent wash. This disparity may be due to the high levels of metal concentrations in BMDE reported by the past study.

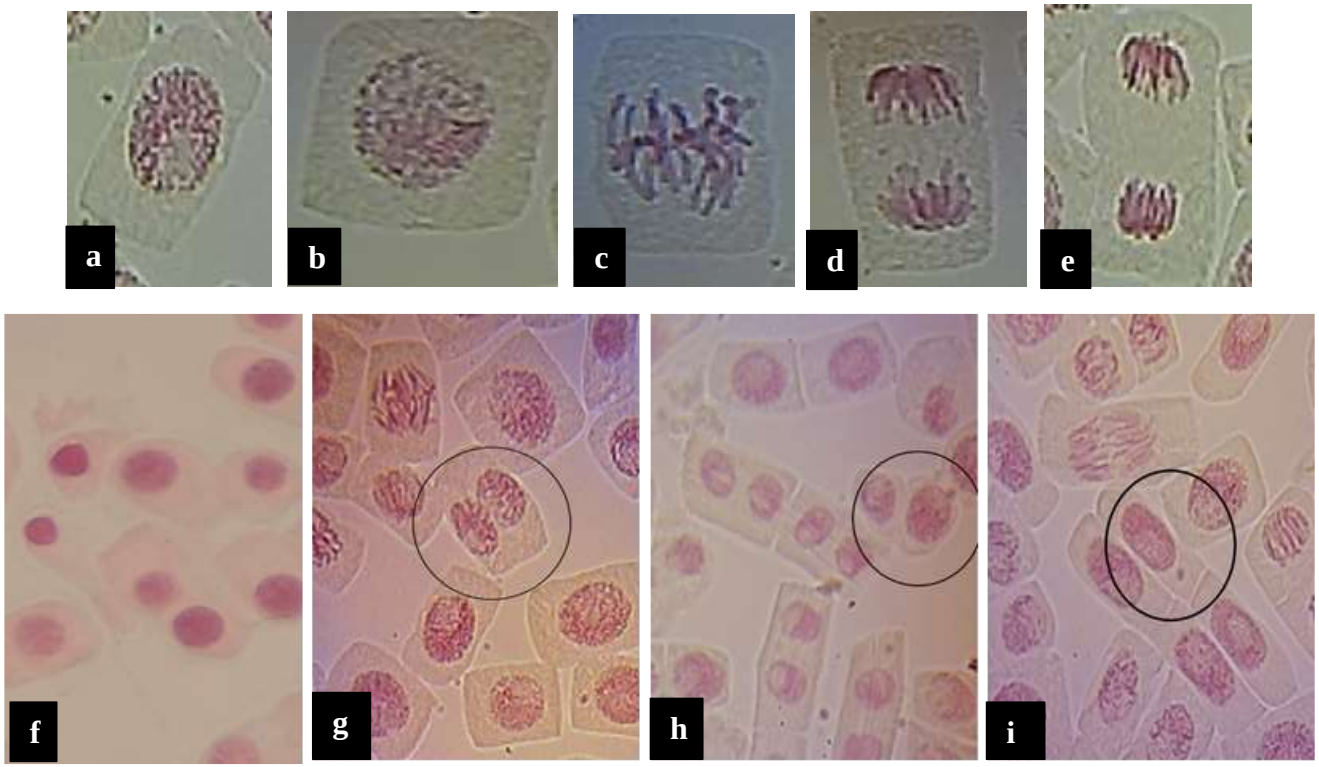

Figure 4.3 upper series shows normal mitotic division of *Allium* root tip cells (a) interphase, (b) prophase, (c) metaphase, (d) anaphase, (e) telophase and lower series shows observed abnormalities of *Allium* root tip cells (f) condensed nuclei, (g) bi-nuclei, (h) nuclear buds, (i) micro nucleus

4.2.3. Occurrence of nuclear abnormalities in the root tip cells of *Allium cepa*

Table 4.5 and Figure 4.4 represents the total nuclear abnormalities whereas **Table 4.6** and represents individual nuclear abnormalities detected in the root tip cells of *A.cepa* when exposed to aged tap water (ATW), 1:8 diluted spent wash (12.5%) and potassium dichromate (PC) in this study. **Figure 4.3 (f** to **i)** shows the types of nuclear abnormalities which were observed in the root tip cells. Total nuclear abnormalities reported significant differences in the root tip cells of *Allium* exposed to all exposure medias (ANOVA, Tukey's test, $p < 0.05$) according to the **Table 4.5 and Figure 4.4**.

When considering the individual nuclear abnormalities according to the **Table 4.6** significant difference was recorded only in condensed nuclei category where the occurrence of condensed nuclei was significantly different from negative and positive control categories medias (ANOVA, Tukey's test, $p < 0.05$). However, the diluted distillery spent wash has shown nuclear abnormalities of all categories when compared with the negative control and positive control.

Nuclear abnormalities and formation of micronuclei are genotoxicity parameters. Nuclear abnormalities are another genotoxicity endpoint that is characterized by the morphological changes occur in the nuclei at the interphase stage of cell resulting due to the action of the exposure media used to test the cytogenotoxic effects. General alterations in the nuclei observed in the previous studies are nuclei with nuclear buds, binucleated cells, polynuclear cells, lobulated nuclei and many others (Leme and Marine-Morales 2009).

In the recent study, observed nuclear abnormalities were micro nuclei, bi nuclei, nuclear buds and condensed nuclei which are depicted in **Figure 4.3 (f** to **i)**. Micronuclei can be identified as similar structure to the parental cells in a reduced size. Nuclear buds can be seen as protruded from the main nucleus. Binucleated cells generate as a result of the inhibition of the cell plate formation and are easily detectable subpopulations. (Leme and Marine-Morales 2009). Condensed nuclei are characterized by deeply stained nuclei with smaller or faded nuclei and disintegrating cytoplasm (Hemachandra and Pathiratne 2015).

Occurrence of micronuclei has been identified as an efficient test in detecting environmental mutagens by past studies (Hemachandra and Pathiratne 2015, Leme and Marine-Morales 2009). Although not statistically significant, induction of micronuclei in the root meristematic cells indicate the potential genotoxic effects of the diluted distillery spent wash.

Presence of micronuclei in the diluted untreated spent wash (0.25%, 0.5%, 0.75%, and 1%) were recorded by Oñate *et al*. 2015. Study suggested that occurrence of micro nuclei had no association with the concentration or type of the sample Furthermore, the genotoxic activity could be identified more conveniently by chromosomal aberrations rather than micro nuclei as the *Allium* test can detect contaminants dissolved in low concentrations. Formation of micronuclei is due to the fragments or whole chromosomes that do not associate with the formation of the main nucleus during cell division. (Hemavathi *et al*. 2015).

Root growth retardation, mitotic index and nuclear abnormalities could be suggested as reliable toxicological endpoints to evaluate the phytotoxic and cytotoxic effects of sugarcane spent wash. Spent wash could contain undesirable toxic compounds and water-soluble recalcitrant polymeric pigments (melanoidins) and heavy metals. Overall effects of these pollutants may have led the root growth retardation, decreased mitotic index and formation of nuclear abnormalities.

Table 4.5 Total nuclear abnormalities in *Allium* root tip cells exposed to ATW, 1:8 diluted spent wash (12.5%) and PC.

Exposure	Total nuclear abnormalities per 1,000 interphase cells
Aged tap water (ATW)	0.25 ± 0.25 (0.00 – 2.00) [c]
1:8 diluted spent wash	15.00 ± 9.03 (5.00 – 42.00) [a]
Potassium Dichromate (PC)	2.63 ± 1.18 (0.00 – 10.00) [b]

Data are presented as mean ± SEM (n = 10). The numbers in parentheses indicate the range. Means indicated by superscript letters are significantly different from each other (ANOVA, Tukey's test, $p < 0.05$).

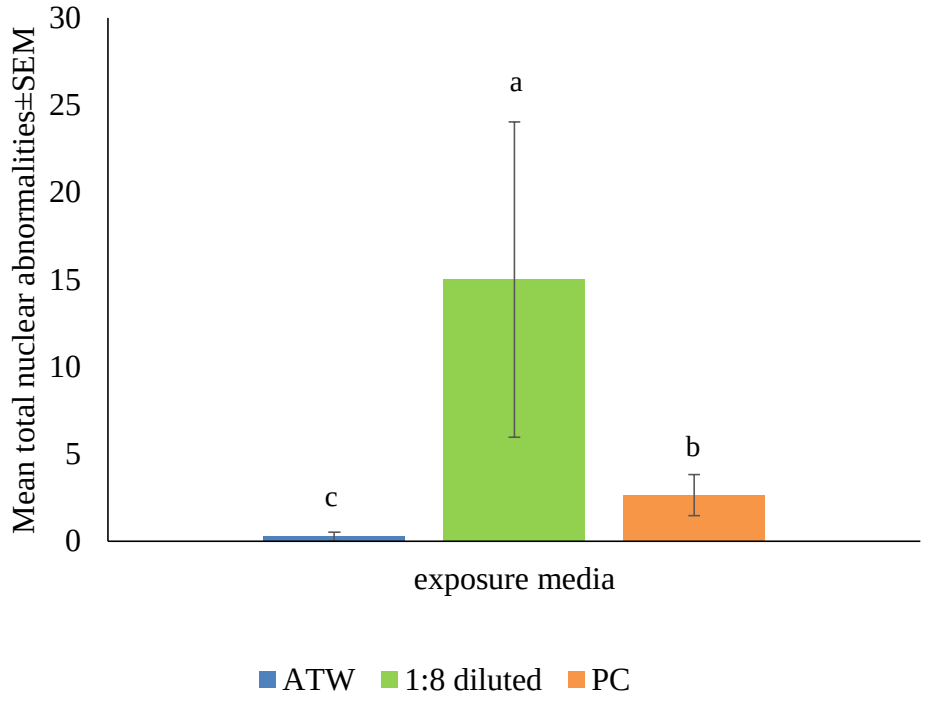

Figure 4.4 Total nuclear abnormalities of *Allium* root tip cells exposed to ATW, 1:8 diluted (12.5%) spent wash and PC. different superscript letters are significantly different from each other (ANOVA, Tukey's test, $p < 0.05$). n=10 for each exposure category.

Table 4.6: Nuclear abnormalities in *Allium* root tip cells exposed to ATW, 1:8 diluted spent wash (12.5%) and PC.

Exposure	Per thousand interphase cells			
	micro nuclei	bi nuclei	nuclear buds	condensed nuclei
Aged tap water	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.25 ± 0.25^{b} (0.0–2.0)
1:8 diluted spent wash	0.75 ± 0.48^{a} (0.0 – 2.0)	3.5 ± 3.5^{a} (0.0– 4.0)	4.5 ± 3.2^{a} (0.0–14.0)	6.25 ± 2.4^{a} (1.0–12.0)
potassium dichromate	1.25 ± 0.56^{a} (0.0–4.0)	0.5 ± 0.27^{a} (0.0–2.0)	$0.75 \pm .0.62^{a}$ (0.0–5.0)	0.12 ± 0.12^{b} (0.0–1.0)

Data are presented as mean ± SEM (n=10). Numbers in parentheses indicate the range. Means indicated by superscript letters are significantly different from each other (ANOVA, Tukey's test, $p < 0.05$).

According to the physicochemical monitoring results and *Allium cepa* bioassay studies of present study, it rejects the first hypothesis which mentioned that the spent wash generated by Pelwatta distilleries limited does not exceed the physicochemical standard for irrigation purpose introduced by CEA and not contaminated with toxic substances which can cause cytogenotoxicity on biota. Nevertheless, application of distillery spent wash at controlled low rates has shown successful results in terms of crop yield and soil fertility when applied to certain crops in literature studies (Mahimairaja and Bolan 2004). With the fair amounts of essential plant nutrients and other trace elements included in the spent wash, current study attempted to find out the nutrient potential of the spent wash as the next objective by using a specific vegetable crop.

4.3. Use of raw spent wash as a nutrition source for the tomato (*Solanum lycopersicon*) crop - open field experiment

Use of raw industrial wastewater or treated wastewater for irrigation of agriculture lands has been identified as a recent trend by many research studies. Disposal of wastewater generate in large quantities is a problem for most industries due to the limited land space. Since most industrial wastewater is rich in nutrients, they could be utilized efficiently to agriculture lands as a resource (Kumar and Chopra 2012).

The distillery spent wash used in the recent study does not undergo any kind of treatment. Currently, the spent wash is being used to irrigate the sugarcane lands. Hence, this study attempted to identify the potential of the spent wash to be used as a nutrition source for the tomato (*Solanum lycopersicon*) crop by measuring some growth-related morphometric attributes of tomato (*Solanum lycopersicon*) plants. Characteristics were measured in the tomato (*Solanum lycopersicon*) seedlings after transplanting the seedlings from the nursery following subjected to various treatments.

4.3.1. Assessment of tomato (*Solanum lycopersicon*) shoot lengths and leaves

Table 4.7 and Figure 4.5 indicate the average shoot lengths of the tomato (*Solanum lycopersicon)* plants in each treatment series viz. tap water as negative control (S0), 0.5 mL (S1), 1.5 mL (S2), 2.5 mL (S3), 5.0 mL (S4), 7.5 mL (S5), 10 mL (S6) and liquid organic fertilizer as positive control (6.25 mL). S1 – S7 were treated with distillery spent wash alone in mentioned doses. Measured shoot lengths after 60 days of transplanting tomato seedlings were significantly different only between average shoot lengths of S1 and S7 treatments (ANOVA, Tukey's test, $p < 0.05$). Average shoot length of other treatment categories did not show any significant difference when compared with the tap water (S0) category (ANOVA, Tukey's test, $p > 0.05$). The amount of spent wash (0.5 mL) that was applied for the S1 series may not be sufficient to support the vegetative growth of tomato (*Solanum lycopersicon)* by fulfilling the nutrient requirements. Total number of leaves were counted from bottom to top of the plant. There were no significant differences recorded among the

average number of leaves of each treatment series according to the **Table 4.8 and Figure 4.6** (ANOVA, Tukey's test, $p > 0.05$).

Table 4.7 Shoot lengths of tomato *(Solanum lycopersicon)* plants 60 DAT.

Treatment	Shoot length (cm)
S0	86.25 ± 3.75 ^{ab} (80.0–95.0)
S1	67 ± 1.22 ^{b} (65.0–70.0)
S2	80 ± 7.07 ^{ab} (60.0–90.0)
S3	76 ± 4.14 ^{ab} (65.0–85.0)
S4	83.25 ± 4.52 ^{ab} (73.0–95.0)
S5	89 ± 4.73 ^{ab} (82.0–102.0)
S6	87.5 ± 8.54 ^{ab} (65.0–105.0)
S7	97.75 ± 6.71 ^{a} (80.0–110.0)

Data are presented as mean ± SEM (n=4). Numbers in parentheses indicate the range. Means indicated by superscript letters are significantly different from each other (ANOVA, Tukey's test, $p < 0.05$).

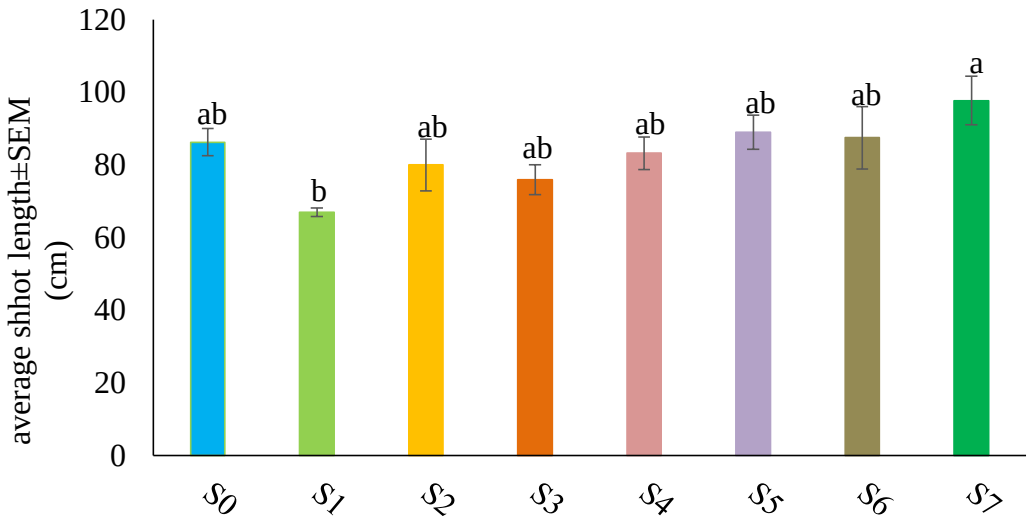

Figure 4.5 Average shoot lengths ± SEM of tomato *(Solanum lycopersicon)* plant for different treatment category 60 DAT.

Table 4.8 Number of leaves in tomato *(Solanum lycopersicon)* plants 60 DAT.

Treatment	Number of leaves
S0	13.50 ± 0.5 [a] (12.0–14.0)
S1	12.0 ± 1.29 [a] (9.0.0–15.0)
S2	17.0 ± 1.78 [a] (12.0–20.0)
S3	14.75 ± 2.21 [a] (10.0–19.0)
S4	17.75 ± 1.11 [a] (15.0–20.0)
S5	13.5 ± 0.86 [a] (12.0–15.0)
S6	15.0 ± 0.5 [a] (15.0–17.0)
S7	17.5 ± 2.47 [a] (12.0–23.0)

Data are presented as mean ± SEM (n=4). Numbers in parentheses indicate the range. Means indicated by superscript letters are not significantly different from each other (ANOVA, Tukey's test, $p > 0.05$).

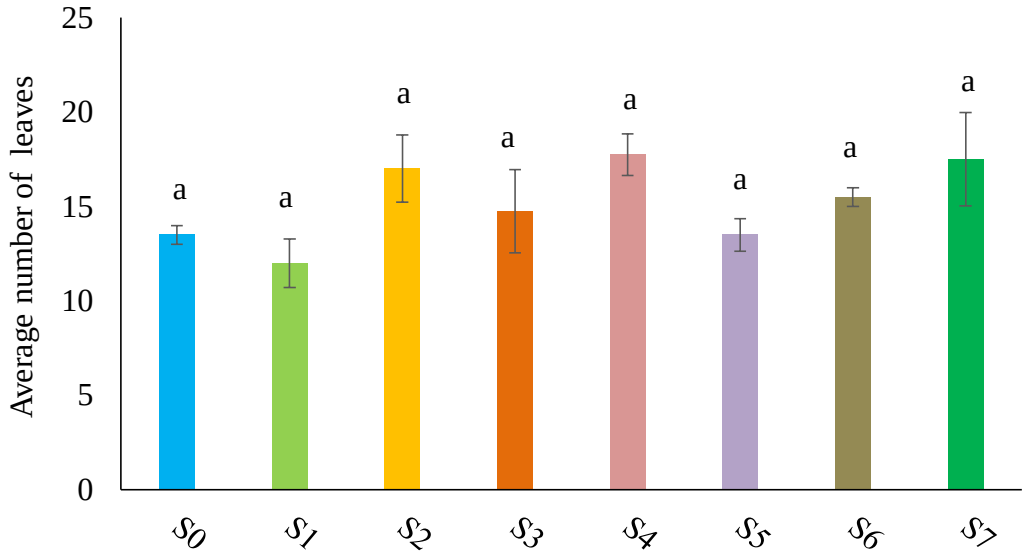

Figure 4.6 Average number of leaves ± SEM (*Solanum lycopersicon*) in each treatment category 60 DAT.

4.3.2. Assessment of tomato (*Solanum lycopersicon*) flower buds and flowers

Flowering stage of a plant is very important as the flowers have to get pollinated to produce fruits /yield eventually. **Table 4.9** shows the average buds and flowers count in each treatment series 40 days after transplanting (40 DAT) the tomato (*Solanum lycopersicon)* seedlings. **Figure 4.7** shows the average buds and flower count and **Figure 4.8** shows percentage of bloomed flowers (total flowers/total buds and flowers) *100 in each treatment series. Number of flower buds and bloomed flowers was visually scored at 40 DAT the tomato seedlings. According to the table 10, significantly different number of buds were recorded in S1 (0.5 mL), S3 (2.5 mL) and S5 (7.5 mL) which were treated with spent wash when compared with the positive control S7 (6.25 mL) (ANOVA, Tukey's test, $p < 0.05$). There was no significant difference among the average number of bloomed flowers in each series (ANOVA, Tukey's test, $p > 0.05$). However, when considering the bloomed flowers percentage according to the **Figure 4.8**, comparatively higher % is recorded by S3 (2.5 mL) and S5 (7.5 mL) series. The reason for this observation could be explained according to the **Figure 4.7** since half of the flower buds counted in these two-treatment series were turned out into bloomed flowers when compared with the other treatment series.

Table 4.9 Number of Flower buds and flowers in tomato plants (*Solanum lycopersicon*) 40 DAT.

Treatment	Buds	Flowers
S0	6.5 ± 1.19^{ab} (5–10)	3.25 ± 1.11^{a} (0–5)
S1	3.5 ± 1.19^{b} (0–5)	1 ± 1^{a} (0–4)
S2	5.25 ± 1.8^{ab} (0–8)	2.75 ± 1.11^{a} (0–5)
S3	3.25 ± 1.49^{b} (0–7)	3.50 ± 1.19^{a} (0–5)
S4	8.5 ± 1.19^{ab} (6–11)	4.25 ± 1.55^{a} (0–7)
S5	2.25 ± 1.31^{b} (0–6)	4.75 ± 1.89^{a} (0–9)
S6	6.0 ± 1.08^{ab} (4–9)	3.0 ± 0.82^{a} (1–5)
S7	10.75 ± 1.8^{a} (6–14)	5.25 ± 0.25^{a} (5–6)

Data are presented as mean ± SEM (n=4). Numbers in parentheses indicate the range. Means indicated by superscript letters are significantly different from each other (ANOVA, Tukey's test, $p < 0.05$).

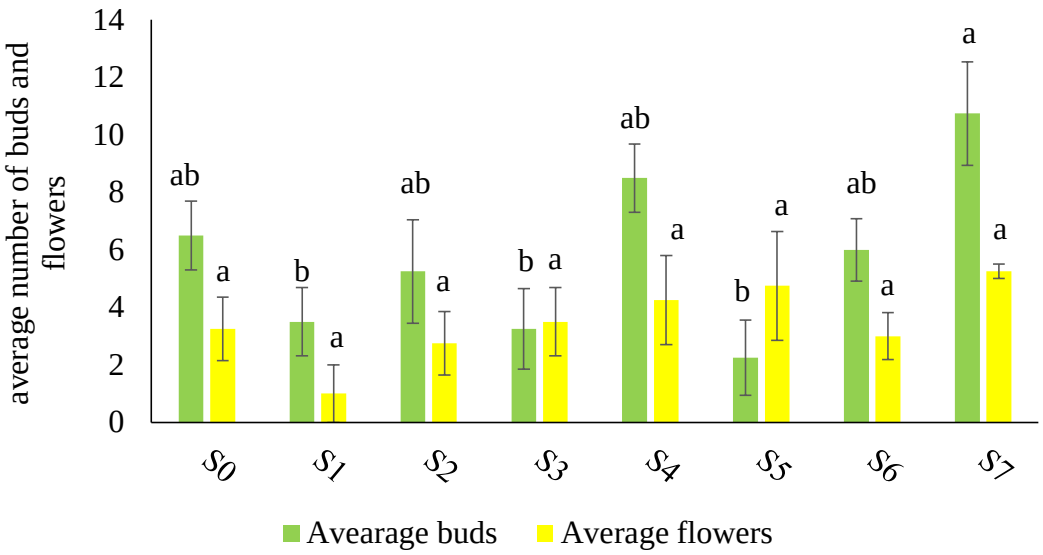

Figure 4.7 Average number of tomato buds (*Solanum lycopersicon*) and flowers of each treatment category 40 DAT

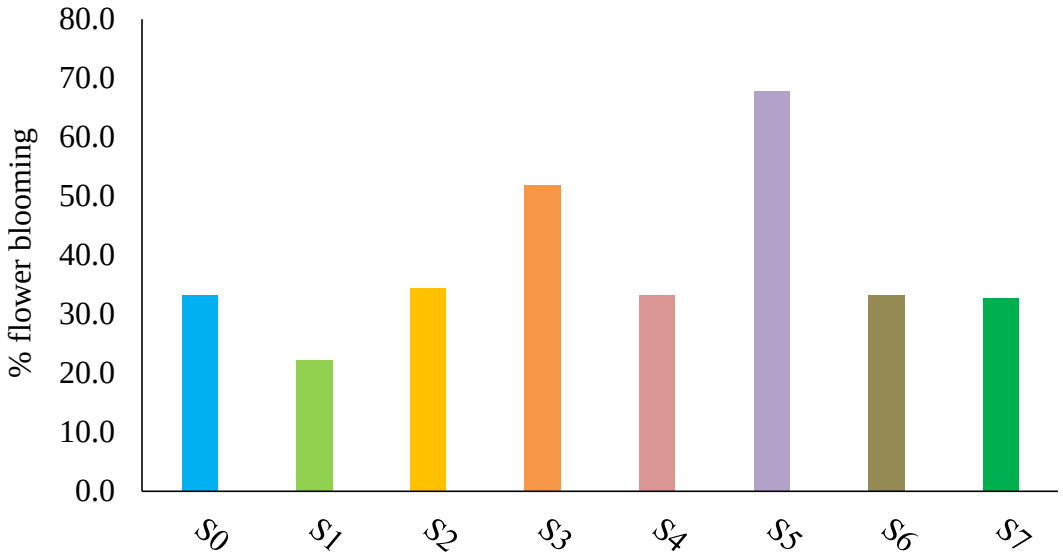

Figure 4.8 Tomato (*Solanum lycopersicon*) flowers blooming % of each treatment category 40 DAT.

4.3.3. Assessment of tomato (*Solanum lycopersicon*) fruit yield

Fruit set generally referred to as the transition of flowers or flower buds into fruits. **Table 4.10** and **Figure 4.9** indicate the average fresh fruit weight (g) of tomato 60 days after transplanting (60 DAT). Significantly different fruit weight was recorded in S6 (10 mL) and S7 (6.25 mL-positive control) when compared with the negative control (S0) (ANOVA, Tukey's test, $p < 0.05$). All the other categories showed a significant difference in average fruit weight when compared with the positive control (ANOVA, Tukey's test, $p < 0.05$). S1 (0.5 mL) series failed to set fruits. When considering the **Table 4.10** and **Figure 4.9**, S0-S6 treatment series (except S1) shows less variations in average fruit weight.

Table 4.10 Fresh fruit weight of tomato (*Solanum lycopersicon*) in each treatment category 60 DAT.

Treatment	Fresh fruit weight (g)
S0	62.5 ± 2.10 ^{c} (58.0–67.0)
S1	NF
S2	65 ± 3.03 ^{c} (56.0–69.0)
S3	68.75 ± 3.50 ^{bc} (60.0–77.0)
S4	75.0 ± 2.38 ^{bc} (70.0–80.0)
S5	72.5 ± 4.59 ^{bc} (60.0–82.0)
S6	80.0 ± 2.71 ^{b} (76.0–88.0)
S7	100 ± 2.20 ^{a} (95.0–105)

Data are presented as mean ± SEM (n = 4). Numbers in parentheses indicate the range. Means indicated by superscript letters are significantly different from each other (ANOVA, Tukey's test, $p < 0.05$).NF-no fruits.

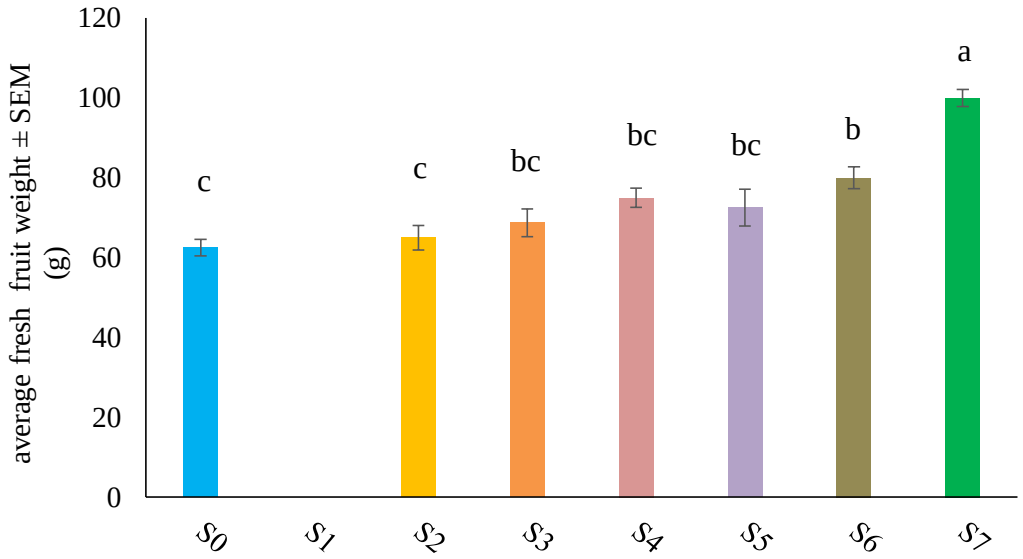

Figure 4.9 Average fresh fruit weight SEM in each treatment category 60 DAT.

4.4. Fertigation of treated /raw spent wash for crops-comparison with the present study

The present study was focused on measuring the morphological characteristics from vegetative stage to early fruiting stage within a time frame of 60 days after transplanting (60DAT) the tomato (*Solanum lycopersicon*) seedlings. Average shoot lengths and leaves which were recorded 60 DAT did not show any significant difference among each treatment except S1 (0.5 mL). This result may be due to the inadequate nutrient supply provided by the smaller volume (0.5 mL) of spent wash.

Average flower buds and flowers recorded 40 DAT also did not indicate considerable differences among each treatment category. However, when considering the average fresh fruit weight/treatment 60 DAT, significant differences were observed among many treatment categories. Though there was a significant difference when compared with the positive control (S7), favorable doses of spent wash may be considered as 2.5, 5.0, 7.5 and 10 mL for the flowering and early fruiting stage.

Choudhary *et al*. 2017 conducted an experiment to compare the effects on yield of tomatoes, number of fruits and plant height by the application of single compost and spent wash (10%) mixed compost revealed that the spent wash mixed compost has given better growth and yield with a maximum fruit weight/plant of 1,013.3 g, maximum fruits/plant of 22.33, maximum height of plant- 108 cm).

Maximum plant height (89 ± 4.73 cm) was shown by S5 (7.5 mL) category which was treated alone by spent wash of the present study. Comparison of the tomato (*Solanum lycopersicon*) height with the above study reflect that the raw spent wash alone may be able to produce more or less similar growth-related morphometric characteristics in tomato (*Solanum lycopersicon*).

A parallel study conducted by Kumar and Chopra, 2012 to analyze the fertigation effects of treated distillery effluent on agronomical practices of Fenugreek (*Trigonella foenum-graecum* L.) was grown in poly bags under open field conditions and irrigated by different effluent dilutions (5%, 10%, 25%, 50%, 75% and 100%)

along with the bore well water as control to evaluate the agronomical characteristics of seed germination stage to maturity stage and analyze the effects on soil characters after fertigation with effluent.

Flowers and number of pods measured after 60 days were not significantly affected by different effluent concentration whereas chlorophyll content and leaf area index measured after 45 days at vegetative stage have shown significant differences at lower dilutions (ANOVA, $p > 0.05$). Shoot lengths, root lengths, number of leaves, dry weight, crop yield/plant, and harvest index were affected significantly at most dilutions when compared with the control. Highly favorable agronomical attributes in each stage were indicated by 50% dilution in most cases.

In the present study, shoot lengths, number of buds and flowers and fresh fruit weights of the spent wash treated categories (S2-S6) did not show significant differences when compared with the negative control category. The result could be due to the less time given for the raw spent wash to naturally oxidize high organic matter contents up to an extent where the plant can uptake the oxidized nutrients. Therefore, some experiments have suggested that pre-plant application of raw spent wash in summer would provide sufficient time to be oxidized naturally (Baskar *et al*. 2013).

Application of crude spent wash at low rate (5 mL/Kg soil) showed increased bud sprouting (10.5 %), settling height (40 %), root number (9.4 %), root length (13.2 %) of sugar cane (Jain and Srivastava, 2012). Favorable effects on the root length, plant height, biomass and vigor index were found by Mahimairaja and Bolan (2004) in some dry land crops (ragi, groundnut, sorghum and green gram) when the seeds were treated by 10% and 20% (v/v) spent wash.

In some other studies, primary treated distillery spent wash has been applied following dilutions (50%, 33%) to assess the yield of some selected top vegetables, leaf vegetables and root vegetables grown in an open field setup. These studies revealed that the yield was increased when irrigated with diluted primary treated spent wash when compared with the raw water control and 33% diluted spent wash produced highest yield. Studies concluded that the diluted spent could be effectively

used for the tested crop types as a fertilizer even without applying any other external fertilizer inputs. Also, diluted spent wash has enriched the soil nutrient levels by elevating essential plant nutrients viz. N, P, K (Chidankumar and Chandraju 2009; Chidankumar *et al*. 2009; Basavaraju and Chandraju 2008).

The current study did not focus on the seed germination of tomato by treating tomato seeds with spent wash at nursery stage. However, a laboratory study conducted by Ramana *et al*. 2001 with the diluted raw spent wash (5%, 10%, 15%, 20%, 25%, 50%, and 75%) to test the germination characteristics of the seeds of some vegetables has suggested that even the lowest concentration (5%) has posed inhibitory effects on the germination percentage of tomato where as other seed species have shown favorable seed germination characteristics at low and middle dilutions.

Vermal and Dhiman (2016) found that the potato seed germination inhibited when treated by different concentrations of untreated spent wash. However, seedling length at lower spent wash concentration showed better effects. Hence the spent was application might be crop specific.

4.5. Sugar cane distillery spent wash as a source and supplement of nutrients in agriculture

Nutritional aspects of the sugar cane distillery spent wash have been investigated through many open field and hydroponic experiments. Through the recent study, physicochemical characteristics of the spent wash were measured and identified that the spent wash included some of the essential plant nutrients as well. As depicted by the **Tables 4.1 and 4.2**, spent wash contains, Nitrate - NO_3^- (255 ± 0.04 mg/L), Phosphate as P (38 ± 0.07 mg/L) and K (3.39 mg/L) which are considered as essential plant nutrients. Even though total N, P and K levels were not measured in the spent wash, similar studies have revealed that the raw distillery spent wash contains ample amounts of essential plant nutrients and trace elements.

A study conducted by Kumari and Phogat (2010) to characterize the raw spent wash and treated spent wash to identify the macro and micro nutrients where it can be used as a manure in agricultural practices. Characterization of raw spent wash collected from several distilleries revealed that spent wash contains highly favorable amounts of N (980-1338 mg/L), P (23-38 mg/L) and K (7375-12187 mg/L).

Heavy metals viz. Zn (0.6 – 0.9 mg/L), Cu (0.16 – 0.27 mg/L), Cd (0.16 – 0.24 mg/L), Pb (2.20 – 2.29 mg/L), Ni (0.16 – 0.21 mg/L) and other metals such as Mn (0.22 – 0.42 mg/L) were recorded. Also, the treated spent wash collected from a distillery treatment plant contained N (341 mg/L), P (12 mg/L) and K (556 mg/L), Zn (0.1 mg/L), Cu (0.02 mg/L), Cd (0.12 mg/L), Pb (0.18 mg/L), Ni (0.1 mg/L) and Mn (0.05 mg/L). Besides the macro and micronutrients, excessive COD, BOD and EC levels of spent wash could be considered as problematic when application for the agricultural crops. However, this could be solved by application after proper dilution or pre-plant application where the spent wash will be provided some time to be naturally oxidize in the crop lands (Baskar *et al*. 2003).

Some past studies also revealed that the composition of the distillery spent wash is more or less similar to the farmyard manure according to the **Table 4.11**. Experiments which have been conducted using the post-methanated distillery spent wash (effluent). These studies suggested that the treated effluent has favorable amounts of macro and micronutrients along with low C:N ratio which helps to accelerate the degradation of soil rather than using the untreated spent wash (Baskar *et al*. 2013).

Table 4.11 Comparison of farmyard manure and raw spent wash.

Parameter	Farmyard manure	Cane distillery spent wash
pH	7.5	3.8
Moisture (%)	22.65	91.1
Organic matter (%)	21.75	31.5 (on solid basis)
Total N (%)	1.44	2.0
Total P (%)	0.83	0.28
Total K (%)	0.79	9.96
Mineral matter (%)	52.5	26.49
C:N ratio	15.10	15.75

(Source: Baskar *et al*. 2013)

Sri Lanka Standard Institution (SLSI) has imposed specification for some physicochemical characteristics which include plant primary nutrients and limits for potentially toxic elements which should be included in a liquid organic fertilizer product (**Appendix 03**). Specification for total N, P, and K are 1, 0.5, 0.5 percent by mass/min consecutively whereas maximum limits for toxic elements in mg/kg for as (0.5), Cd (0.5), Cr (0.5), Pb (1.0) and Hg (0.5). The present study has revealed that some plant nutrients and amounts of NO_3^- (255 mg/L), total P (38 mg/L), and K (3.39 mg/L) of the spent wash were in trace levels. The distillery industry needs Urea (600 kg/day) and Di ammonium phosphate (12 kg/day) as chemical inputs for the production process (Production manager, personal communication, pelwatta distillery).

Considering the levels of above-mentioned chemical inputs combined with "molasses", which generates as a by-product of sugar crystallization and function as the major feed substrate for the fermentation process of ethanol production, it could be justified that the spent wash may contain the levels of N, P, K and other plant nutrients in satisfactory amounts. Sugarcane molasses has a plant-based origin which composed of variety of nutrients and organic acids (Basso *et al*. 2009). However, further studies should be conducted in Sri Lanka to confirm the nutritional composition of the spent wash.

Use of spent wash as a nutrient supplement in fertilizers has been experimented by various literature studies (Kulasekra *et al*. 2021; Kaushik *et al*. 2005). In the present study, positive control used in the tomato (*Solanum lycopersicon*) experiment was a certified liquid organic fertilizer (Biotin -V, Fish Hydrolysate) made of fish leftovers, *Gliricidia* and sugar media. "Molasses" is used as the sugar media which is somewhat expensive in local market and could be non-economical to be used as an input ingredient. Molasses is the major input ingredient in alcohol production of the distillery spent wash. Therefore, the spent wash might be used as a more economical alternative input besides the use of molasses for production of organic fertilizers. The fact that some spent wash doses used in the current study itself have shown favorable on some evaluated growth-related morphometric characteristics of tomato (*Solanum lycopersicon*) could be taken as an illustration for the production of liquid fertilizers by incorporating spent wash as a supplementary nutrient source.

However, caution must be taken to identify the effects on soil characteristics and bio accumulation of heavy metals in plants when the spent wash is directly used without any prior treatments. Frequent monitoring should be carried out to evaluate the biological effects on plants by introducing "*Allium cepa*" plant bioassay with applicable modification for the crops which are fertigated using sugarcane distillery spent wash.

Nevertheless, sugarcane distillery spent wash is enriched with nitrates and phosphate whereas long-term excessive application may cause eutrophication of water bodies which eventually block sunlight penetration and depletes the available dissolved oxygen level essential to maintain biological activities (Fito *et al*. 2019[a]). Long term application of raw spent wash may also promote salt accumulation problems in soil. Hence management of spent wash should incorporate with technical experimentation followed by systematic monitoring to reduce environmental threats forced by exceeding levels of nitrates and phosphate, meanwhile improve the potential utilization as a valuable nutritional source resource for agriculture (Mahimairaja and Bolan, 2004).

The field experiment conducted using tomato (*Solanum lycopersicon*) in the current spent wash generated by Pelwatta distilleries has a potential use as a liquid nutrition source for the selected agricultural crop. Hence, the second hypothesis can be accepted. But unacceptable physicochemical properties and cytogenotoxic effects in first experiment suggested the utility of spent wash under strict and continuous physicochemical and biomonitoring approaches.

5. CONCLUSIONS AND RECOMMENDATIONS

The present study assessed the physicochemical characteristics of the raw sugarcane distillery spent wash and its cytogenotoxicity using *Allium cepa* bioassay for the first time in Sri Lanka. In addition, this study attempted to find out an environmentally friendly solution for the sugarcane distillery spent wash using it as a liquid nutrition source for selected agricultural crop.

The physicochemical characterization of the sugarcane distillery raw spent wash revealed that the spent wash has an acidic nature with extreme levels of COD, BOD, EC, TSS and TDS, trace amounts of NO_3^-, PO_4^- , K, Mn, and heavy metals. Concentrations of the COD, BOD, TDS and pH values exceeded the tolerance limits for industrial wastewater discharged on land for irrigation purpose stipulated by Central Environmental Authority **(Appendix 01)**. Concentrations of As, Cd, Cr, Pb ad Cu do not exceed the tolerance limits **(Appendix 01)**. The root growth restriction, mitodepressive effects and nuclear abnormalities induced by the toxicity assessment conducted for raw sugarcane distillery spent wash using *Allium cepa* standard bioassay indicates that the raw spent wash may contain cytotoxic substances and may need an advance treatment prior disposal. According to the physicochemical monitoring results and *Allium cepa* bioassay studies of present study, it rejects the first hypothesis which mentioned that the spent wash generated by Pelwatta distilleries limited is not exceed the physicochemical standard for irrigation purpose introduced by CEA and not contaminated with toxic substances which can cause cytogenotoxicity on biota. Further physicochemical characterization of the spent wash is essential to affirm the levels of heavy metals and trace metals in the spent wash.

Measured morphometric attributes of tomato (*Solanum lycopersicum*) tended to be more favorable towards applying the doses of 2.5, 5.0, 7.5 and 10 mL of raw spent wash along with tap water as required. Technical experimentations should be designed to confirm whether the spent wash application is favorable in all growth stages of a crop or more favorable in specific stage/s (i.e; vegetative stage, flowering stage, fruiting stage). The field experiment conducted using tomato (*Solanum*

lycopersicon) revealed that eventhough unacceptable physicochemical properties and cytogenotoxic effects were associated with the spent wash generated by Pelwatta Distilleries, it can be used as a liquid nutrition source for agricultural crops. Hence the second hypothesis was accepted with specific recommendations.

Growth related morphometric attributes which were measured in this study indicated that the plants treated with different spent wash doses performed more or less similar characteristics in relation to the positive control category. Since all the treatment categories were provided with similar external conditions (soil and weather), sugarcane distillery raw spent wash might has functioned as a nutrition source and caused the observed growth morphometric of the spent wash treated tomato (*Solanum lycopersicon*) categories. Findings of the present study should be solidified by characterization of the nutritional quality of the soil before and after treated with spent wash.

Despite the sole application, it would be more appropriate to incorporate the spent wash as a supplementary nutrient source with other organic fertilizer ingredients to make improved organic fertilizers or modify as a soil amendment to obtain a better crop output. More related studies should be conducted in terms of assessing toxicity and possibility of the sugarcane distillery spent wash to be utilized as a nutrition source for crops. Judicious use of sugarcane spent wash could assist in minimizing the unbounded chemical fertilizer application on local crops.

However, continuous monitoring should be carried out to evaluate the effects on physicochemical and biological characteristics of the soil irrigated by spent wash. Extensive experiments should be conducted to confirm the bioaccumulation effects of the metals in plant tissues treated by the spent wash directly or indirectly. Methodology of *Allium cepa* bioassay could be used as a standard bioassay for test wastewater. Evaluation of seed germination and root growth characteristics also should be incorporated into the future comparative studies to cover all the crop development stages of a specific crop and to get a better conception about the effects on morphometric and soil attributes of the crops treated with spent wash application.

Experiments could be conducted to different crop types under different climatic zones on spent wash applications to identify the crop and climatic zone specificity.

With the current local policy on promoting organic manure and banning of chemical fertilizer use, spent wash would be a timely fitted solution as a source of nutrients for the crops. Besides the nutritional qualities, wise use of spent wash in agriculture crops would save the cost of chemical fertilizers as well as reduce undesirable environment impacts of chemical fertilizer applications.

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APPENDICES

Appendix 1 Tolerance limits for industrial waste discharge on land for irrigation purposes, by CEA.

No	Parameter	Unit type of limit	Tolerance Limit
1	Total dissolved solids	mg/ L, max	2,100
2	pH at ambient temperature	mg/L, max	5.5–9.0
3	Biochemical Oxygen Demand (BOD_5 in five days at 20 °C or BOD_3 in three days at 27 °C)	mg/L, max	250
4	Oils and greases	mg/L, max	30
5	Chemical Oxygen Demand (COD)	mg/L, max	400
6	Chlorides (as Cl)	mg/L, max	600
7	Sulphates (as SO_4)	mg/L, max	1,000
8	Boron (as B)	mg/L, max	2
9	Arsenic (as As)	mg/L, max	0.2
10	Cadmium (as Cd)	mg/L, max	2.0
11	Chromium (as Cd)	mg/L, max	1.0
12	Lead (as Pb)	mg/L, max	1.0
13	Mercury (as Hg)	mg/L, max	0.01
14	Sodium Adsorption Ratio (SAR)	mg/L, max	10 – 15
15	Residual Sodium Carbonate (RSC)	mg/L, max	2.5
16	Electrical conductivity	S/cm, max	2,250
17	Fecal coliform	MPN 100 mL, max	40
18	Copper (as Cu)	mg/L, max	1.0
19	Cyanide (as CN)	mg/L, max	0.2
20	Ratio Active Material (a) Alpha emitters (b) Beta emitters	Micro curie/mL, max	10–9 10–8

Appendix 2 Preparation of the solutions for physicochemical analysis and bioassay.

Preparation of the solutions for Biochemical Oxygen Demand (BOD).

Alkali-iodide-azide reagent

Sodium Hydroxide (NaOH, 50 g) and Sodium Iodide (NaI, 13.5 g) was dissolved in deionized water and diluted to 100 mL. Sodium azide (NaN_3, 1 g) was dissolved in deionized water (4 mL) and added to the diluted solution.

Sodium Thiosulphate titrant (0.025M)

Sodium Thiosulphate ($Na_2S_2O_3$, 6.205 g) was dissolved in deionized water. Solid NaOH (0.4 g) was added and mixes thoroughly to dissolve. The solution was diluted to 1000 mL.

Preparation of the solutions for Chemical Oxygen Demand (COD).

Digestion solution

Distilled water (500 mL) was added to Potassium Dichromate (10.216 g, primary standard grade previously dried at 150 $^{\circ}$C for 2 hours). Sulphuric acid (167 mL) and Mercuric Sulphate (33.3 g) were added and dissolved. Allowed to cool to room temperature and diluted to 1000 mL.

KHP solution-500ppm

Weighed Potassium Pthalate Monobasis ($H_5C_8O_4K$, 0.425 g) and heated in 110^0C for 30 minutes. Dissolved in distilled water to make 1000 mL solution.

H_2SO_4 solution

Concentrated sulphuric acid (2.5 L) was added to silver sulphate (25.3 g) and allowed to stand for 12 hours.

Preparation of the solutions for Nitrate

Stock Nitrate solution (100mg/L)

Potassium nitrate (KNO_3) was dried in oven at 105 $^{\circ}$C for 24 hours and a portion of dried KNO_3(0.7218 g) was dissolved in distilled water and diluted to 1000 mL. The solution was preserved with $CHCl_3$ (2 mL).

Standard solution series

Different volumes of stock nitrate solution were diluted to 50 mL with distilled water.

Preparation of the solutions for Phosphate (as P).

Sulfuric acid reagent for the digestion

Concentrated Sulfuric acid (300 mL) was carefully added to distilled water (600 mL) and diluted to 1L with distilled water.

Preparation of Combined reagent

1. 5N Sulfuric acid

Concentrated Sulfuric acid (70 mL) was diluted to 500mL with distilled water.

2. Potassium antimonly tartrate solution

Potassium antimonly tartrate solid (1.3715 g) was dissolved in distilled water (400 mL) and diluted to 500 mL with distilled water.

3. Ammonium molybdate solution

Ammonium molybdate (20 g) was dissolved in distilled water (100 mL).

4. Ascorbic acid solution (0.1M)

Ascorbic acid (1.76 g) was dissolved in distilled water (100 mL).

Combined reagent (100 mL) was prepared by mixing the above regents in the following order and amounts.

1. Sulfuric acid (50 mL)
2. Potassium Antimonyl tartrate solution (5 mL)
3. Ammonium molybdate solution (15 mL)
4. Ascorbic acid solution (30 mL)

Preparation of Phosphate solutions for the construction of the standard curve

Stock Phosphate solution (50 mg/L)

Anhydrous Monopotassium phosphate (KH_2PO_4, 219.5 mg) was dissolved in distilled water and diluted to 1,000 mL.

Standard Phosphate solution (2.5 mg/L)

A portion of the stock phosphate solution was taken (50 mL) and diluted to 1,000 mL with distilled water.

Preparation of the solutions for *Allium cepa* bioassay

Acetocarmine solution

Carmine (1 g) and distilled water (55 mL) were boiled in a flask and allowed to cool. Then Acetic acid (45 mL) was added and boiled again. The mixture was allowed to cool and filtered.

Appendix 3 Proposed SLS standards for liquid fertilizer.

SI No.	Characteristic	Requirement	Method of test
i	pH (1:100)	6 – 8.5	SLS 1526
ii	Electrical conductivity (1:5), dS/m, max	20	ISO 11265
iii	Total Nitrogen content as N, percent by mass, min	1	SLS 645: part 1
iv	Total Potassium content as K_2O, percent by mass, min	0.5	SLS 645: part 4
v	Total Phosphorus content as P_2O_5, percent by mass, min	0.5	SLS 645: part 5
vi	Total primary nutrient, (N+ K_2O+ P_2O_5) percent by mass, min	2.0	SLS 645: part 1 SLS 645: part 4 SLS 645: part 5
viii	Organic carbon percent by mass, min	5.0	

Proposed SLS requirements for limits of potentially toxic elements.

SI No.	Element	Limits, mg/Kg maximum	Method of test
i	Arsenic as As	0.5	AOAC 2006.03 and ICP-MS
ii	Cadmium as Cd	0.5	
iii	Chromium as Cr	0.5	
iv	Lead as Pb	1.0	
v	Mercury as Hg	0.5	