Two-Phase Anaerobic Reactor Coupled with Microalgae Photobioreactor for Slaughterhouse Wastewater Treatment and Bioenergy Production

Dejene Tsegaye

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Supervisor: Professor Seyoum Leta

Addis Ababa University

Center for Environmental Sciences

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Professor Seyoum Leta Supervisor	___________	____________
__________________________ External Examiner	____________	____________
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ABSTRACT

Two-Phase Anaerobic Reactor Coupled with Microalgae Photobioreactor for Slaughterhouse Wastewater Treatment and Bioenergy Production

Dejene Tsegaye Bedane, PhD thesis

Addis Ababa University, 2023

*In Ethiopia, the transformation from agricultural to agro-industrial based development contributed to an increase in the number of agro-processing industries such as slaughterhouses, which discharge its process wastewater to the environment with partial or no treatment. Uncontrolled and regular disposal of partially treated or untreated wastewater into the water body has significant impacts on the environment and humans. The objective of this study was therefore to evaluate the pollutant removal efficiency and bioenergy (biogas and biodiesel) production potential of an integrated biological treatment system composed of two-phase anaerobic reactor and microalgae photobioreactor treating slaughterhouse wastewater. The experiment was conducted using two consecutively connected forty-liter galvanized metal anaerobic batch bioreactors (hydrolytic-acidogenic and methanogenesis reactors) integrated with a 9000-cm^3 microalgae photobioreactors. From the results of the hydrolytic-acidogenic phase, a hydraulic retention time (HRT) of three days and an organic loading rate (ORL) of 1788.81 mg COD/L*day offered optimal reactor stability and performance. Thus, highest degree of hydrolysis of 63.92%, degree of acidification of 53.26%, soluble chemical demand (3430.2 ± 80.44mg/L), and total volatile fatty acids (1176.50 ± 81.66 mg/L) were achieved for hydrolytic-acidogenic reactor operating at indicated condition. The NH_4^+-N concentration varied from 278.67 to 369.46 mg/L, which is not in a range that would affect the reactor's stability. The results from methanogenesis phase also revealed improved stability and performance of the system with peak operating parameters: total volatile fatty acid (TVFA) = 520 ± 19 mg/L; Total Alkalinity (TotA) = 1424 ± 10 mg/L; TVFA: TotA. Ratio = 0.36; salinity = 1172 mg/L, pH = 6.92) were found at a HRT of six days and an OLR of 298, mg COD/L*day. Additionally, highest removal efficiency for COD = 81%; volatile solid (VS) = 95%; biogas production = 185 ± 4 mL; and methane yield = 0.03 per mg COD consumed were achieved at HRT of 6 days and OLR of 298 mg COD//L*day. Moreover, the system showed good performance in terms of methane yield (67%) and methane production rate (123 mL/day). The results from the two-phase anaerobic digestion operated at optimal* *hydrolysis and methanogenesis reactors operating conditions showed substantial organic matter (TCOD (82.87%), TDS (93.32%), turbidity (98.0%6)) but low nutrient (TN (57–63%) and TP (27.57%)) removal efficiency. At the polishing step using microalgae photobioreactor, removal efficiencies above 85% were found for COD, TN, NO_3^-N, NH_4^+-N, TP, and PO_4^{-3}–P. The average crude lipid content of Chlorella, Scenedesmus, and co-culture microalgae biomass was 15.70 ± 1.27%, 17.75 ± 1.08%, and 21.65 ± 1.03%, with 72-79%, 67-78%, and 71-81% biodiesel content, respectively. The variation in lipid and biodiesel content was significant ($p \leq 0.05$) between the treatments. In general, the integrated two-phase AD and microalgae photobioreactor showed removal efficiencies of greater than 90% and 95% for nutrients (TN, NH_4^+-N, NO_3^-N, TP, PO_4^{-3}–P, and SO_4^{-2}) and organic matter (BOD, SCOD, and TCOD), respectively. The bioenergy produced from the integrated system in terms of methane and biodiesel were 128.40 mL/day and 67-81%, respectively. Furthermore, a two-phased AD system integrated with Chlorella, Scenedesmus species, and the co-culture exhibited final effluent concentrations below the permissible discharge limit for slaughterhouse effluent standards of Ethiopia for all pollutants. Therefore, it can be concluded that the two-phase AD system integrated with microalgae photobioreactor used to treat slaughterhouse wastewater in this study has implications for circular economy approach by converting this biowaste into valuable products. The treated final effluent can also be reused for non-potable domestic purposes such as floor cleaning, irrigation, and greenery purposes, which in turn guarantee sustainable practices.*

Keywords: Biogas yield, microalgae co-culture, microalgae-based wastewater treatment, slaughterhouse wastewater, two-phase anaerobic digestion

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ACRONYMS

AD	Anaerobic Digestion
APHA	American Public Health Association
BOD	Biological oxygen demand
CE	Circular economy
COD	Chemical oxygen demand
CW	Constructed wetlands
DA	Degree of acidification
DH	Degree of hydrolysis
DO	Dissolved oxygen
EC	Electrical conductivity
EPA	Environmental protection authority
HR	Hydrolytic-acidogenic reactor
HRT	Hydraulic retention time
MR	Methanogenisis reactor
NH_4^+-N	Nitrogen ammonium
OLR	Organic loading rate
ORP	Oxidation reduction potential
RE	Removal efficiency
SCOD	Soluble chemical oxygen demand
SHWW	Slaughterhouse wastewater
TCOD	Total chemical oxygen demand
TDS	Total dissolved solid
TKN	Total nitrogen
TSSs	Total suspended solids
TVFAs	Total volatile fatty acids
VFAs	Volatile fatty acids
Sp.	Species
PPP	Polluters pay principle
WWTP	Wastewater treatment plant

PUBLICATIONS

1. Tsegaye, D.; Khan, M.M.; Leta, S. (2023). Optimization of Operating Parameters for Two-Phase Anaerobic Digestion Treating Slaughterhouse Wastewater for Biogas Production: Focus on Hydrolytic–Acidogenic Phase. ***Sustainability***, ***15*** **(6)**, 5544. https://doi.org/10.3390/su15065544.
2. Tsegaye, D.; Leta, S. (2022). Optimization of operating parameters for biogas production using two-phase bench-scale anaerobic digestion of slaughterhouse wastewater: Focus on methanogenic step. ***Bioresources and Bioprocessing***, ***9*** **, 125**. https://doi.org/10.1186/s40643-022-00611-6.
3. Tsegaye, D.; Leta, S. (2023). Evaluation of biogas production and pollutant removal efficiency of two-phase anaerobic digestion treating slaughterhouse effluent. ***Biofuels*****,** ***14*** **(4)**, 1–9. https://doi.org/10.1080/17597269.2023.2185728.
4. Tsegaye, D.; Leta, S. (2023). Microalgae and co-culture for polishing pollutants of anaerobically treated agro-processing industry wastewater: The case of slaughterhouse. ***Bioresources and Bioprocessing***, 10 **(1)**. https://doi.org/10.1186/s40643-023-00699-4.
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CHAPTER ONE

1. INTRODUCTION

1.1. Background

The volume of wastewater produced globally has been steadily increasing due to the improved living standards because of economic growth, an increase in population, and growing agro-processing industry with huge fresh water consumption. Every year, global wastewater production reaches about 360–380 billion m^3 (Jones et al., 2021). The mismanagement of this huge volume of wastewater and overuse of water put maximum stress on freshwater bodies like rivers, lakes (lotic and lentic), seas, and oceans and also caused a decrease in the quality of aquatic ecosystem services (Minnesota Pollution Control Agency, 2008; Zemene Worku and Seyom Leta, 2017). The wastewater generation rate globally is projected to 24% and 51% in 2030 and 2050, respectively, showed the increasing trend.

In Ethiopia, the transformation from agriculturally based to agro-industrial based economy contributed to an increase in the number of agro-processing industries such as slaughterhouses, which consume huge amounts of freshwater at the same times discharge it to the environment with partial treatment or no treatment at all. The slaughterhouses and the meat processing sector are growing in Ethiopia, and on the world (Haddis et al., 2014; US EPA, 2002). The slaughterhouse industry's investment, both for local service and export, is increasing in Ethiopia, which is mainly associated with the livestock resources of the country, as Ethiopia ranked first and 2nd in the Horn of Africa and the whole of Africa, respectively in terms of the livestock population (Bustillo-Lecompte and Mehrvar, 2015a; US EPA, 2002; Zemene Worku and Seyom Leta, 2017). In 2018, there have been more than thirty-five meat export and two hundred ninety-six municipal slaughterhouses (BCaD, 2015; Eshetie, 2018). The municipal slaughterhouses are mainly found in regional major cities, zonal towns, and some at woreda level and are managed by the government, while the export slaughterhouses are privately owned and mainly found around cities in the rift valley such as Modjo, Adama, Batu, and Jigjiga. The number is increasing from time to time due to the government-oriented agro-processing industry's expansion for the transformation from an agriculturally based to an industrial based economy. Between 2015 and 2020, the amount of wastewater generated by the meat processing industry in Ethiopia and world were increased from 0.011 to 0.127 billion m^3 and 49-76 billion m^3, respectively (Figure 1). Steady growth at the global level is predicted, necessitating treatment facilities for a sustainable and safe discharge. The main driving forces for the increase in wastewater from meat processing industries or slaughterhouses are urbanization, meat demand, and economic growth. Besides, the transition/policy direction from an agricultural-based economy to an agro-industrial development strategy in Ethiopia additionally contributes to the increase in industrial growth and, consequently, wastewater production from slaughterhouses (Burkhart et al., 2018; Philipp et al., 2021; Ritchie et al., 2017; Wazir Shafi, 2021), which open up market opportunity for wastewater treatment from slaughterhouse processing industries in particular and agro-processing industries in general, primarily in developing countries such as Ethiopia.

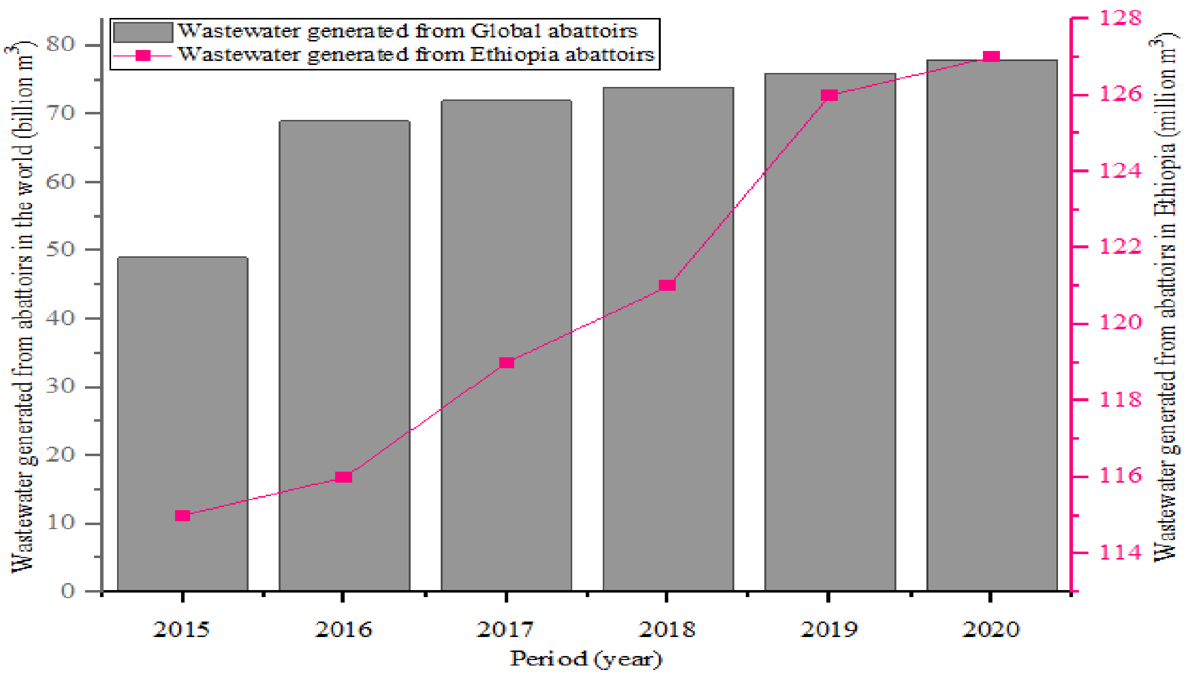


Figure 1: Wastewater generated from slaughterhouses in Ethiopia and world (2015-2020)

Source: (Burkhart et al., 2018; Philipp et al., 2021; Ritchie et al., 2017)

Padilla-Gasca et al. (2011) reported that the amount of wastewater generated per animal type varies from 200 to 300 liters, with an additional 25 percent of this for further processing of the edible parts. The main sources of wastewater in the slaughterhouse processes are livestock receiving and washing (care), slaughtering operations, separation of the carcass from offal products, cleaning of stomach materials and intestines, sanitation, and other services like floor washing, though the amount generated from each source or stage depends upon the type of livestock slaughtered (Widiasa, 2011; Minnesota Pollution Control Agency, 2008). Hence, slaughterhouses are categorized among industries characterized by water-consuming agro-processing industries. The wastewater is mainly composed of manure and urine, blood, stomach materials, and wash waters. Due to this it is high in suspended solids (SS) (3,835.33–8,000 mg/L), insoluble and soluble organic concentrations that exhibit high chemical oxygen demand (COD) (4,000–1,546.67 mg/L), and biological oxygen demand (BOD) (1,200–4,500 mg/L), and is categorized as strong wastewater (Mittal, 2006a). Moreover, it is also high in phosphorous (30–202 mg/L) and nitrogen (95.2–1,200 mg/L) contents (Aleksić et al., 2020; Dyan et al., 2015; Kundu et al., 2013; Nweke et al., 2014; Widiasa, 2015).

Organic Export Abattoir is a private limited company found in Modjo, 70 km away from Addis Ababa, which is dedicated to processing and exporting mainly sheep and goat organic meat. About 800-1200 sheep and goats (each) per day are being slaughtered at this slaughterhouse, for which a total of 400 L of water per sheep or goat is being used. An almost equivalent amount of wastewater is discharged into the nearby Modjo River without proper treatment, increasing the pollution load of TCOD, SCOD, BOD, ammonia, phosphorous, nitrogen, sulfate, nitrate, and nitrite on Koka Lake/dam, the destination of the Modjo River (Mulu and Ayenew, 2015; Tsegaye, et al., 2023).

In Ethiopia, wastewater management practices in many slaughterhouses involve disposing of it in landfills or nearby water bodies, which in turn poses major environmental challenges like bad odor, leachate management, eutrophication of water bodies, and greenhouse gas emissions. Furthermore, earlier studies showed that about 90% of agro-industries in Ethiopia are discharging their effluent directly into the nearby water bodies or environment, thereby putting respective ecosystems at risk (Alemu et al., 2018; Leta et al., 2003), indicating that wastewater treatment facilities at agro-industries in Ethiopia can be taken as non-existent, and if there are any, they are mismanaged, i.e., the facilities are there but the functionality, efficiency, and sustainability are questionable (Genet et al., 2018; Haddis et al., 2014; Zemene Worku and Seyom Leta, 2017).

Hence, continuous improvement and affordable wastewater management provides opportunities for both clean water supply augmentation and pollution reduction, which at the same time promote sustainable development and support the transition to a circular economy (CE) (Directorante & Papers, 2000; Jones et al., 2021). This shows that the mechanisms that go beyond abatement of pollution and seek value from wastewater are a timely demand. Among the biological slaughterhouse wastewater treatment systems, anaerobic digestion (AD) represents an alternative for agro-processing industry wastewater, with high biodegradable organics, sufficient alkalinity, adequate phosphorous, nitrogen, and micronutrient concentrations for bacterial growth, and lower toxic compound level via biogas production (Duarte et al., 2021; Dyan et al., 2015; Padilla-Gasca et al., 2011).

The efficiency of anaerobic digestion is highly dependent on the characteristics of the waste, the reactor configuration, and other operational parameters (Lindorfer et al., 2008; Siegert & Banks, 2005). In traditional single-stage anaerobic digestion practice, both the hydrolytic-acidogenic and methanogenesis take place in one reactor. In such reactors, acidogens grow faster and are less sensitive to pH variations than methanogens or acetogens, resulting in the accumulation of volatile fatty acids, a lowering of pH, and the suppression of methanogens, resulting in reactor failure or instability (Koutrouli et al., 2009). Separation of the HR and MR is mandatory as it permits the optimization of the OLR and HRT based on the requirements of the microbial consortiums of each phase and can prevent the imbalance due to the groups of anaerobic bacteria occurring in single-phase reactors (Ghosh et al., 1995; Koutrouli et al., 2009).

Hence, possible overloading of a methanogenic reactor can be detected at the hydrolytic-acidogenic phase and prevented by the supply of the acidified effluent from HR at optimal employment of methanogenic activities present in MR (Wilson, 2009). The HR also serves as a buffering reactor by reducing the easily floating grease and oil and partially degrading the organic matter in the agro-industrial wastewater (Ghorbanian, 2014). This in turn increases the stability of the methanogenesis reactor by avoiding the accumulation of total volatile fatty acids (TVFA) through a sudden increase in OLR as acetogens grow slower than acidogenics (Tanarat & Hanjai, 2020). Furthermore, the second reactor is methanogen-rich and obligatory anaerobic, which makes it sensitive to variations in operating conditions such as OLR and HRT. This, therefore, necessitates the optimization of the methanogenesis phase operating conditions (Dinopoulou, 1988; Van et al., 2020).

Furthermore, regardless of their current importance and upcoming potential, anaerobic wastewater treatment systems have not always enjoyed auspicious standing due to the high organic matter and nutrients, mainly nitrogen and phosphorus (McCarty, 1964; Praveen et al., 2018), which need further treatment prior to release to the environment (Alayu and Leta, 2020; Alemu et al., 2019; Figueroa-Torres et al., 2021; Tsegaye and Leta, 2022b; Yirgu et al., 2020; Zemene Worku and Seyom Leta, 2017), which otherwise cause eutrophication of the receiving water bodies (Arbib et al., 2014; Figueroa-Torres et al., 2021).

Activated sludge technique is among the numerous post-treatment categories that minimize the AD digestate nutrient contents. Nevertheless, it is expensive and produces large volume sludge (Craggs et al., 2014). In this regard, microalgae have been suggested by scholars as a post-AD treatment system (Koutrouli et al., 2009; Mallick, 2002). This is attributed to their role in the self-purification of polluted waterbodies by naturally assimilating nutrients (nitrogen and phosphorous) found in the agro-industry effluent (Radin et al., 2017). *Scenedesmus* and *chlorella* species were reported as potential candidates for both agro-processing industry effluent treatment and biomass yield, mainly for bioenergy production (Abdel-Raouf et al., 2012).

Integrating microalgae with wastewater treatment facilities, mainly AD facilities treating agro-processing industry wastewater systems for dual purposes of nutrient removal as well as biomass production, was extensively studied (Onay, 2018; Onay and Aladag, 2022). Most of the studies used synthetic media, microalgae acquired from laboratories, and microalgae growing media such as Bold’s Basal Medium (BBM) (Miranda, Passarinho, 2012; Mamo and Mekonnen, 2020). There is also research conducted using raw or partially treated (AD effluent or other) effluent for nutrient and organic matter removal as well as bioethanol and biodiesel production: *Scenedesmus* sp. (Yirgu et al., 2020) for brewery AD effluent and *Chlorella*, *Scenedesmus,* and their co-culture (Asmare et al., 2014) for raw municipal wastewater. Yet, the two-phase anaerobic digestion system with microalgae collected from local freshwater bodies and cultivated in a photobioreactor to treat slaughterhouse wastewater via biofuel production has not been explicitly studied. In addition, there is also an information gap in the optimization of the operational parameters of a two-phase AD system fed only with slaughterhouse wastewater. Therefore, the objective of this study was to develop an integrated biological wastewater treatment system containing AD and microalgae photobioreactors for SHWW, focusing on sustainable biowaste management through bioenergy production.

1.2.Statement of the Problem

Between 2015 and 2020, the amount of wastewater generated by the meat processing industry in Ethiopia (annually) was 95.31 (15.89) m^3 million, with steady growth at the global level predicted. Urbanization, meat demand, economic growth, and industrialization are the main driving forces behind the increase in wastewater from meat processing industries or slaughterhouses. Besides, the transition from an agricultural-based economy to an agro-industrial development strategy in Ethiopia additionally contributes to the increase in industrial growth and, consequently, wastewater production. Likewise, recent research findings indicated that the annual global wastewater production reached about 360–380 x 10^9 m^3 and tends to increase at a rate of 24% and 51% in the years 2030 and 2050, respectively (Burkhart et al., 2018; Jones et al., 2021; Philipp et al., 2021; Ritchie et al., 2017).

In the recent decade, municipal and export slaughterhouses in Ethiopia have increased from time to time. Most slaughterhouses do not have wastewater treatment plants and directly discharge their effluent to nearby water bodies or the environment, though some may have physically installed treatment plants that do not efficiently treat the wastewater (Mulu & Ayenew, 2015; Zemene Worku and Seyom Leta, 2017). The wastewater mainly contains manure and urine, blood, stomach materials, and wash-water (Hernández et al., 2018). Due to this, the wastewater is high in suspended solids (SS) (3,835.33–8,000 mg/L), insoluble and soluble organic concentrations that exhibit high chemical oxygen demand (COD) (4,000–15,460.67 mg/L), and biological oxygen demand (BOD) (1,200–4,500 mg/L), and is categorized under strong wastewater. Moreover, it also contains high levels of phosphorous (30–202 mg/L) and nitrogen (95.2–1,200 mg/L) (Aleksić et al., 2020; Bustillo-Lecompte et al., 2014; Kundu et al., 2013; Mulu and Ayenew, 2015; Nweke et al., 2014). A previous study reported that 90% of agro-industries in Ethiopia discharge their effluent directly to nearby water bodies or the environment, thereby putting these ecosystems at risk (Leta et al., 2003). Discharging partially treated or raw effluent with a high pollution load as well as volume causes eutrophication of water bodies, pollutes soil and air, and decreases the aesthetic value of the natural environment (Alemu et al., 2018). Improper disposal of abattoir waste not only affects air and water quality but also increases threats to human health due to the presence of pathogenic microbes (Tolera and Alemu, 2020). Furthermore, wastewater treatment facilities at agro-industries in Ethiopia can be taken as non-existent, and if there are any, they are mismanaged, i.e., the facilities are there, but the functionality, efficiency, and sustainability are questionable (Haddis et al., 2014). Hence, continuously improving and affordable agro-processing industry wastewater management that offers chances for both clean water supply augmentation and pollution reduction, which at the same time encourage sustainable development and backing the switch to a circular economy, should be in high demand (Directorante and Papers, 2000; Jones et al., 2021).

Slaughterhouse wastewater treatment via biogas production was comprehensively studied by different scholars (López-Sánchez et al., 2022; Vavilin et al., 2008; Zemene Worku and Seyom Leta, 2017). Furthermore, most of those studies were mainly focused on single-phase anaerobic reactor systems to treat poultry, pig, and cattle slaughterhouse wastes through resource recovery options. However, Abraham and Seyoum (2021) studied two-phase anaerobic digestion of slaughterhouse wastewater with a main focus on biogas production only with co-digestion of varieties of vegetable waste and fruit. The conclusions from all the studies were that the AD system can potentially remove most of the organic matter, TN, and TP, but not to the level of the country's wastewater discharge limits set for slaughterhouses and other agro-processing industries (Alemu et al., 2018, 2019; Kundu et al., 2013; Zemene Worku and Seyom Leta, 2017). Therefore, an additional treatment system is required to lessen or remove the organic matter, nitrogen, and phosphorus in the two-phase AD effluent to the country's discharge limits. In this regard, Zemene Worku and Seyoum Leta (2017) studied an integrated single-phase anaerobic reactor with a constructed wetland to remove both organic matter and nutrients from a slaughterhouse wastewater. However, very few studies have integrated AD effluent treatment using microalgae and biomass production (Onay and Aladag, 2022), and most of the studies use synthetic or microalgae growing media such as Bold’s Basal Medium (BBM) (Miranda, Passarinho, 2012; Mamo and Mekonnen, 2020). But little research has been done using raw or partially treated effluent for nutrient and organic matter removal as well as bioethanol and biodiesel production using *Scenedesmus* species (Yirgu et al., 2020) using brewery AD effluent and *Chlorella*, *Scenedesmus* sp., and their mixture (Asmare et al., 2014) using raw municipal wastewater. Therefore, a system that can address the existing challenges in the agro-processing industry's wastewater treatment area is timely. Hence, a circular economy concept approach for agro-processing industries such as slaughterhouse wastewater treatment that can recover resources in the form of energy, clean water, and nutrients should be framed. The overreaching objective of this study is therefore to develop an integrated biological slaughterhouse wastewater treatment system composed of anaerobic digestion and a photobioreactor as a polishing step for biogas and nutrient recovery as an exercise of circular bioeconomy transformation.

1.3. Objectives of the Study

1.3.1. General objective

The general objective of this study was to investigate the potential of two-phase anaerobic digestion coupled with microalgae photobioreactor for the slaughterhouse wastewater treatment and bioenergy (biogas and biodiesel) production.

1.3.2. Specific objectives

The specific objectives:

1. To determine the effect of organic loading rate (OLR) and hydraulic retention time (HRT) on hydrolytic-acidogenic and methanogenesis process stability and performance.
2. To investigate the biogas production and pollutant removal performance of two-phase anaerobic digestion of slaughterhouse wastewater.
3. To determine the pollutant removal efficiency and biomass production potential of microalgae culture cultivated in a photobioreactor treating phased AD effluent as a polishing phase.
4. To investigate the pollutant removal efficiency of the integrated system treating slaughterhouse wastewater.

1.4. Scope of the Study

The study mainly focuses on the development and evaluation of an integrated treatment system composed of two-phase AD and microalgae photobioreactors for slaughterhouse wastewater treatment and biofuel (biogas and biodiesel) production. Accordingly, the scope of this thesis was:

- Evaluation of the effect of HRT and OLR on hydrolytic-acidogenic and methanogenesis process stability and performance (optimization of operating parameters of HR and MR),
- Evaluation of biogas production and pollutant removal performance of two-phase anaerobic digestion treating slaughterhouse wastewater,
- Polishing of nutrient and organic matter-rich phased AD effluent using microalgae cultivated in a photobioreactor and microalgae biomass yield,
- Evaluation of the integrated two-phase AD and microalgae photobioreactor performance in terms of pollutant removal and biofuel production.

1.5. Thesis organization and Framework

The PhD thesis is centered on four major objectives, from which scientific papers were produced and published in reputable international journals in the thematic area of the study. Objective one focuses on the effect of HRT and OLR on hydrolytic-acidogenic and methanogenesis process stability and performance parameters (optimization of operating parameters of HR and MR). Objective two deals with the overall performance of a phased anaerobic reactor in terms of pollutant removal and biogas production. Objectives three and four present microalgae-based treatment for polishing nutrient-rich AD effluent through biomass production for biodiesel and the overall integrated system performance in terms of pollutant removal and renewable energy (biofuel) production as an exercise of circular bioeconomy, respectively.

In general, there are five chapters in this PhD research that are organized as follows;

Chapter one provides a summary of the background and motivations of the research. The general and specific objectives, milestones, scope, and problem of the statement of the research are stated in this section. The entire research work's content and framework are presented in this chapter.

Furthermore, the overview of the thesis and interconnection of the included papers is indicated in this part and is shown in Figure 2.

A comprehensive review of most published scientific papers in the major focus areas of the study or research is presented in this part. Accordingly, slaughterhouse wastewater characteristics, major sources of wastewater in the slaughtering process, expansions, its environmental impacts, and basic treatment technologies are discussed in detail. In addition, factors affecting the AD process and performance enhancement techniques like operational parameter optimization, co-digestion, and others are also discussed in this chapter. Furthermore, the merits of integrated wastewater treatment techniques are also discussed in detail in chapter two.

Chapter three presents the methodology for the experiment to achieve the objectives while chapter four provides a detailed result with discussions on the optimization of the operating parameters of HR and MR, biogas production, methane yield, and pollutant removal. Two microalgae and their co-culture evaluation results for AD effluent treatment through biomass production, from which biodiesel was produced, are also presented with detailed discussions in this section. Furthermore, the overall AD coupled with microalgae photobioreactor performance in terms of pollutant removal efficiency and comparison of the parts are also presented in the last part of this chapter. A generalized conclusion from the findings of the research and foreseen recommendations are provided in chapter five.

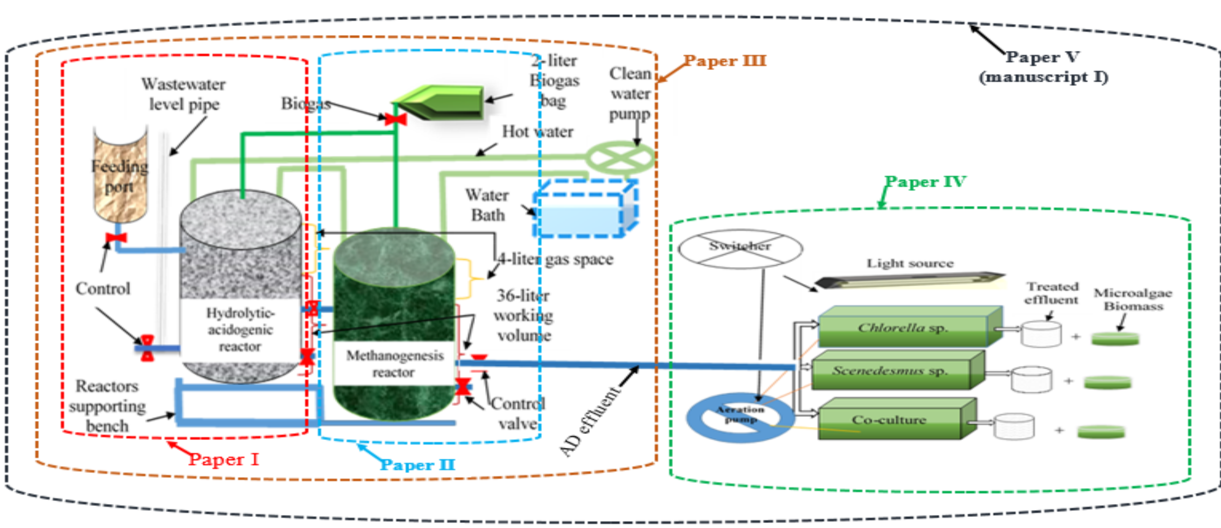


Figure 2: Overview of the thesis and the interconnection of the published papers

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Slaughterhouse Industry Wastewater Generation Trends

A slaughterhouse, commonly known as an abattoir, is a facility where animals are slaughtered for human consumption. The world animal protein increment in recent decades, predominantly in developing countries, has resulted in a global livestock revolution with significant effects on human health and the environment (Matheyarasu et al., 2015; Pandey, 2006). Consequently, the world meat demand from 2000 to 2010 increased by 24 percent, and the projection shows it is increasing at an alarming rate (by 22 percent from 2010 ahead) (Rabobank, 2011). Similarly, from 2015 to 2020, the amount or volume of wastewater generated from the slaughterhouses or meat-processing industry in the world was 41.53 billion m^3, with an annual average of 6.92 billion m^3, and substantial growth at the global level was predicted. The core driving forces for the growth of wastewater from slaughterhouses or meat processing industries are meat demand, urbanization, and economic growth. Besides, the switch from an agriculture-based economy to that of an agro-industrial development strategy in Ethiopia is an extra contribution to the increase in industrial growth and subsequently to wastewater generation. Moreover, according to recent research findings, the annual worldwide wastewater production is about 360-380 x 10^9 m^3 and has a tendency to increase at a rate of 24% and 51% in the years 2030 and 2050, respectively (Burkhart et al., 2018; Jones et al., 2021; Philipp et al., 2021; Ritchie et al., 2017).

Besides, worldwide, the agro-processing industry is known for accounting for 29% of the total freshwater consumption budget in the agricultural sector. Consequently, the increasing number of slaughterhouse facilities results in a predictable higher wastewater volume from slaughterhouses that must be managed before being released to the environment (Mekonnen and Hoekstra, 2012; Philipp et al., 2021). In addition, the United States Environmental Protection Agency (USEPA) classified SHWW among the most damaging agro-processing industrial wastewaters to the receiving environment if there is no proper disposal. Henceforth, the slaughtering and processing activities in the slaughterhouse generate a complex mixture of wastewater high in both organic and inorganic pollutants, which are potent for the environment if released without treatment (Padilla-Gasca et al., 2011). This indicates that the slaughterhouse industries are among the agro-industries that have a significant contribution to environmental degradation, with byproducts of nearly 150 million tons per year (Jones et al., 2021; Ritchie et al., 2017; US EPA, 2002).

2.2. Major Slaughterhouse Process Generating Wastewater

Slaughtering as well as further processing of meat products is a multi-step activity that consumes much water and, as a result, produces huge amount of wastewater in each respective step. To minimize the water consumption in the basic slaughtering process, steps has become more efficient, and automated in recent years, mainly in hide removal, evisceration, stunning, and splitting systems (Ruiz et al., 1997). Livestock and animal care, killing, hide (hair) removal, gutting (eviscerating), carcass washing, trimming, as well as clean-up activities are the main sources of wastewater in the slaughterhouse or meat processing industry. As the consumption of water in slaughterhouses varies based on the operation and the type of animal, so does wastewater generation (Mittal, 2006b; US EPA, 2002; Villarroel Hipp and Silva Rodríguez, 2018). The basic steps and type of effluent of the slaughtering processes are indicated in Figure 3. All the water spent at the slaughterhouse in due course becomes wastewater or effluent at the end. High blood content is the largest particular source of wastewater pollutant burden in the meat processing or slaughterhouse industries (Kundu et al., 2013; Zealand et al., 2000). It was also estimated that every slaughtered pig, bird, and cow can produce 700, 15, and >330 liters of wastewater, respectively, which contain a high concentration of biodegradable organics, suspended and colloidal particles, and microbes (COWI, 2000; Padilla-Gasca et al., 2011). To this end, the details of the slaughtering processes contributing to the wastewater are presented in subsequent sub-sections.

Lairages/Pre-handling of cattle: This is a room or place where the animals are rested for one or two days before being slaughtered after the truck arrives in the abattoir and is unloaded into holding pens. Every slaughterhouse must have shelters allowing animal grouping and recuperation before slaughtering and an ante-mortem veterinary inspection. Any unclean (dirty) cattle should be washed in this holding or room. In addition, paths and runs from the holding pens or lairs to the slaughtering room need to be planned without protuberances to avoid damage to the moving animals (Shende et al., 2022).

Slaughtering and Bleeding: This is the 1st step where the animals are slaughtered: stunning, lifting, and killing occur. The slaughtered animals' blood, fibrin, and albumin are recovered. The blood and sanitary water are the main sources of wastewater from this step.

Viscera handling and hide processing: To remove the viscera, the carcasses are opened and washed. Edible parts (lungs, tongue, heart, and liver), beef, and paunches are cleaned and kept for consumption in cold storage or a space for rapid chilling. High salt-containing wastewater results from washing and curing hides, as expected. The head is cleansed using high-pressure water, and the tongue and brain are removed for use as edible parts. The hides are then isolated from the process room and taken to the place where they are preserved by chilling or salting before they are shipped for further processing as leather (Naila, 2008).

Cutting and meat preparations: In this unit, carcasses are cut and trimmed, then cuts are cured and deboned, packed for dissemination, and some carcass pieces stick to saw blades and conveyors. At this stage, the wastewater is the result of materials or equipment washing, curing solutions, and spills from cooking equipment. The waste materials arising from the floor and equipment washing contribute to the waste burden of the slaughterhouses or meat processing industry (USEPA, 1974).

Rendering/ Carcass dressing: This step is where edible and non-edible slaughtered animal parts are formed. Fats are separated from other animal parts, tissues, and water. Tumbles coming from cooking materials, collection tanks, and discharge from the washing of tissues and other sanitary items contribute to wastewater at this step (Shende et al., 2022). The meat and fat particles obtained from the operations are normally washed into the sewer line of the slaughterhouse or meat processing industry (USEPA, 1974).

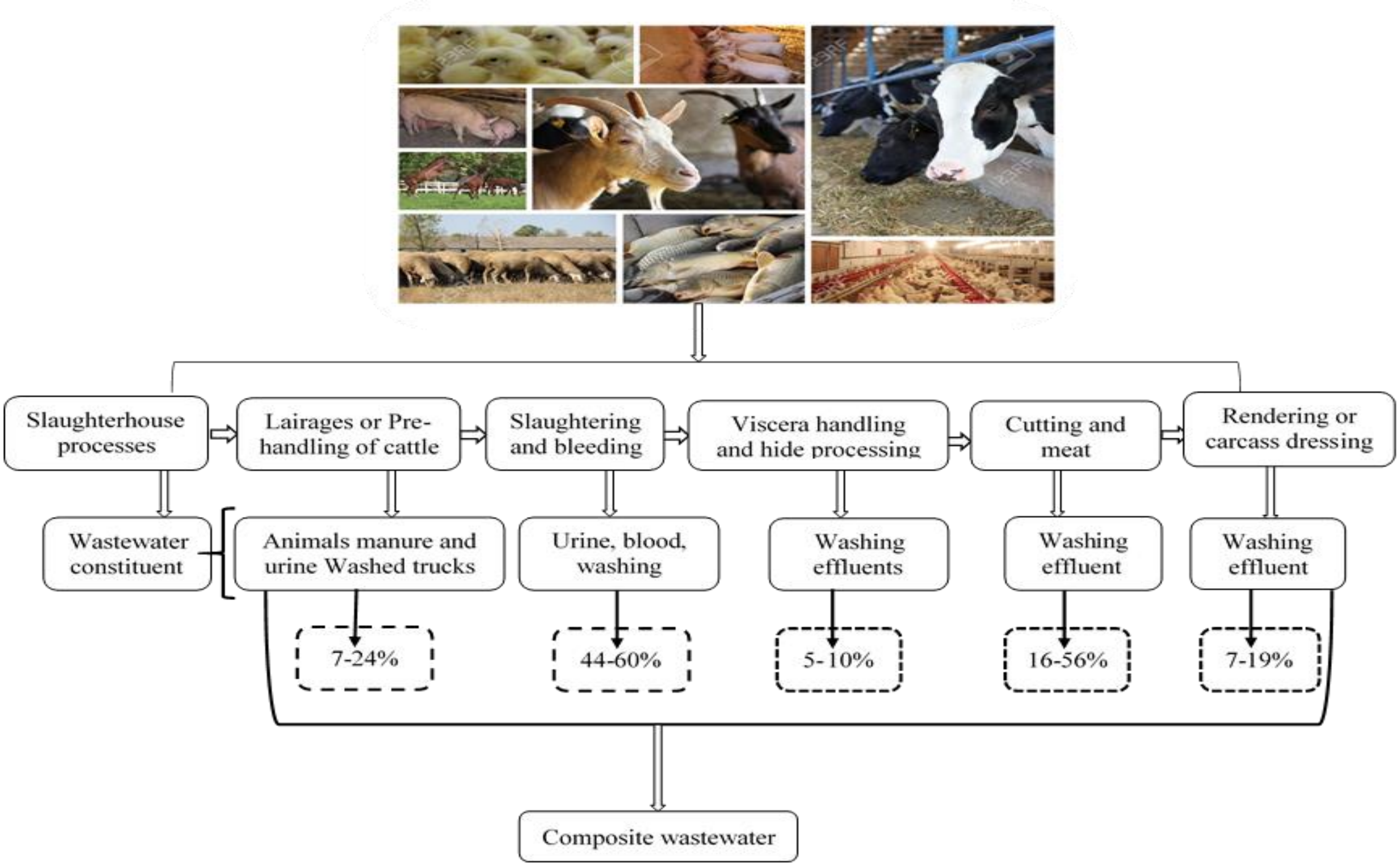


Figure 3: Slaughterhouse processes steps and type of effluent generated

Modified from (Mozhiarasi & Natarajan, 2022)

2.3. Slaughterhouse Industries Wastewater Management Practices in Ethiopia

Similar to the agro-processing industries in Ethiopia, the slaughterhouse and meat processing industry sectors are increasing, even if it is still considerably less than it would be assumed for the livestock resource potential. In Ethiopia, the local milk and meat demand consumption projected between 2015 and 2050 was estimated to grow by about 5.5 and 0.9 million tons, respectively (FAO, 2012). This demand, coupled with the agro-centered industrial development strategic plan, again increases the number of meat processing facilities, such as slaughterhouses, both for local consumption and export. For example, with the fast increase in the world's meat product demand, Ethiopia's opportunity to export fresh meat to Middle Eastern and North African countries have been growing. As a result, the meat export sub-sector of the agro-processing industry is becoming at the forefront of the Ethiopian Government's development goals in order to boost the sub-sector's productivity and overall economic contribution. The sub-sector has surely supported the country's poverty-reduction efforts by producing foreign currency and creating job opportunities. The country has a number of significant chances to influence the meat and live animal industries, notably in the export sector. The number of contemporary animal products firms, particularly meat export firms, that have been developed in the country is growing (Eshetie, 2018; Haddis et al., 2014). There are more than 35 export slaughterhouses and abattoirs, and the establishment of most of the export abattoirs was in five to six rift valley towns, including Modjo (Eshetie, 2018). In Ethiopia, the municipal slaughterhouses are increasing both in number and in capacity. For example, a new export abattoir with a large capacity and the latest standards has started in eastern Ethiopia, mainly targeting the export market. Its capability to slaughter more than 100 camels and 3000 sheep and goats per day indicates the development of the industry.

Moreover, backyard slaughtering has been transformed into industrial-scale production of meat, resulting in massive wastewater discharges into the environment that are not treated, endangering human health and the environment (AAE, 2016). It is almost tolling to more than three hundred (300) serving the local meat demand, mainly in federal, regional, and zonal cites, with some at woreda or special zone towns. Apart from that, illegal animal slaughtering is also highly experienced for domestic meat consumption purposes in the backyards of all households, especially for shoats (BCaD PLC, 2015).

Henceforth, slaughterhouses are known for their huge water consumption, which comes out as wastewater overdue to slaughtering processes, though the amount of wastewater generation fluctuates depending on the process used and type of animals slaughtered (Silva et al, 2012; Mittal, 2004). The sector is one of the most environmentally damaging agro-industries because of the wastewater it produces, in terms of both quality and quantity as well as nature and composition. In slaughterhouses and the meat processing industry, wastewater of 1.6-9-L and 4-4.2 grams of COD are released into the environment for every kilogram of edible cattle carcass produced (European Commission, 2005). The same study results also confirmed that 0.5-4.6 grams of TN are emitted to produce one kilogram of poultry meat. The volume of wastewater generated from the slaughterhouses in Ethiopia between 2015-2020 was 95 million cubic meters (Gashu et al., 2022; Jones et al., 2021). According to figures from the FAO (2012) and the European Commission (2005), Ethiopia's slaughterhouse industries produced roughly 18.2 million m^3 of wastewater by the year 2018. This is based on the FAO's (2012) wastewater release per kg of carcass and the European Commission's (2005) wastewater release statistics. The majority of the water used at slaughterhouses is eventually turned into effluent, and the slaughtering process is the main cause of waste burden in meat processing facilities (Kundu et al., 2013). The effluent from rendering often represents the single most important source of pollution in slaughterhouse effluent at those plants where rendering takes place.

Therefore, the situation on the ground and literature indicate that the slaughterhouse development in the country both in the export and municipal sectors, is increasing in capacity as well as in number and complexity of pollutants, including emerging pollutants that are usually released to waterbodies without treatment (Ng et al., 2022; Olaniran et al., 2018), leading to environmental and public health interference. This production of meat at the export and large municipal scale discharges huge amounts of wastewater with no treatment to the surrounding environment, and causing severe environmental and health problems for humans (Gashu et al., 2022; Jones et al., 2021).

In general, most slaughterhouses, either export or municipal, do not have wastewater treatment of the standard or not at all (Mulu and Ayenew, 2015) and cannot properly treat their wastewater before discharging it to the environment, regardless of the pollution load. Few of them have non-functional stabilization ponds or other primary treatment facilities. According to our observation, the slaughterhouses in Ethiopia are advanced in meat processing units or facilities but not in the associated environmental issues. The slaughterhouses, mainly for export, standardize the meat production process, but a lot of work should be expected to advance their wastewater treatment facility as well.

2.4. Challenges of Slaughterhouse Industry Wastewater

In numerous studies, it was highlighted that slaughterhouse wastewater is one of the most challenging types of agro-processing industry waste, requiring substantial management locally as well as globally. The main issues with slaughterhouse wastewater are mainly due to the high levels of blood from slaughtered animals, suspended particles from rumen contents, undigested food, feathers, flesh fragments, bone fragments, and microbiological species. The impacts may be amplified by the tendency toward industrialized, intense production of confined sheep, goats, and poultry. The main challenges due to the slaughterhouses are categorized under the following broad umbrella: environmental, social, economic, and biodiversity loss or ecological impacts (Bustillo-Lecompte and Mehrvar, 2015b; COWI, 2000; D, 2015).

Table 1: Major environmental impacts due slaughterhouse wastewater

Receiving media or disposal technique	**Major impacts**
Water bodies	◦ Eutrophication due to high nutrient load ✓ Nitrogen, phosphorous ◦ High inorganic and organic pollutants load ✓ BOD, COD, and TS ◦ Global warming and climate change ✓ CH_4, CO_2, CFC, NO_2 ✓ Pathogenic organism, FC and TC ◦ Biodiversity loss and ecosystem value decrease ✓ Loss of fishes breeding and death ◦ Inappropriate for irrigation ◦ Odor
Land (soil)	• Contamination of soil (can be persistent) • Odor • Potential for transporting of heavy metals to food chain • Bio-magnification, Bio-concentration, and Bioaccumulation
Evaporated pond	• Toxicants or odor emission • Organics and inorganics load • Pathogens (disease causing agents)
Infiltration	• Loss of ground water quality due to contamination • Long-term effects in the fauna and flora. • Aquifer clogging

Source: (Bustillo-Lecompte and Mehrvar, 2015b; Matheyarasu et al., 2015)

2.5. Prospects for Slaughterhouse Wastewater as Resource

The primary forces behind Ethiopia's and Eastern Africa's economic growth are thought to be agriculture and shifting to agro-processing industries. Ethiopian slaughterhouse effluent is primarily unused and untreated, and is therefore typically dumped into bodies of water. Aside from their part in the emission of greenhouse gases (GHG) that cause climate change, these practices deplete bioresources. Simultaneously, the use of fossil fuels, which increases greenhouse gas emissions, has drawn attention from all quarters, calling for the creation of cleaner, alternative bioenergy sources that are renewable. Even though they pose a risk to the environment, organic wastes can be used as energy carriers and can be regenerated to meet the demand raised due to the increasing population (Ng et al., 2020). Furthermore, the high biodegradability of slaughterhouse wastewater makes it easily used by micoorganisms, either in aerobic or anaerobic conditions (Speece, 1996). Utilizing resources from slaughterhouse effluent for energy recovery shows promise in addressing environmental issues. The use of this agro-industry wastewater as feedstock for biorefinery processes has drawn a lot of attention in the context of environmental sustainability. In this sense, the waste from slaughterhouses can produce a significant quantity of valuable items, like bioenergy, power, and other byproducts. For instance, one gram of COD degraded at 35 degrees Celsius and one atmosphere would provide 395 milliliters of CH_4 if all of the COD in the trash could be converted to methane with equivalency (Speece, 1996). Put another way, one cubic meter of wastewater will yield 2.472 ± 0.451 m^3 of total biogas if it is assumed that 60% of the biogas contains methane. Additionally, if wastewater discharged was equal to 0.9 m^3/slaughtered animal unit, total biogas production will be 2.225 ± 0.406 m^3/animal unit slaughtered. Above all, bioenergy production is a major component of the circular bioeconomy agro-industrial waste management option (Cosmas and Stephen, 2023).

Similar to this, treated wastewater from a slaughterhouse can be reused for a variety of purposes, contingent on the requirements and quality of the treated water:

> Agricultural irrigation: If the treated water satisfies quality standards and regulatory requirements established for the protection of human and environmental health, it can be used for irrigation of agricultural crops.

Internal process water: Treated water can be reused within the slaughterhouse for cleaning, equipment washing, toilet flushing, or other non-potable uses that do not require potable quality water.

Industrial use: Sometimes treated water can be used in industrial processes that do not call for potable water, like equipment cooling, manufacturing, or industrial cleaning activities that do not encompass direct interaction with people or food (Laura, 2023).

Therefore, safe management of wastewater from slaughterhouses reduces pollution in the environment and shields the economically active population from diseases associated with waterborne infections. In addition to the three main effects (bioenergy production, wastewater treatment, and nutrient/water reuse), recovering bioenergy from slaughterhouse waste also helps to meet domestic and industrial energy needs, reduces the rate of deforestation for charcoal and firewood, and lowers mainly indoor air pollution and respiratory illnesses because biogas burns smokelessly (Fantozzi and Buratti, 2009).

2.6. Slaughterhouse Industries Wastewater Treatment Techniques

Even though the freshwater consumption of the slaughterhouse industry varies widely, a significant amount of wastewater is generated and released from various processes and facility cleaning. As a result, water reuse as well as the recovery of valued by-products from slaughterhouses or meat processing effluents are the primary focus of the agroindustry’s efforts toward cleaner production centered on biogas production, nutrient and fertilizer recovery for land applications, and high-quality effluents (Bustillo-Lecompte and Mehrvar, 2015b). SHWW and meat processing industries wastewater treatment techniques are mostly similar to municipal wastewater treatment techniques and mainly include primary, secondary, and advanced (tertiary) treatment. Following preliminary treatment, SHWW treatment techniques can typically be classified or categorized into four (4) broad categories, such as physicochemical treatment, AOPs, biological treatment, and combined processes (Philipp et al., 2021).

2.6.1. Preliminary treatment

The preliminary slaughterhouse wastewater treatment step is mainly used to screen or isolate large particles (10-30 mm) and solids from the SHWW. At this stage, there is a reduction of up to 30% of the slaughterhouse BOD content. Sieves, screeners, and strainers are the most commonly employed operational unit materials in the SHWW preliminary treatment. Other types of preliminary treatment techniques applicable to wastewater from agro-processing industries include homogenization, flotation, equalization, catch basins, and settlers (Mittal, 2006a). The effluent from preliminary treatment, should be further subjected to primary (1^0), secondary (2^0), and tertiary (3^0) treatment as the purpose of this unit is mainly to control the effluent budget and remove bulky solids (Musa & Idrus, 2021).

2.6.2. Physicochemical treatment

Physicochemical treatment methods are predominantly employed for the reduction of fat, oil, grease (FOG), TSS, and BOD (Al-Mutairi et al., 2008) and include coagulation-flocculation and sedimentation, membrane processes, dissolved air flotation (DAF), and electrocoagulation. This technique is the most popular and widely used type of conventional agro-processing industrial wastewater treatment, including slaughterhouse or meat processing wastewater, by screening, catch basin, and flow equalization (USEPA, 2008). Similarly, grit chambers, screens, and settling tanks are also widely used for the removal of SS, colloidal particles and fats from SHWW. Chemical coagulation or flocculants such as ferrous sulfate ($FeSO_4$), aluminum sulfate ($ALSO_4$), ferric chloride ($FeCl_3$), and magnesium chloride ($MgCl_2$) are used to decrease COD and suspended solids (SS) loads as well as other toxic substances. At this agro-processing industry wastewater treatment stage, approximately 65% of FOG, 25-50% of BOD, and 50-75% of SS can be removed (Lofrano et al., 2018).

2.6.2.1. Coagulation-flocculation and sedimentation

This technology is a physicochemical technique used to treat agro-processing industry wastewater as well as SHWW based on the addition of coagulant to a bioreactor to promote the formation of colloidal particles. A schematic illustration of coagulation flocculation and sedimentation processes is indicated in Figure 4. In the process, the colloidal particles in the SHWWs' congregated into almost negatively charged larger particles, called flocs. This helps them be stable and tough to aggregate. Therefore, positively charged ions and coagulants to be added in order to disrupt the negatively charged colloidal particles and smooth the process of sedimentation. The process is mostly accomplished by adsorption, using a coagulant chemical composed of metal-organic complexes, and removed by precipitation (Ng et al., 2022). The coagulants used are mainly classified into inorganic, chemical, and natural. The most commonly used metal based inorganic coagulants are aluminum chlorohydrate, aluminum sulfate, poly-aluminium chloride, ferric chloride, and ferric sulfate, with removal efficiencies for COD, BOD, and TSS up to 80% (de Sena et al., 2008). Furthermore, previous reports also showed that the use of poly-aluminium chloride coagulant showed REs of up to 75.0, 99.9, and 88.8% for COD, TP, and TN, respectively, via reducing sludge volume by 42% (de Sena et al., 2008; Mittal, 2006a). The most common shortcomings of this technology are its environmental impacts due to the added chemical coagulants (if chemical coagulants are used), which include diseases upon prolonged use, toxic sludge, and chemical residuals (Abouelenien et al., 2022). Switching to natural coagulants can reduce ecological contagion and threats to well-being due to chemical coagulants. The natural coagulants obtained from plants are consistently found in large amounts or abundantly, accessible, and distinguished as being nontoxic (Ribau Teixeira et al., 2017).

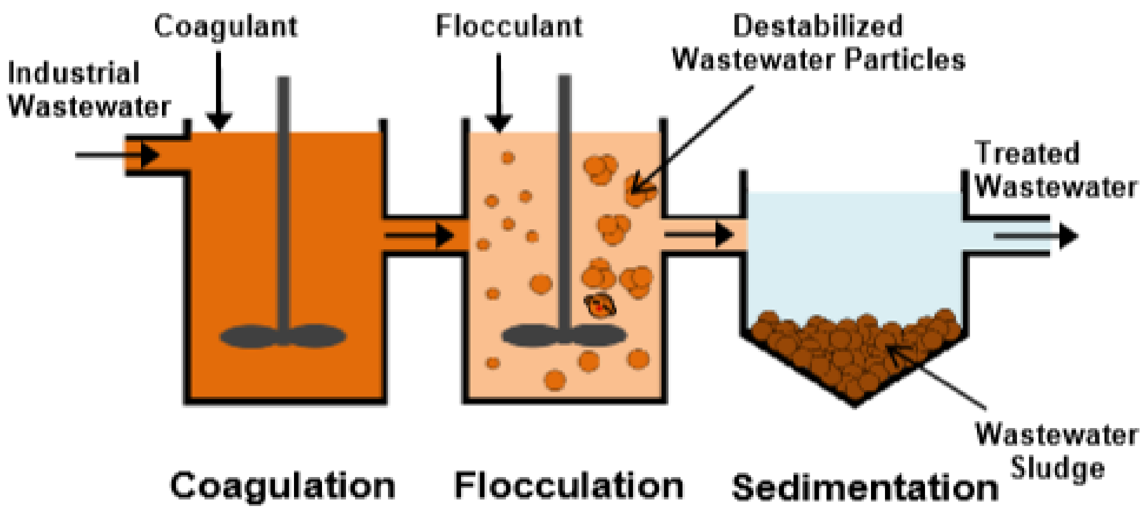


Figure 4: Graphic illustration of coagulation-flocculation and sedimentation processes

Source: (Musa & Idrus, 2021)

2.6.2.2. Membrane bioreactors

Membrane bioreactors (MBRs) have become a well-established, mature, and promising technology, with several full-scale plants worldwide for both the treatment and reuse of industrial and domestic wastewater (Krzeminski et al., 2017; Xiong et al., 2019). In recent decades, compared to conventional biological treatment techniques, membrane technology has attracted more attention in the treatment of wastewater, long activated sludge HRT, small footprint wastewater, and due to governmental concerns to meet stringent water and effluent quality requirements (Feng et al., 2022; Xiong et al., 2019). The most common membrane technologies recently applicable for agro-industrial wastewater treatment are reverse osmosis (RO), ultrafiltration (UF), microfiltration (MF), and nanofiltration (NF). In general, membrane technology can remove particles, colloids, and macromolecules based on the pore size of the membrane and is used to remove organic matter and bacteria from SHWW (Belsky et al., 1999). For example, 2° effluent SHWW pre-treated by activated sludge using RO can achieve a removal efficiency of 97.5, 90.0, 85.8, and 50.0% for TP, TN, COD, and BOD, respectively, and it is concluded that RO is a suitable technique as a post-treatment of SHWW effluent. The main shortcoming of membrane technology is fouling when used for high-strength wastewater, the cost of frequent membrane cleaning, and the fact that it is not a standalone system (it needs other conventional treatment systems) (C. Bustillo-Lecompte and Mehrvar, 2017; Meyo et al., 2021; Xiong et al., 2019). Membrane fouling is a practice in which the foulants progressively accrue on the surface of the membrane, causing clogging of the membrane pore, which necessitates cleaning or replacing the membrane with high operation and maintenance costs (Xiong et al., 2019).

2.6.2.3. Dissolved air flotation

This technology represents or refers to the separation of liquid and solid by the introduction of air from the bottom of the bioreactor. In this technique, a sludge blanket is formed from the light solids together with FOG on the top surface of the wastewater in the reactors and can be continuously removed by scraping the scum. Furthermore, in treating SHWW using DAF, treatment performances can be enhanced by adding flocculants and blood coagulants (polymers), namely aluminum sulfate and ferric chloride, to ease the precipitation and aggregation of proteins other than FOG and light solids floatation (Abouelenien et al., 2022; Bustillo-Lecompte et al., 2016; Bustillo-Lecompte and Mehrvar, 2017; Cvetković et al., 2014; Musa and Idrus, 2021). DAF technology is capable of achieving 70 to 80% BOD, 30 to 90% COD, and moderate to high nutrient removal (Bustillo-Lecompte and Mehrvar, 2017; De Nardi et al., 2011; Mittal, 2006a; Musa and Idrus, 2021). The main drawbacks of DAFs are relatively frequent system malfunctioning or failure, moderate nutrient removal, and lower (inefficient) TSS separation (Tetteh et al., 2017).

2.6.2.4. Electrocoagulation

Electrocoagulation (EC) necessitates the production of in-situ coagulants by electrically dissolving aluminum or iron electrodes. The anode generates metal ions, while the cathode emits hydrogen gas. The hydrogen gas would also assist in the removal of flocculated particles from the air. The electrodes can be configured in either monopoly or bipolar modes.

Plates or scraps, such as those from steel turning and milling made of aluminum or iron, are used as electrodes. For the EC process, electrodes such as Pt, Al, Fe, SnO_2, and TiO_2 are sometimes used, though Fe and Al are the most widely used ones. The metal ions that can form plus three cations (M^{+3}) is usually produced on-site in the EC process using sacrificial anodes that can interact with an OH^- ion in an alkaline medium or hydrogen ions in an acidic medium. Studies have shown that the system can remove organics, pathogens, nutrients, and even heavy metals from SHWW by introducing an electric current without the use of chemicals (Bayar et al., 2011; Emamjomeh and Sivakumar, 2009). In treating SHWW using electrocoagulation, Fe, as an electrode material, is able to remove a maximum of 98% oil and grease, whereas Al is responsible for removing up to 93% COD (Kobya et al., 2006; Musa and Idrus, 2021). Furthermore, slaughterhouse wastewater treatment using electrocoagulation achieved RE (%) of color (96), COD (85), BOD (80), TSS (81), and TN (84), which is mainly attributed to the operating time, electrode material, pH, and current density of the system (Bayramoglu et al., 2006; Kobya et al., 2006; Musa and Idrus, 2021). The EC process is a cutting-edge, efficient, and cost-effective treatment technology that has recently been applied to the treatment of SHWW (Meiramkulova et al., 2021; Musa and Idrus, 2021).

2.6.3. Advanced oxidation processes

Advanced oxidation processes (AOPs) are mainly applied for the removal of non-biodegradable and trace pollutants not removed by biological techniques. It is also employed as both an alternate and accompaniment to optimize SHWW treatment in order to enhance biodegradability, remove non-biodegradable and recalcitrant pollutants, and conceivably reach the discharge limit of treatment for wastewater reuse (Bustillo-Lecompte and Mehrvar, 2017). Studies on AOPs have shown that the highest pollutant removal arises from employing AOPs in SHWW treatment as either a pre-treatment or a post-treatment in combination with other biological treatment technologies. AOPs are effective treatment techniques due to their capability for mineralization rather than degradation (Ng et al., 2022). Consequently, AOPs have emerged manageable enough to be recognized as cutting-edge water reuse, degradation, and pollution control processes, indicating outstanding overall results as complementary treatments (Bustillo-Lecompte et al., 2014).

2.6.4. Biological SHWW treatment technologies

Based on the characteristics of the particular agro-processing industrial wastewater, numerous techniques can be used for its treatment. Thus, biological treatment techniques are thought to be the most appropriate for wastewater burdened with high FOG, organic matter, suspended solids, macronutrients, and pathogens (Bustillo-Lecompte and Mehrvar, 2015b). Primary treatment and physicochemical treatment processes usually do not completely treat SHWW to the degree of discharge limit set by the regulation body. Hence, 2º treatment system should be used for the remaining soluble organic compounds not treated at 1º treatment. The forefront biological treatment techniques used as 2º treatment of nutrient and organic RE of up to 90% are lagoons with aerobic, anaerobic, or facultative microorganisms, trickling filters, activated sludge (AS) bioreactors, as well as constructed wetlands (CWs) (Bustillo-Lecompte & Mehrvar, 2015b).

2.6.4.1. Anaerobic treatment

Anaerobic digestion (AD) is the ideal method for SHWW treatment due to its efficiency in treating high-strength agro-processing industrial wastewater via the production of CO_2 and CH_4 in an anaerobic condition by degrading biodegradable organic contents by anaerobic bacteria. The existence of biogas showed organic matter decomposition by four interconnected biological phases: hydrolysis, acidogenesis, acetogenesis, as well as methanogenesis, and biogas is the result of the methanogenesis process (Gerardi, 2003). AD also involved numerous microorganisms to support the four-step process. The decrease in COD concentration and production of biogas was a signal of the accomplishment of the anaerobic process (Oktavitri et al., 2019). Typical anaerobic processes for the treatment of meat processing or slaughterhouse effluents include anaerobic baffled reactors (ABR), anaerobic lagoons (AL), septic tanks (ST), anaerobic digesters, anaerobic filters (AF), and up-flow anaerobic sludge blankets (UASB) (Mittal, 2006a). AD systems have the benefit or advantage of achieving less sludge production, reducing pathogens, executing higher loading rates, having the lowest energy requirements with possible resource recovery, excellent organic matter, and substantial production of biogas (Aziz et al., 2019, 2022; Odekanle et al., 2020). Nevertheless, anaerobic treatment hardly meets current agro-processing industry effluent discharge limits. Complete stabilization of the organic compounds is difficult due to the high organic strength of SHWW. Therefore, an extra treatment stage or phase is suggested to remove organics, pathogens, and nutrients that remain after anaerobic treatment. In contrast, anaerobic treatment necessitates a higher space and a higher HRT to attain high overall treatment efficiency, disturbing the economic sustainability of anaerobic treatment alone (Chan et al., 2009; Zemene Worku and Seyom Leta, 2017).

2.6.4.2. Aerobic treatment

Aerobic treatment technology, conversely, is a less sensitive process that can efficiently remove nutrients from wastewater with a lower start-up period. Before being anaerobically treated, wastewater is released to waterbodies, where it is aerobically treated to remove the major residual BOD and suspended solids and to oxidize H_2S and NH_3 to less harmful forms called sulfate and nitrate, respectively. In the presence of air, aerobic bacteria consume organic matter for cell synthesis. The organic contents are converted into CO_2, nitrogen, or nitrate ions.

2.6.4.3. Phytoremediation for SHWW treatment

Constructed wetlands (CWs) are an alternative to conventional SHWW treatments and are getting attention as a biological treatment process due to their cost-effectiveness (low operational and management costs), simplicity in design, as well as relatively limited effects on the environment (Alemu et al., 2019; Bustillo-Lecompte et al., 2014; Chan et al., 2009). CW mimics the processes of the natural wetlands for the purification of water while also integrating biological, chemical, and physical processes that occur when atmosphere, soil, microorganisms, plants, and water interact. Sedimentation, adsorption, filtration, biodegradation, photo-oxidation, photosynthesis, and successive nutrients and organics uptake by the system from the interactions (Vymazal, 2014). Previous studies indicate CWs (both vertical subsurface and horizontal flow) have revealed an organic matter removal of 85-99% and TN removal of about 80% though the effluent quality depends on wetland design, wetland plants, microbial community, and operational conditions (Alemu et al., 2019; Bustillo-Lecompte and Mehrvar, 2017; Gutiérrez-Sarabia et al., 2004; Vymazal, 2014). Hence, CWs as a biological treatment technique have become an eye-catching substitute for conventional treatment of SHWW due to their simplicity, low operation and maintenance costs, and limited adverse impacts on the environment. It was indicated that in most cases, CWs were employed as a polishing treatment technique for agro-industrial wastewater treatment, including SHWW (Alemu et al., 2018; Gutiérrez-Sarabia et al., 2004; Sultana et al., 2015; Vymazal, 2014; Zemene Worku and Seyom Leta, 2017).

2.6.5. Integrated or combined treatment methods

This technique integrated the various wastewater treatment techniques for the better pollutant removal as well as value creation along the treatment. In recent decades, this type of wastewater treatment technologies is getting due attention as they treat the agro-processing industry effluent to the required level. This enables the industries to re-integrate into its own production cycle – one of the most important advantages of industrial wastewater treatment. Hence it contributes to the reduction of freshwater resource use as the industry uses it only for the high-quality purposes (Chen et al., 2022).

2.7. Overview of Anaerobic Digestion in Agro-processing industry Wastewater Treatment

Anaerobic digestion is considered a natural process in which numerous microorganisms break down complex organic matter into smaller compounds in an oxygen-free environment. AD is the stepwise breakdown of a decomposable organic substrate by a conglomerate of mutually dependent groups of microorganisms. It undergoes four major biochemical processes: a) hydrolysis: hydrolytic microorganisms decompose polymers into monomers; b) acidogenesis: acidogenic bacteria transform monomers into short carboxylic acid, hydrogen, CO_2, and alcohol; c) acetogenesis: the products of the previous phase are decomposed into acetic acid; and d) methanogenesis: methane is built from the acetic acid (Environmental Protection Agency, 2002; Mekonnen and Hoekstra, 2012). The most important environmental advantage of the AD process in treating SHWW is the production of biogas (a renewable energy alternative), which can be used for fuel in the internal combustion engines, for direct heating, and, under better efficiency, in cogeneration, for electricity production as well (Abuaku et al., 2009; Eslava, 2021). Another factor contributing to the importance of the AD system are governmental policies on financial incentives for renewable energy facilities, climate change, landfills, and an increasing energy need (Michael et al., 2016). The digestate of the AD can be applied to agricultural lands as valued fertilizer, supporting the recycling of the most important nutrients for agricultural production, which marks an extra environmental benefit (Eslava, 2021; Holm-Nielsen et al., 2009; Michael et al., 2016). The biogas produced by the AD process is a blend comprising mainly methane (CH_4 > 60% by volume), carbon dioxide (CO_2 < 40% by volume), and smidgeon of hydrogen sulfide (H_2S), carbon monoxide (CO), hydrogen (H_2), nitrogen (N_2), oxygen (O_2), water vapor (H_2O), or other gases and vapors of various organic compounds. Due to this, literature indicates a rapid increase in AD systems both at large and small scales, though they are facing two major challenges: digestate quality and operational instability (Wang, 2015; Holm-Nielsen et al., 2009).

Anaerobic digestion biotechnologies play an infinite role in the handling or treatment of agro-processing industrial effluents. Agro-processing industrial wastewater decomposable organic pollutants are broken down by microbes under oxygen-free conditions to form CH_4 and CO_2 and have worldwide applications (Liu, 2001). Agro-processing industrial effluent treatment using AD technology is increasingly recognized as a core technique of advanced technology for protection of the environment and resource preservation. When combined with other techniques, it can be used for sustainable biowaste management (Seghezzo, 2004). Enhanced water quality alteration due to large amounts of agro-processing industrial wastewater discharge with a high toxic and nutrient content into the environment has become a problem in developing countries. Sustainable wastewater treatment technologies are attracting the attention of industries and policymakers for their ability to meet pollution standards set by national regulators and preserve natural resources. Adoption of environmentally friendly and economically viable sewage treatment systems is essential for the best possible treatment, recovery, and utilization in both industrialized and developing nations (Dhoble and Ahmed, 2018). In recent decades, advanced AD technologies have gained attention for agro-processing industry effluent treatment via value creation (Alayu and Yirgu, 2018; López and Borzacconi, 2017).

Anaerobic digestion of wastewater treatment techniques can capably biodegrade organic matter, are still being intensely established, and are mainly used in agro-processing industries that produce high quantities of biodegradable effluent. This is mainly due to their numerous advantages over conventional techniques such as biofilters and aerobic-activated sludge processes. In this regard, the most frequently quoted welfares are the effective treatment of high-OLR wastewater, comparatively small space and size requirements, the ability to accommodate high OLRs, noticeably lesser operation and investment costs related to aerobic systems or technologies, and the capability to halt and recommence operation (which is a priority for intermittent or seasonal producers) (Kazimierowicz, 2023).

2.7.1. Biochemical principles or process of anaerobic digestion system

Anaerobic digestion (AD) is considered a natural process in which several microorganisms break down complex organic matter into simpler compounds in an oxygen-free environment. The anaerobic digestion process consists of four major biochemical steps: Hydrolysis: hydrolytic bacteria remove polymers to monomers; acidogenesis: acidogenic bacteria remove monomers to short carboxylic acid, CO_2, hydrogen, and alcohol; acetogenesis: the products of the previous phase are removed to acetic acid; methanogenesis: methane is built from acetic acid (Adekunle and Okolie, 2015; Widiasa, 2011; Merlin Christy et al., 2014).

Hydrolysis

The first phase of the AD process consists of the breakdown and hydrolysis of complex organic polymers such as proteins, carbohydrates, and lipids into soluble compounds such as amino acids (aa), simple sugars, long-chain fatty acids, and alcohols, respectively, which can pass through the cell membrane. This is known as the hydrolytic step. In this phase, enzyme-mediated conversion of higher molecular compounds and insoluble organic materials into soluble and other simple organic compounds, which are suitable for energy and cell carbon sources, is carried out; furthermore, strict anaerobes (clostridia and Bacteroides) and facultative bacteria (streptococci) carry out the transformations (Adekunle and Okolie, 2015; Merlin Christy et al., 2014). The hydrolysis phase is very essential since large organic molecules are just too large to be absorbed directly and utilized by microorganisms as a food source. Hence, certain microorganisms secrete several diverse varieties of enzymes known as extracellular enzymes to complete biodegradation. The extracellular enzymes breakdown the larger materials or molecules into smaller parts that the microorganism can then take into the cell and use as a source of nutrition and energy. Extracellular enzymes such as proteases, lipases, and cellulases are the catalysts in the hydrolytic steps (Batstone et al., 2000). Literature indicates that hydrolysis can be the rate-limiting step of the entire AD process (Fernandes, 2010; Harirchi et al., 2022; Vavilin et al., 2008). The common chemical reactions taking place in this step are indicated in equation 1.

$$(C_6H_{10}O_5)_n + nH_2O \longrightarrow nC_6H_{12}O_6 + nH_2 \qquad (1)$$

Acidogenesis

In the acidogenic step, monomers or soluble compounds produced in the hydrolysis stage are converted into the fermentation of products like hydrogen, alcohols, VFA, ammonium, and carbon dioxide by acidogenic (obligatory and facultative) anaerobic bacteria. Generally, in this stage of the hydrolytic-acidogenic phase, simple sugars, fatty acids, and amino acids are transformed into organic acids and alcohols (Gerardi, 2003). However, in methanogenic environments, in which the electron acceptors are only CO_2 and protons, hydrogen transfer among interspecies microorganisms mainly mediates LCFA degradation. During such conditions, the LCFA compounds are degraded by a mandatory syntrophic proton-reducing acetogenic consortium of bacteria, converting them into hydrogen and acetate, while the methanogenic archaea that consume hydrogen produce methane by reducing bicarbonate (Biebl, 1999; Schink, 1997). Most of the bacteria involved in the hydrolysis step are also able to perform this step, and they belong to the taxonomic groups *Bacilli*, *Bacteroidetes*, *Clostridia*, and *Actinobacteria* (Deublein and Steinhauser, 2011; Krause et al., 2008). The two basic biochemical reactions at the acidogenesis step are indicated by equations 2 and 3.

$$\left.\begin{aligned} &C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2 && (2)\\ &C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O && (3)\end{aligned}\right\} \text{Acidogenesis}$$

Acetogenesis

Acetogenesis is the stage at which the intermediate compounds produced in the acidogenic step are oxidized by proton-reducing acetogenic microorganisms into a proper substrate for the methanogenic step microorganisms, namely hydrogen, acetic acid, and carbon dioxide (Aslanzadeh et al., 2013). Furthermore, in this stage, products that methanogenic bacteria could not directly convert into methane are converted into VFA; methanogenic substrates as well as alcohols are converted also to methanogenic substrates such as H_2, CO_2, and acetate; and VFA containing carbon chains longer than one unit are oxidized into hydrogen and acetate (Gerardi, 2003; Teodorita A. et al., 2008). Therefore, it is crucial for the organisms that undertake the anaerobic oxidation reactions to cooperate with the methane-forming bacteria in the next stage of the AD process. Therefore, during oxidation in the anaerobic system, the final electron acceptors are protons, which leads to H_2 production. This symbiotic liaison condition creates inter-species H_2 transfer (Chandra et al., 2012; Deublein and Steinhauser, 2011; Schink, 1997; Jarvis, 2009). This requirement is attained by syntrophic connotation with hydrogenotrophic methanogenesis that withstands the hydrogen partial pressure at low levels, permitting syntrophic acetogenesis to be active. Therefore, in order to preserve the favorable thermodynamics for the transformation of VFAs to acetate, the hydrogen partial pressure would be set to low. System inhibition may occur if hydrogen is not consumed in the acetogenesis step, causing VFA accumulation, which in turn decreases the pH and inhibits methanogenesis. A special kind of acetogenic microbe is the homoacetogenic, which consumes CO_2 and H_2 to produce acetate. Two examples are the *Clostridium aceticum* and *Acetobacterium woodii* microorganisms (Espinosa, 2011; Hattori, 2008; Weiland, 2010). Equations 4-8 show the basic chemical reactions taking place in the acetogenesis step.

$$\left.\begin{array}{lr}
2CH_3CH_2COOH + 2H_2O \longrightarrow 3CH_3COOH + H_2 + 2CO_2 & (4) \\
2CO_2 + 4H_2 \longrightarrow CH_3COOH + 2H_2O & (5) \\
4CO_2 + 2H_2O \longrightarrow CH_3COOH + 2CO_2 & (6) \\
4HCOOH \longrightarrow CH_3COOH + 2CO_2 + 2H_2O & (7) \\
4CH_3OH + 2CO_2 \longrightarrow 3CH_3COOH + 2CO_2 & (8)
\end{array}\right\} \text{Acetogenesis}$$

Methanogenesis

This is the final stage in the process of AD in which methane is produced by the methanogenic microorganisms using a limited number of substrates such as hydrogen, CO_2, acetic acid, formic acid, methanol, carbon monoxide, and methylamines under strict anaerobic conditions (Aslanzadeh et al., 2013). In the intact AD process, due to the slowest biochemical reaction of the AD processes, methanogenesis is a thoughtful stage (Gerardi, 2003; Teodorita et al., 2008). The methanogenic microbes are divided into two main groups: 1) acetoclastics, methane-forming microorganisms from acetic acid or methanol; and 2) hydrogenotrophic, based on their affinity for the substrate, which produces methane from carbon dioxide and hydrogen. This step of the AD can also be rate limiting if the wastewater is not complex. The most sensitive microbes' group in the AD process, called methanogens, are categorized in one of the orders *Methanopyrales*, *Methanomicrobiales*, *Methanosarcinales*, or *Methanococcales*. *Methanosarcinales* have the microbes with the widest range of substrate consumption. Furthermore, this taxonomic group is allocated into two families called Methanosarcinaceae (higher growth rate) and Methanosaetaceae (more sensitive to pH) (Augusto & Chernicharo, 2007; Deublein and Steinhauser, 2011).

$$CH_3COOH \longrightarrow CH_4 + CO_2 \qquad (9) \qquad \text{Acetate splitting reaction}$$

$$CO_2 + 4H_2 \longrightarrow CH_4 + H_2O \qquad (10) \qquad \text{Carbon dioxide reduction}$$

$$2CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2CH_3COOH \qquad (11)$$

Methanogenesis

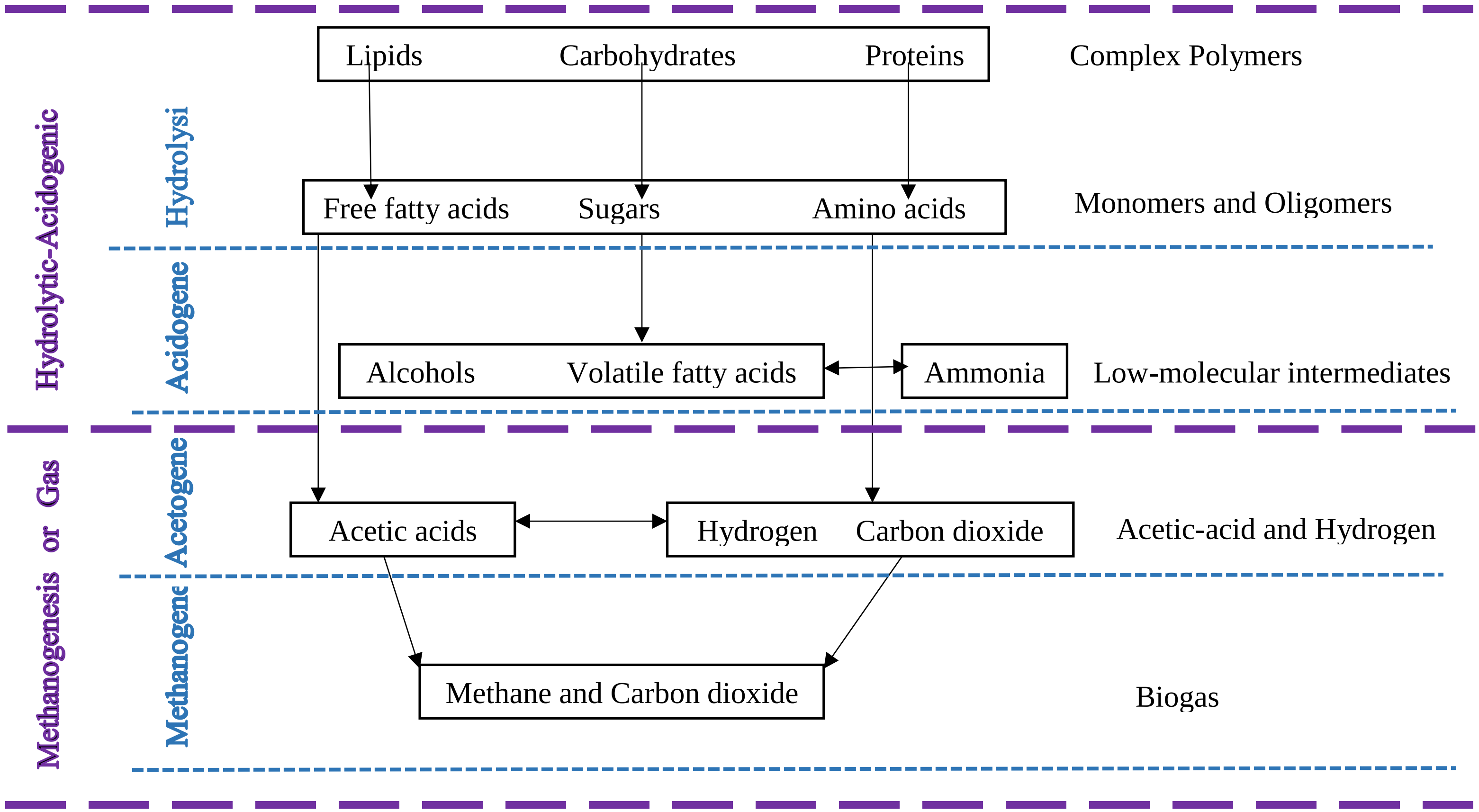


Figure 5: The key process stages of anaerobic digestion pathway

Modified from: (EPA, 2006; Teodorita Al Seadi, Domiik Rutz, Heinz Prassl, Michael Kottner, Tobias Finsterwalder, Silke Volk, 2008)

2.7.2. Factors affecting anaerobic digestion process

2.7.2.1. Environmental factors

The anaerobic digestion process is governed by numerous different parameters for optimal enactment. In order to keep all the microorganisms in balance for the multifarious groups of microbes taking part in methane production, favorable environmental conditions have to be established. AD is a biological process that is strongly influenced by operational conditions or environmental factors like temperature, C/N ratio, pH, alkalinity, and toxicity (Adekunle and Okolie, 2015; Esposito et al., 2012).

2.7.2.2. Operational parameters

Hydraulic retention time and OLR are the main AD system operational parameters. HRT is the average time over which the feedstock organic matter is decomposed into metabolic products like amino acids, monosaccharides, and polysaccharides by a consortium of microorganisms, while OLR is the organic matter amount found in the feedstock and expressed in terms of COD or VS of the feedstock that needs to be handled or treated by a definite size of anaerobic reactor in a given period and directly affects the biogas production and overall system efficiency (Dobre, 2014; Harilal, 2012). In anaerobic systems, the organic matter decomposition is slow, which results in a long time for the digestion to be completed, though it depends on the microbial type and the temperature range (Harilal, 2012). The organic matter content in the feedstock needs to be controlled throughout the experimental period to avoid an organic material overload that may create a shock in the system (Beccari et al., 2005). Even though VFA is an important intermediate compound formed in the metabolic path of methane production, it is vital to regulate the amount of VS or COD added into the reactor to prevent an accumulation of VFA (Babaee and Shayegan, 2011). VS overloading can induce the rapid growth of an acidifying consortium of bacteria at an exponential rate, which may lead to an accumulation of VFA (Harilal, 2012). Shorter HRT or high OLR, cause the active microbial colony to be subjected to washout, but longer HRT or lower OLR, needs a larger digester volume, which will increase the system's operational cost (Nges and Liu, 2010; Yadvika et al., 2004).

2.7.2.3. Anaerobic digestion process inhibitors

In AD process shyness due to the existence of toxic substances such as VFA and ammonia may occur in various intensities, affecting biogas production and organic matter removal upset up-to digester letdown. These inhibitors can be found as constituents of the feedstocks such as sulfur compounds, heavy metals, and antibiotics (Boe, 2006) or produced in the reactor as byproducts of the metabolic activities of microorganisms consortium (VFA and ammonia) (Schon, 2009). Recent papers on AD process indicate most of the inhabitants toxicity or inhibition level is varies due to the significant effect of microbiological mechanisms namely: synergism, antagonism, and acclimation (Chen et al., 2008, Figure 6). Synergism is an escalation of the toxic upshot of one substance in the presence of another while antagonism is a lessening of the toxic upshot of one substance in the presence of another. Acclimation is the capability of microorganism to readjust or reorder to their metabolites to overwhelm the metabolic blockage formed by the toxic or inhibitory substances after the concentration level of the compounds is gently augmented within the investigational environment. The main substances with toxic or inhibitory prospective to AD process are heavy and light metal ions, sulfide, ammonia, and organic substances.

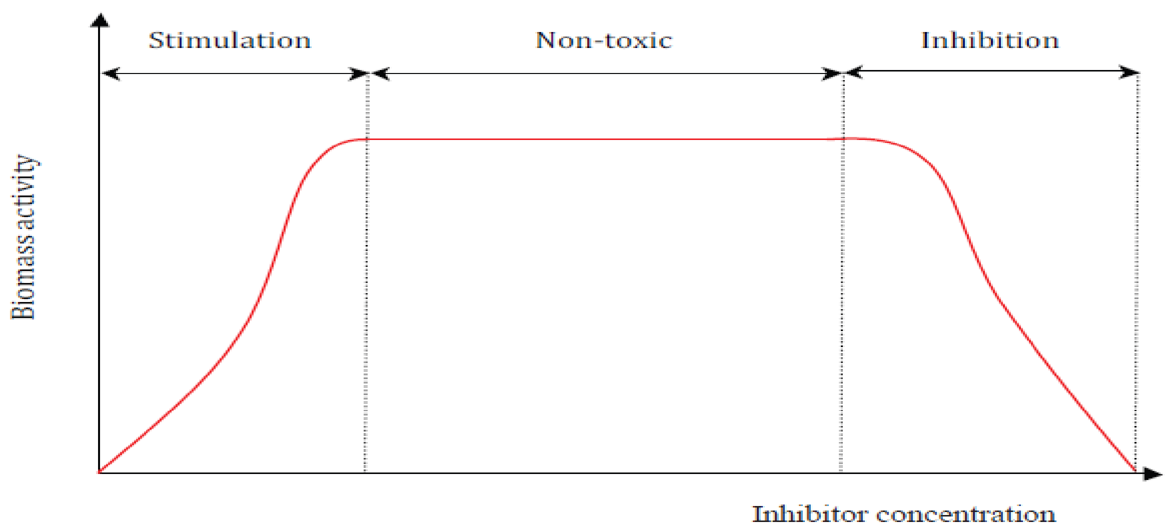


Figure 6: AD inhibition phenomena schematic representation

Source: (Angelidaki and Ahring, 1994; Weinrich and Nelles, 2021)

2.7.3. Anaerobic digestion process performance improvement techniques

Resource recovery from the AD process can be accomplished with four basic metabolic stages: hydrolysis, acidogenesis, acetogenesis, & methanogenesis. Type of substrates buildup-of, volatile fatty acids, and ammonia inhibition are only a few examples of the variables that might have an impact on these AD metabolic phases. The microorganism groups in the AD process are susceptible to a number of variables, including changes in some monitoring parameters, such as alkalinity, pH, and VFAs, as well as some operating parameters (temperature, hydraulic retention duration, substrate composition, and organic loading rate (OLR)). As an outcome, digester disruption in the AD process can be brought on by inadequate substrate composition, unintentional inclusion of harmful substrates, an incorrect loading rate, or a number of other process parameters. Hence, reduced biogas output and perhaps complete process failure are caused by process instability.

The AD method has proven to be the best way to treat bio-waste and is frequently used for industrial, municipal, and agricultural wastes. Therefore, optimization or enhancement of the AD system mainly focuses on setting these parameters at an optimal range to achieve better biogas yield and pollutant removal. Furthermore, numerous researchers have demonstrated that some alterations to the feeding components (co-digestion), the addition of surfactant and nanoparticles to the feeding stream, recycling of sludge or effluent, pond design, pre-treatment, separation of phases, and optimization of operational parameters for HR or MR may help to overcome many challenges that the AD process may encounter (Berhe and Leta, 2018; Berhe Shifare and Seyoum, 2017; Hailu et al., 2020; Lindorfer et al., 2008; Tsegaye and Leta, 2022a, 2022b, 2023; Zemene Worku and Seyom Leta, 2017). Techniques mainly endorsed for the purpose of AD process optimization, better biogas yield, and pollutant removal are: operational parameter optimization (OLR and HRT), addition of additives, feedstock or substrate pre-treatment, and co-digestion (Lindorfer et al., 2008).

Operational parameter optimization, mainly OLR and HRT, is at the forefront of AD process enhancement, as an extreme change in OLR and HRT can adversely disturb the AD system, which may lead to system failure. A significant rise in OLR or decline from the suitable range is a main cause for the VFAs accretion, resulting in a decreased rate of biogas production. Therefore, these operating parameters should be within an appropriate range so that high yields of biogas and organic matter can be achieved in a particular AD system (Shifare and Seyoum, 2017; Hailu et al., 2020; Tsegaye and Leta, 2022b, 2023).

Another technique for AD process optimization or system enhancement is introducing additives or adsorbents. Additives are substances that are used to increase the AD system's performance in order to produce a high level of biogas as well as remove considerable organic matter from the wastewater. These additives can be biological (microbial cultures, zeolite, and crop residues), chemical (inorganic salts), or biological (enzymes) and are mainly used in AD system enhancement or optimization (Gunaseelan, 1987), though their suitability is anticipated to be extremely reliant on the type of feedstock or substrate (Shimizu, 1992; Lin et al., 2013).

Pretreatment is another method used for improving the AD process's performance and stability. In the AD system, hydrolysis phase is the rate-limiting phase. In this phase, the solubilization of particulate matter as well as the biological breakdown of organic polymers into monomers or dimers are taking place. The main pre-treatment methods during the optimization of the AD process reported so far are chemical, biological, mechanical, and thermal, including their combinations. The techniques facilitate the disintegration, which in turn increases the surface area of the sludge cells to release intracellular matter that anaerobic microorganisms easily access and utilize to henceforth or improve the whole AD process (Muller, 2000; Elliot and Mahmood, 2012).

The recycling of the digested slurry or effluent is also another method in which the washed-away microbes are reintroduced into the reactor, thus providing a larger microbial populace that can enhance or boost the performance of the AD system (Santosh et al., 1999). Recycling of the plug flow AD sludge or effluent increases the biogas yield by 20-65% and avoids problems due to the precipitation of substrate, which increases the acidity or alkalinity as well as the toxicity of ammonia (Kanwar and Guleri, 1994; Malik and Tauro, 1995).

The use of fixed film is another technique that increase the efficiency of agro-processing industry wastewater treatment facilities or plants increasing the surface area for the microorganisms to attach to and grow on in the form of a fixed film on the medium used. This further increased the microorganisms' population and their presence in the reactor even after the completely digested substrate slurry or effluent drifted out (Kloss, 1991). Previous study results revealed a 25% reactor volume reduction and a 75% HRT reduction by using plastic support as a fixed film in the AD of cattle manure when compared with a conventional AD system (Weiland and Peters, 1992). This tool is important for holding methanogenic biomass, which enhances methanogenesis and decreases the start-up period in anaerobic biofilms.

Another approach that improves the AD process is co-digestion, in which two or more types of waste are mixed in the same digester. It is considered an attractive option to increase methane yields and organic matter reduction owing to the proven positive synergisms of the substrates, dilution of inhibitors, increased biodegradable organic content, nutrient imbalance, fast acidification, enhancement of the essential moisture content, increased lessening of greenhouse gases in the air, and economic benefits realized because of equipment and cost sharing in the reactor (Mata-Alvarez et al., 2000; Martinez-Garcia et al., 2007; Alatriste-Mondragón et al., 2006). According to Shifare and Seyoum (2017), Castillo et al. (2006), and Hailu et al. (2020), the use of a co-substrate that contains waste with low nitrogen and lipid content boosts the production of biogas due to the complementary traits of both types of waste, thereby lowering issues related to the accumulation of intermediate volatile compounds as well as high ammonia concentrations.

Phase separation, or multi-phase anaerobic digestion, is used in the optimization of AD performance by arranging or separating the reactor configuration. This is to favor the diverse consortium of bacteria in the AD system for optimum biogas production and organic matter removal. The anaerobic microorganisms have varying nutrition requirements and growth parameters, which can be fulfilled by separating the AD processes into different phases rather than conducting the four AD processes in one single reactor (Dhanalakshmi, 2012). Earlier studies indicated that multi-phase bioreactors are required instead of single-phase systems, which could be verified profitably in recent years and can handle high OLR by reducing 90-93.5% SS. Furthermore, a 91.5% biodegradation of sugar beet pulp using a two-phase AD system (with differentiation of hydrolysis-acidogenesis and acetogenesis-methanogenesis stages) was reported in a pilot-scale system. The first reactor serves as a shock absorber, so substantial (high) biogas and organic matter reductions were achieved during the AD of organic biowaste (Hutnan et al., 2001; Bouallagui et al., 2004). Hence, compared to single phase, two-phase had high flexibility, stability, and performance; no risk of breaking down the methanogenesis; a digester environment; and no or less foam formation (Tsegaye and Leta, 2022b, 2023).

2.8. Microalgae for Anaerobic Digester Effluent Treatment and Nutrient Recovery

Anaerobic digestion is among the best broadly used biological processes for converting biodegradable organic matter to renewable energy known as biogas. Literature shows that the efficiency of the AD process in removing TN, organic matter, and TP is limited. Furthermore, the failure of AD systems to meet effluent discharge standards has constrained the application of these systems as a standalone or complete treatment technique (Cormier, 2010; Foster, 2019; Tsegaye and Leta, 2022b; Zemene Worku and Seyom Leta, 2017). Thus, by coupling other biological treatment units to the AD system to minimize or recover organic matter, TN, TP, and other pollutants at the same time to meet the discharge standards in the SHWW treatment, microalgae are viewed as an alternative and sustainable biological treatment that is capable of removing nutrients and other pollutants from most anaerobically treated agro-industry wastewater. Microalgae are photosynthetic microbes which, are commonly occurs in marine environment, utilize water, sunlight, and carbon dioxide for growth (Ruiz-Martinez et al., 2012). Microalgae-based agro-processing industry effluent treatment has various benefits than traditional biological methods. Concurrently microalgae's' eliminate nutrients originated from nitrogen and phosphorus, lessen operating and energy costs, capture CO_2, lessen the formation of sludge, release oxygen sufficient effluent, and produce valuable biomass (Arbib et al., 2013). In addition, microalgae also play a substantial role in the self-purification of polluted waterbodies by naturally assimilating nutrients (nitrogen and phosphorous) found in the agro-industry effluent (Li et al., 2014; Radin et al., 2017).

The microalgae-based agro-industry wastewater treatment is accomplished on the basis of phosphorous and nitrogen assimilation for their growth and then lessening the nutrient content of the final effluent (Mobin and Alam, 2014; Rani et al., 2021). Besides, the biomass formed in the microalgae-based agro-processing industry wastewater treatment could be recycled or used as an input or raw material for crude lipid, from which biodiesel is produced, and many other useful products (carbohydrate and protein) (Chen et al., 2015; Li et al., 2019; Rani et al., 2021). Hence, the agro-processing industry wastewater would be either anaerobically digested or diluted before microalgae-based wastewater treatment. Due to this, anaerobically treated effluent treatment by microalgae was proposed by many researchers without any chemical addition (Ji et al., 2013; Wang et al., 2010; Craggs et al., 1997), as consolidating microalgae-based agro-processing industry wastewater treatment with value-added product such as microalgae biomass generation for bioenergy feedstock. It has been a potentially preferred feedstock for biofuels in comparison to many other agricultural plants (Frac et al., 2010; Miranda et al., 2016). This is predominantly because of its extraordinary photosynthetic efficiency, fast growth, and high biomass production, which allow it to improve strain growth on non-agricultural land and produce other valuable coproducts for instance pigments, carbohydrates, lipids, and proteins (Kightlinger et al., 2014; Li, Chen, et al., 2011; Mubarak et al., 2015). The biomass produced will be further exploited as a source for protein accompaniments and food additives, energy (fuels and biogas), agriculture (soil conditioners or fertilizers), cosmetics, pharmaceuticals, and other valuable chemicals (Cormier, 2010; Mallick, 2002).

The consumption of anaerobically treated agro-processing industries, for example, slaughterhouses, wineries, olive mills, vegetable processing, and brewery wastewater, by microalgae and the utilization of microalgae biomass for biofuel production have been reported by scholars, showing numerous promising compensations (Elalami et al., 2021; Foster, 2019; López-Sánchez et al., 2022; Yirgu et al., 2020). They produce oxygen (O_2), which could reduce the biological treatment aeration cost. Additionally, they can also utilize inorganic phosphorus and nitrogen for their growth, decrease nutrients, and confiscate heavy metals (Cai et al., 2013; Ummalyma et al., 2021). The microalgae biomass will contain both the energy from wastewater (internal energy) and the energy from photosynthesis (solar energy). Microalgae growth under different environmental conditions is indicated in Figure 7. Previous studies found the wastewater treatment plant's (WWTP) energy balance might be enhanced by cultivating microalgae for biofuel (biodiesel, biogas, ethanol, etc.) in the effluent stream. In particular, microalgae are a multifarious group adaptable to several environs, and several species are compatible to succeed in wastewater environments (Cormier, 2010; Ummalyma et al., 2021).

Though many microalgae' species have been utilized in the phycoremediation process of wastewater, *Scenedesmus* sp. & *Chlorella* sp. are the most well-known and comprehensively used microalgae. *Scenedesmus* sp. is known for its wide-ranging temperature and high potential to endure acidic eutrophic water conditions (Radin et al., 2017), while *Chlorella* sp. is among the most broadly used microalgae for nutrient removal because of its capability to grow in high inorganic nutrient concentrations and its appropriateness for use as crop fertilizer, which have been shown to have positive impacts on the environment in regards to soil and plants health (Fornarelli et al., 2017). In addition, among numerous species of microalgae, *Scenedesmus* and *Chlorella* sp. are common in the making of skin health products, used for the, animal feed supplements, fertilizers, and other applications (Foster, 2019; Pulz and Gross, 2004; Radin et al., 2017). Likewise, according to Canovas et al. (1996), Morais et al. (2022), and Masseret et al. (2000), the two microalgae species are generally considered the predominant phytoplanktonic communities in the high-rate algal ponds and oxidation ponds, respectively.

Furthermore, as diverse species have the capability to consume different pollutants found in partially treated agro-processing industry wastewater using anaerobic digestion or other biological treatment techniques, the use of microalgae syndicates tends to be preferred over monocultures because they are apt to be more effective than pure or monoculture (Fornarelli et al., 2017; Taşkan, 2016; Ye et al., 1988). Many studies have been undertaken, mostly using pure or syndicated microalgae cultures for agro-processing industry wastewater nutrient treatment (Asmare et al., 2014). For example, D. Hernández et al. (2016) reported that the microalgae-bacterial syndicate used for the treatment of diluted piggery slaughterhouse wastewater showed a high capacity to remove nutrients and organic matter. In addition, recent studies have also shown that *Chlorella* sp. and *Scenedesmus* sp. have the ability to grow on anaerobically treated wastewater (Ayre et al., 2017; Fornarelli et al., 2017). Furthermore, the use of *Chlorella* sp. culture in agro-processing industry wastewater treatment to lessen nutrients via the production of microalgae biomass is an encouraging approach for the production of biofuels as renewable-energy in addition to the pollutant removal in the wastewater. Hence, the integration of anaerobically treated slaughterhouse wastewater with microalgae-based treatment techniques can deliver nutrient-depleted effluent or water that can be reused on the site provided it meets the standards as well as safe disposal (Foster, 2019; Taşkan, 2016; Zhu et al., 2013).

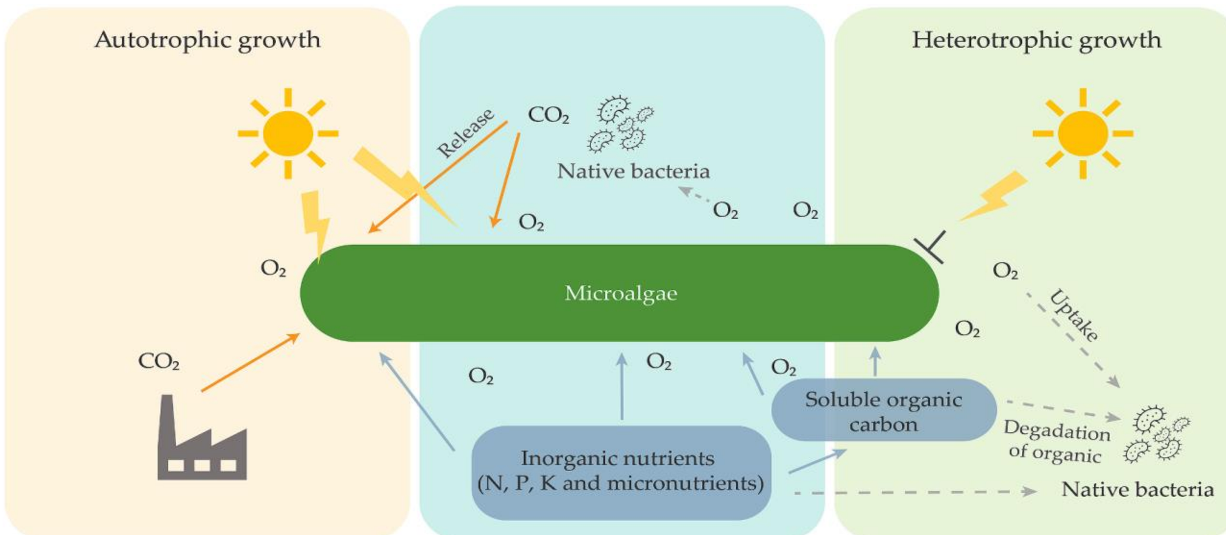


Figure 7: Microalgae growth regimes under different environmental conditions.

Source: (Geremia et al., 2021; López-Sánchez et al., 2022).

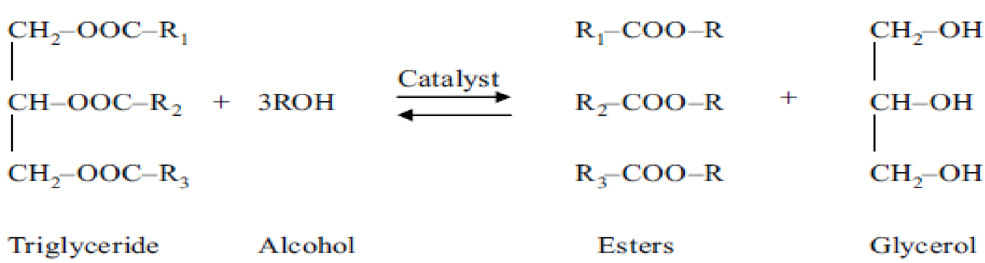
2.9. Biodiesel Production Process

Microalgae grow or are cultivated in areas (marshy areas, brackish water areas, waste water, sewage, or marine environments) where crops do not grow, giving them the unique ability to produce biodiesel in its natural form without the need for complicated, expensive processing methods and technologies [7]. They also do not compete with other food crops for agricultural land. Because microalgae do not compete with other food crops for agricultural land, they may be more advantageous than lignocellulosic biofuel source crops [8]. As a result, recovering oil from microalgae and turning it into biodiesel is expensive. Some extraction techniques, such ultrasonic, catalytic, and supercritical fluid extraction, need huge volumes of solvents and extended extraction times, which can be expensive and energy-intensive [9].

Following the completion of the lipid/oil extraction step, triglycerides (TAGs) and methanol are directly transesterified using a catalyst to make biodiesel, with the byproduct creation of glycerol. The process of transesterification involves reacting an alcohol with the triglyceride molecules found in microalgae oils. According to Indhumathi et al. (2014), the transesterification procedure reduces the oil's viscosity. An enzyme or an acid-base catalyst is frequently used to catalyze the reaction (Utoma, 2013). According to Anna et al. (2013), biodiesel is created by transesterifying TCG with methanol or ethanol, and it can be either methylic or ethylic esters of fatty acids. According to Park et al. (2014), transesterification is an equilibrium reaction that needs extra alcohol to propel it nearly to completion.

According to Li et al. (2011), biodiesel is defined as the mono-alkyl esters of fatty acids (FAMEs) obtained from vegetable or animal fats (Patil, 2011). A new substance known as a fatty acid alkyl ester is created when alcohol and vegetable or animal fat are chemically reacted in the presence of a catalyst, such as sodium or potassium hydroxide. Patil (2011) states that the approximate response proportions are as follows:

Furthermore, the method is really straightforward. As seen in equation 12, transesterfication transforms viscous and raw microalgal lipid fatty acid alkyl esters when a catalyst is present.



equation 12

2.10. Merits of Integrated Biological Agro-Processing Industry Wastewater Treatment

Even though the AD system is efficient in removing most of the organic matter, mainly COD and BOD, in the agro-processing industries effluent, such as slaughterhouses, it is not up to the country's (Ethiopia) industry discharge limit set for nutrients and organic matter (Alemu et al., 2018; Kundu et al., 2013; Tsegaye and Leta, 2022b, 2023; Zemene Worku and Seyom Leta, 2017). Hence, integrating additional biological treatment systems is mandatory. In recent decades, microalgae have been given extra consideration in practical biotechnological research for different aspects such as water, energy, and high-value-added bioproducts. Cultivation of microalgae in anaerobically treated agro-processing industry wastewater and associated feedstocks is a conspicuous research areas or fields inspiring the scholars in the world (Park, 2016; Whitton et al., 2015), because they could be used as nutrient and organic matter sources for microalgae due to the high phosphorus and nitrogen content (Kandimalla et al., 2016), thus decreasing the load on the treatment facility or plant, plus microalgae produce oxygen during photosynthesis that support the oxidative activities of microbial (Nagy et al., 2018), and also remove heavy metals in the wastewater via accumulation (Zeraatkar et al., 2016). The conventional agro-industrial wastewater treatment mechanism is a sequenced process; primary, secondary, and tertiary treatments target removing or reducing solid materials, organic matter, and nutrients and are used to polish the final effluent, respectively, and are referred to as the water line. In a wastewater treatment system, AD processes are categorized by a water line. Though sludge volume, TP, TN, and organic matter reduction can occur, nutrient and organic matter content are above the country's legal threshold in most cases (EEPA, 2005), regardless of their precedence in CE. Therefore, the pairing of another biological treatment system that can potentially reduce the nutrients and organic matter content of AD effluent below the legal requirement or discharge limit level is required. The effluent of AD is a favorable substrate for microalgae cultivation due to its substantial organic matter (carbon) and nutrients (TP and TN) content. It was also reported that microalgae are capable of removing the excess amount of nutrients in the AD effluent, saving operational costs and capacity by producing a value-added product as microalgae biomass. To this end, the CO_2 in biogas from AD is often recycled for microalgae biomass production as a carbon source. On top of this, attempts have been undertaken or made to integrate microalgae into the secondary stage too. Many fruitful experimental findings in flasks, sterile, controlled laboratory conditions using synthetic media, and a photobioreactor (PBR) also outlined the benefits of microalgae cultures used as tertiary or polishing stages (Ge et al., 2013; Morais et al., 2022). In addition, microalgae coupling to wastewater treatment as a tertiary stage is a sustainable alternative due to low GHG emissions compared to conventional systems. Furthermore, it adds value to the waste and the raw material as the sludge produced is a biomass that can be improved for bioproducts that can contribute to the CE (value-added product and re-use), contribute to the clean water scarcity for the coming generation, and reduce various environmental impacts (Boretti and Rosa, 2019; Gururani et al., 2022; Morais et al., 2021).

CHAPTER 3

3. MATERIALS AND METHODS

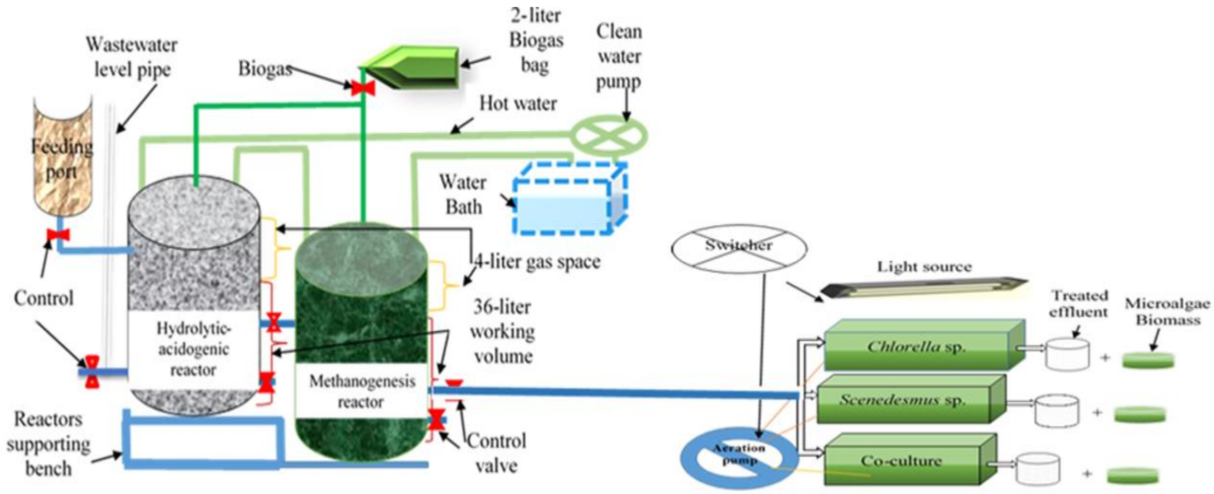
3.1. Experimental design and Setup of the Integrated System

3.1.1. Two-phase AD and microalgae photobioreactor bench-scale experimental setup

The integrated bench-scale biological system consisting of a two-phase anaerobic reactor and microalgae photobioreactor for the treatment of slaughterhouse wastewater was established at the Center for Environmental Science laboratory. Figure 8 shows the schematic diagram of the integrated biological system setup used for SHWW treatment via value-added product production: biofuel (biogas from two-phase AD and biodiesel from microalgae grown to recycle the nutrient) and treated effluent. The two anaerobic sequential batch reactors (ASBR) have a total volume of 40 L (36 and 4 L, working and headspace, respectively) and were connected consecutively with a pipe. The microalgae photobioreactors have a dimension of 30*20*15 cm^3. The complete two-phase AD system had a total volume of 80 L, while the microalgae photobioreactor had a total volume of 9 L. To create an anaerobic condition, the AD reactors were connected consecutively, sealed with a gasket maker, and strengthened by tensioning bolts. The first and second reactors were used as HR and MR, respectively. In order to assure anaerobic conditions and dissolve the gases in both reactors, the reactors were flushed with inert gas (nitrogen gas) before starting the experiment.

The temperature of the reactors was maintained at 37.5 ± 0.1 °C using hot water circulated from a thermostatic water bath (Hangzhou West Tune Trading Co., Ltd, Zhejiang, China). A clean water pump (inGCO Inc., Zhejiang, China) was used to circulate the hot water to maintain the reactors' temperature. The pipes used for hot water circulation were composed of stainless steel inside the reactor to assure the transmission of temperature and ¾ PPR for the extension of the pipe outside the reactor. The two-phase AD reactors have wastewater feeding and discharging, level regulation, and sludge discharging ports with a control valve at each port (Figure 8).

The microalgae photobioreactors were illuminated with two fluorescent lamps (each 20 watts, Philips) with a determined light illumination of 150–300 μmolm^{-2}s^{-1} above the surface of the photobioreactor (Li et al., 2014). Both photoperiod and light illumination were controlled by an electric timer switch at a 12:12 light/dark cycle at almost room temperature. An aquarium air pump (SB-648, China) was used to supply air and CO_2 at a flow rate of 250 mL/minute and 100 mL/minute, respectively. The photobioreactor used to grow microalgae culture was covered from the top by transparent glass to avoid the entrance of potential contaminants.



(a)

(b)

Figure 8: Schematic diagram (a) of the bench-scale two-phase AD experimental setup and (b) photo

3.1.2. Study design

This study was conducted primarily to investigate the potential of two-phase anaerobic digestion coupled with a microalgae photobioreactor for slaughterhouse wastewater treatment and bioenergy (biogas and biodiesel) production. For this, the physicochemical characteristics of slaughterhouse wastewater had been determined as a pre-requisite to feed a two-phase AD system. Then the wastewater was fed to HR for the determination of the optimal operating conditions. Then the HR effluent at its optimal operating condition was fed to MR to determine optimal operating conditions. The two-phase AD system was then evaluated for its bioenergy production and pollutant removal efficiency. Lastly, the effluent from two-phase AD was further treated in microalgae photobioreactors, in which *Chlorella* and *Scenedesmus* species and their co-culture were cultivated for evaluation of pollutant removal efficiency as well as microalgae biomass production.

3.2. Sources of Wastewater and Inoculum

Slaughterhouse wastewater (SHWW) collected from Organic Export Abattoir was used as a feedstock for the purpose of this study. Organic Export Abattoir belongs to the conglomerate that is at the forefront of export-based activities in the Ethiopian meat export market. It has a capacity of slaughtering more than 800 sheep and goats (each) per day and a total of about 400 L of water utilized per sheep or goat. Almost equal volumes of wastewater are discharged into the nearby Modjo River, increasing the pollution load on Koka Lake, the destination of the Modjo River. A composite wastewater was collected using an acidified 20 L polyethylene plastic jerrycan and transported to the laboratory and stored in the refrigerator at 4 °C until fed to the reactor.

Rumen contents (cud) from the same abattoir and Saint George Brewery Factory, a subsidiary company of Brasseries et Glaciers International's (BGI) up-flow anaerobic sludge blanket (UASB) wastewater treatment plant effluent sludge, were used as inoculum for the HR and MR, respectively. For both phases, a 1:1 ratio in volume of the inoculums to the respective feedstock (substrate) was acclimatized for a month and used as a source for the crucial microbes to initiate the experiment. The inoculums were stored in the refrigerator at 4 °C until fed to the reactor.

3.3. Start-up and Operation of the Integrated System

3.3.1. Start-up and operation of hydrolytic-acidogenic reactor

The hydrolysis-acidogenesis reactor operation was fed with a 1:1 ratio of inoculum to slaughterhouse wastewater to initiate the system and was set to be acclimatized for fifteen days. Then the experiment was started by the gradual addition of the slaughterhouse wastewater up to the reactor's working volume (36 L). HR stability and performance evaluation were conducted for HRT of 6, 5, 4, 3, 2, and 1 day and OLR varying from 1789 to 894.49 mg COD/L*day at a mesophilic temperature of 37.5 ± 0.1 °C. The HR experiment started with the highest HRT of 6 days and the lowest OLR of 894.41 mg COD/L*day. The detailed operating conditions of the hydrolytic-acidogenic reactor are presented in Table 2. The optimal operating parameters (OLR and HRT) for the hydrolytic-acidogenic reactor were determined by comparing the performance of the process at six different HRTs. The HR efficiency indicator parameters considered during the optimization were TCOD, SCOD, TVFA (the key parameters as it is the main acid-phase end product indicating the organic matter that has been hydrolyzed), and NH_4^+–N (inhabitant of the reactor or the system). HR's optimum OLR and HRT are set to yield the relatively highest VFA and SCOD concentrations under favorable conditions (Rincón et al., 2008).

Table 2: Operating/working condition of HR and MR at different HRT and OLR.

HRT of HR (days)	OLR (mg COD/L*day)	Flow Rate (L/day)	HRT of MR (days)	Overall HRT (days)	Flow rate of MR (L/day)
6	894.41	6	12	15	2.4
5	1073.27	7.2	9	12	3
4	1341.61	9	6	9	4
3	1788.81	12	3	6	6
2	2683.22	18			
1	5366.43	36			

3.3.2. Start-up and operating conditions of methanogenesis reactor

HR effluent operated at a 3-day HRT or OLR of 1879 mg of COD/L*day previously optimized as shown in Tsegaye D. et al. (2023) was used as a feedstock for the MR phase. Then, HR effluent from a 3-day HRT/OLR of 1789 mg/L*day and the inoculum from Saint George Brewery Factory UASB wastewater treatment plant effluent sludge with a 1:1 ratio based on volume were fed to MR and acclimatized for fifteen days to conduct the optimization of the MR experiment. The MR was optimized at four HRTs (12, 9, 6, and 3 days) and respective OLRs (149, 199, 298, and 596 mg of COD/L*day). The optimization process of MR was also started at the highest HRT or lowest OLR and gradually moved to the lowest HRT or highest OLR. Optimum methanogenesis phase stability (TVFA, TotA, TVFA/TotA Ratio, salinity, NH_4^+–N, and pH) and performance (pollutant removal efficiencies and total biogas and methane production) indicator parameter conditions were evaluated at OLRs of 149, 198, 298, and 596 mg COD/L*day and respective HRTs of 12, 9, 6, and 3 days.

Feeding both reactors (HR and MR) was done once a day. MR effluent was discharged 1st and fed to a microalgae photobioreactor or stored in the collection tank, followed by an equal amount from the HR by opening the control valves, and finally feeding the same volume of SHWW to the HR manually in order to keep the semi-continuous mode. The volume discharged or fed to or from the reactors was calculated based on the OLR or HRT and the volume of the reactors in both phases.

HR and MR effluent samples were taken every three days for each HRT and OLR to study the trends of the parameters of TVFA, NH_4^+-N, TCOD, SCOD, TotA, salinity, and pH, during the course of the experimental process. Biogas production and methane content were measured every three days. The average MR effluent values of all the parameters under study were obtained from the readings taken at steady-state conditions. The steady-state condition was assumed to be achieved when the concentrations or values of the parameters under study were within 10% variation, and five consecutive measurements were taken after the steady-state condition was realized. Finally, the performance evaluation of the two-phase AD system in terms of biogas production, methane yield or content, and reduction of organic matter, TN, and TP was evaluated based on readings and analyses of the parameters done at the steady-state condition. Biogas volume, methane yield, and removal efficiencies of the pollutants were used for the evaluation of the two-phase ASBR system. The effluent of the optimized two-phase AD was evaluated for its performance in terms of pollutant removal efficiency and biogas production, as well as its composition, which is further fed to the microalgae photobioreactor used as a polishing step.

3.3.3. Start-up, and operation of microalgae photobioreactor

3.3.3.1. Microalgae sample collection, isolation and cultivation

The microalgae (*Chlorella* and *Scenedesmus* species) used in the study were collected from a local freshwater body, Awassa Lake, Awassa, Ethiopia, and transported to the laboratory. The collected sample was relocated to the closed flasks on arrival in the laboratory to avoid contamination, enriched in BBM, and then incubated using petri dishes for 5 days at 22 ± 3 °C with a radiance of about 40–50 $\mu molm^{-2}s^{-1}$, as described in Chalivendra (2014). The two microalgae in the freshwater body were grown under controlled circumstances, colonized, and picked from the plate based on their morphology and color using agar plating with pipetting and serial dilution combinations based on their morphology using a light microscope. The picked or isolated colonies were again cultured in fresh BBM (the constituents of BBM are shown in appendices) until the required amount was obtained and kept in the refrigerator at 4 °C till used for the experiment for the treatment of two-phase anaerobic reactor effluent). The BBM solution was always autoclaved for 15 minutes at 121 °C before use. The two microalgae isolated were checked against the previously identified species using a light microscope based on their size, color, morphology, and shape, as indicated in Andersen and Kawachi (2005), Dolganyuk et al. (2020), K. Lee et al. (2014), Kumaran et al. (2023), and Ogbonna (2015).

3.3.3.2. Start-up and operation of microalgae photobioreactor

As indicated in Figure 8, the MR reactor was integrated into three microalgae photobioreactors for the evaluation of nutrient and organic matter removal. Two microalgae's (*Chlorella* species and *Scenedesmus* species) and their co-culture were used for the nutrient removal of the two-phase AD effluent as a polishing step. The two microalgae's (*Chlorella* species and *Scenedesmus* species) and their co-culture were evaluated for organic matter, NH_4^+-N and PO_4^{-3}–P removal. The two-phase effluent was filtered using 21-mm Whatman filter paper before being fed to the photobioreactors. The photobioreactors were illuminated with 2 fluorescent lamps (each 20 watts, Philips) with a maximum light illumination of 150-300 $\mu molm^{-2}s^{-1}$ above the surface of the photobioreactor, as indicated in Li et al. (2014). Both the photoperiod and light illumination were controlled by an electric timer switch at a 12:12 light/dark cycle at room temperature. An aerator was used to supply air and CO_2 at a flow rate of 250 mL/minute and 100 mL/minute, respectively. The filtered AD effluent was added to the working volume of the photobioreactor (8 mL). Regarding the mixture microalgae culture, an equal volume of individual microalgae was mixed and added to the photobioreactor, and the MR effluent feeding was followed in the same way for the individual microalgae. The microalgae cultivation was conducted for 20 days. After the twenty-day incubation period, or end of the experiment, the microalgae's biomass was harvested by evaporation. The effluent from the photobioreactor was analyzed for TCOD, TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P every four days to investigate the trends in the concentrations. The microalgae's pollutant removal efficiency was then evaluated. At the end of the experiment, or when the exponential algal growth phase reached its peak, the microalgae were harvested by evaporation. The microalgae biomass harvested was air-dried first followed by oven at 60 °C by evaporation. Then it was placed in dissector until constant weight achieved and ready for its crude lipid content determination.

3.4. Reactors Operating, Performance, and Stability Indicator Parameters

3.4.1. Reactors operating parameters

Hydraulic retention time

The HRT is the time the feedstock/substrate spent in the specified bioreactor so that the degradation of the organic matter is complete. It is calculated as indicated in (Ekama and Wentzel, 2008) using the equation 12.

$$\mathrm{HRT} = \frac{\mathrm{V}}{\mathrm{Q}} \tag{12}$$

Where, V and Q are the reactor volume (m^3) and the influent flow rate (m^3/day)

Organic loading rate

OLR is the organic matter concentration fed into the reactor in a definite period. In this study, the OLR was calculated according to (Ekama and Wentzel, 2008) based on equation 13.

$$\mathrm{OLR} = \frac{\mathrm{Q}*\mathrm{S}}{\mathrm{V}} \qquad (13)$$

Where, Q, S, and V are wastewater flow-rate (m^3/day), the inflow COD concentration (g/m^3) and the working volume of the reactor (m^3), respectively.

3.4.2. Reactor's stability parameters

Degree of acidification and hydrolysis

Degree of acidification is a parameter used to determine the degree of completion of acid fermentation, which used to evaluate the performance of the reactor was calculated equation 14.

$$\mathrm{DA}\ (\%) = \frac{\mathrm{Sf}}{\mathrm{Si}} * 100 \qquad (14)$$

Where, DA represents degree of acidification, S*i*: initial feedstock concentration expressed in mg/L of COD, S*f*: Net VFA produced (Final-initial) expressed as theoretical equivalent of COD concentration in mg/L. The COD equivalent of each VFA: Acetic acid (1.066), Butyric acid (1.816), Propionic acid (1.512), Valeric acid (2.036) and Caproic acid (2.204) (Yilmaz and Demirer, 2008).

DH defines the degree of solubilization of organic matter and is calculated according to (Demirer and Chen, 2004) as per equation 15.

$$\mathrm{DH}\ (\%) = \frac{\mathrm{Ss}}{\mathrm{Si}} * 100 \qquad (15)$$

Ss is the HR effluent SCOD measured and Si represents the initial Feedstock concentration expressed in mg/L of TCOD (tCODi).

3.4.3. Reactor's performance efficiencies

Removal Efficiency

The systems, and specific reactors performance in terms of RE (%) was determined as demonstrated in Abdelhakeem et al., (2016) using the formula sown by equation 16 for the parameters; BOD_5, TCOD, SCOD, TN, NH_4^+-N, NO_3–N^-, TP, PO_4^{-3}–P, S^{-2}, and SO_4^{-2}.

$$\text{RE}\ (\%) = \frac{\text{Ci}-\text{Cf}}{\text{Ci}} \text{x}100 \qquad (16)$$

Where RE = removal efficiency, Ci and Cf = initial and final reactors effluent concentrations

Methane yield

Specific methane yield (SMY) is the amount of methane formed comparative to organic matter removed or added to anaerobic digester. The SMY during the experimental study was calculated using the formula indicated in Angelidakia et al., (2008) using equation 17 and expressed in mL/mg COD removed.

$$\text{MY} = \frac{\text{VCH4}}{\text{COD consumed}} \qquad (17)$$

Where, MY, V_{CH4} and $COD_{consumed}$ are the specific methane yield (mL/g COD consumed), volume of methane production and $COD_{consumed}$ in mg/L.

Microalgae biomass yield and productivity

Microalgae biomass yield and productivity were determined according to Lee et al. (2013) by measuring the optical density (OD) at 680 nm (OD680). The OD of microalgae was measured using a Jenway spectrophotometer.

$$\text{Microalgae biomass concentration} = 0.95 \times \text{OD680} - 0.04 \qquad (18)$$

The biomass productivity during the fermentation (experimental) period, PB (mg/ L * day), was calculated by using equation 19 (Zhu et al., 2013).

$$Microalgae\ BP = \frac{Ct - C0}{Tt - T0} \tag{19}$$

Where C_t and C_0 are microalgae biomass concentration at time (t) and the initial concentration at time (0) in mg/L*d.

The absorbance (ABS) of microalgae wastewater suspension was measured at wavelength of 680 nm against blank (distilled water) using a spectrophotometer. The measured absorbance was used to calculate the microalgae's growth rate (Gr) using the following equation 20.

$$\mathrm{Gr} = \frac{\ln ABS\ (t) - \ln ABS\ (0))}{t} \tag{20}$$

Where, ABS (0) and ABS (t) are absorbance of the suspension at time 0 and t.

Pollutant removal rate

The nutrient removal rate was calculated based on equation 21.

$$Rr = \frac{Ct - Co}{Tt} \tag{21}$$

Where Rr is the nutrient (COD, N, and P) removal rate, Ct and Co represents the nutrient concentration at time (t) and initial time (days), respectively.

3.4.4. Biochemical composition analysis of microalgae biomass

Crude Lipid Extraction

The microalgae's crude lipid content was measured at various growth periods for the determination of lipid productivity. The microalgae crude lipid extraction was done based on Bligh and Dyer's procedure (Bligh and Dyer, 1959). Accordingly, 50 mg, 12.5 mL, and 25 mL of air and oven dried microalgae biomass, chloroform, and methanol (a 1:2 chloroform and methanol ratio in volume) were added to a flask, respectively, followed by the addition of 12.5 mL of water, carefully mixed for about 25 minutes with shaker adjusted to 160-rpm, and then placed overnight in room temperature. Isolation of the biomass from the mixture solution was done with Whatman-number-one filter-paper. Sodium chloride (1%) was added after the formation of the layer to wash the lipid-containing layer of chloroform, which was lastly, isolated of the water-methanol mix stratum by separatory-funnel. The lipid part then poured into the pre-weight beaker and dried (water or moisture removed from the lipid) at 60 °C in an oven up to constant weight was realized and for subsequent biodiesel analysis.

$$\text{Crude lipid } (\%) = \frac{\Delta W}{W} * 100 \tag{22}$$

Where, ∆W is weight loss after extraction of lipid with chloroform and methanol followed by drying, W is original weight of microalgae biomass sample taken.

3.4.5. Biodiesel production

A 250-mL round-bottom volumetric flask with a three-neck and a condenser were used. The reaction temperature was monitored using a hot plate. The crude microalgae lipid and pre-heated methanol were poured into a flask and stirred at 1500 rpm with the magnetic stirrer for about 3 hours on a hot plate subjected to heat at 60 °C. After a 3-hour reaction, the microalgae's oil and methanol mixture was transferred into a separating funnel, shaken, and allowed to stand overnight. The biodiesel was collected using a pre-weighed beaker from the three phases: solid catalyst (0.1% KOH), glycerol, and biodiesel, heated to 80 °C to remove any unreacted methanol, purified, weighed, and the result presented in percent.

3.5. Laboratory Sample Analyses

The physicochemical characteristics of SHWW, HR, MR, and microalgae photobioreactor effluent samples used for the determination of stability, performance, and efficiency indicating parameters before and during the experimental period were all analyzed using standard methods. Accordingly, TCOD, SCOD, BOD_5, TN, NH_4^+-N, NO_3^-–N, TP, PO_4^{-3}–P, S^{-2}, and SO_4^{-2} were analyzed following HACH instructions using a spectrophotometer (HACH DR/3900 HACH, Germany). Oxidation-reduction potential (ORP) and pH were analyzed using a pH meter (JENWAY, Manchester, UK). Resistivity, salinity, electrical conductivity (EC), and total dissolved solids (TDS) were analyzed by a multi-meter (EUTECH Instruments, Madrid, Spain). TS and VS were analyzed according to Standard Methods for the Examination of Water and Wastewater (APHA, 2017) using an oven at a temperature of 105 ℃ and 550 ℃, respectively. TVFA and TotA were analyzed using titration according to the APHA (2017) standard method. Total biogas production was measured by sucking the biogas collected in a 2-L glucose bag using a 100-mL airtight syringe. The biogas composition was measured using a gas analyzer (Geotechnical Instrument Gas Analyzer, Leamington Spa, UK). Microalgae biomass production was determined according to Lee et al. (2013) by measuring the optical density (OD) at 680 nm (OD680) using a Jenway spectrophotometer (model). Microalgae biomass production was determined according to Lee et al. (2013) by measuring the optical density (OD) at 680 nm (OD680). The OD of microalgae was measured using a Jenway spectrophotometer.

3.6. Data Analyses

The data generated from the analysis during the study were entered into the MS Excel spreadsheet based on the objectives set for further statistical analysis. The statistical analysis for mean, standard deviation, correlation, and one-way analysis of variance (ANOVA) at 95% confidence intervals followed by a post hoc analysis was also performed using the Excel Statistical Package to compare the performance of the HR and MR at different HRTs, and microalgae's photobioreactor performance evaluation and Origin 2022 software were used to draw figures. All the sample values analyzed for the parameters under study were taken in at least triplicate to ensure the reproducibility of the experiment.

CHAPTER 4

4. RESULTS AND DISCUSSIONS

4.1. Characteristics of Abattoir Wastewater

The main characteristics of the Organic export abattoir wastewater are presented in Table 3. The pH of the organic export abattoir effluent was nearly in the neutral pH range (6.80-7.39). The temperature and ORP were in the range of 28.9-30.5 ℃ and -62.50 to -101.10 mV, respectively. The EC, TDS, salinity, and resistivity of the slaughterhouse wastewater used as feedstock during the study were between 1345-1970 ppm, 1160-1688 ppm, 1210-1628 ppm, and 290.90-425.00 Ω, respectively. As indicated in Table 3, the TCOD value of slaughterhouse wastewater was 5366.43 ± 83.80 mg/L, of which about 60-90.24% (4842.21 ± 83.81 mg/L) was in soluble form and the other in suspended particulate matter. The average concentrations of TVFA and NH_4^+ were 816.60 ± 38.67 and 338.40 ± 58.13 mg/L, respectively. The dissolved ions (NH_4^+–N, SO_4^{-2}, and NO_3^-–N) in slaughterhouse wastewater are primarily responsible for the high EC, salinity, and TDS contents obtained, which is also consistent with the previous finding (Padilla-Gasca et al., 2011). This high organic matter content in terms of TCOD is mainly due to the grease and fat, blood, manure, and indigested food content in the slaughterhouse wastewater (Bustillo-Lecompte and Mehrvar, 2015b; González-Fernández et al., 2016). Mulu and Ayenew (2015); Ren et al. (2014); and Zemene Worku and Seyom Leta (2017) reported COD values of 4752.66 ± 1156.27 mg/L and 6942.59 ± 152.98-7079.69 ± 226.89 mg/L, respectively. Furthermore, slaughterhouses and meat processing facilities generates a large volume effluent with organic matter measured as TCOD between 500 and 15900 mg/L (Bustillo-Lecompte and Mehrvar, 2015b), TVFA between 435 and 1197 mg/L (Tsegaye and Leta, 2022b), and NH_4^+ between 186 and 6407 mg/L (Anyango et al., 2022; Zemene Worku and Seyom Leta, 2017), which coincide with the present study results. This high concentration of organic matter necessitates a greater amount of oxygen to be oxidized into carbon dioxide and water, which may contribute to an increase in the COD and BOD of the receiving water body (Abdullahi et al., 2015).

The average TN, NH_4^+–N, NO_3^-–N, TP, PO_4^{-3}–P, & SO_4^{-2} contents of slaughterhouse wastewater were 1198 ± 12, 338.4 ± 58.13, 453.58 ± 81.47, 105 ± 1215, 62.333 ± 15.948, & 410.33 ± 15.61 mg/L, respectively (Table 3). The TN level of the slaughterhouse obtained is also comparable with the previous study's results (1050-1536 mg/L) (Adou et al., 2020; Kundu et al., 2013). The slaughterhouse wastewater NH_4^+–N, NO_3^-–N, and TP concentration consistent with this study findings varying between 86-640, 113.38-186.57, and 135.01-171.01 mg/L, respectively, were also addressed by Anyango et al. (2022) and Zemene Worku and Seyom Leta (2017). The BOD/COD and COD/SO_4^{-2} ratios of the organic export abattoir wastewater were 0.46 and 13, respectively, which indicate the biodegradability of the wastewater. SHWW BOD/COD, COD/SO_4^{-2}, SO_4^{-2}, and S_2^{-2} values in the range of 0.4-0.5, 11.9-12.4, 180-400 mg/L, and 0.24-1.83 mg/L, respectively, were reported in previous studies, supporting the current finding (Widiasa, 2011; Zemene Worku and Seyom Leta, 2017).

The average metal concentration in the SHWW is shown in Table 3. The concentrations of Fe, Cu, Zn, Mg, Ni, Mn, Na, K, and Ca in this study were in the range between 1.08, 0.01-0.05, 0.09-0.71, 25-69, 0.05-0.19, 0.18-0.68, 324.83-594.83, 0.01-0.05, and 493.79-821.55 mg/L, respectively. The slaughterhouse wastewater metal concentration values obtained were in range with the previously reported values as well as in the range that does not affect the anaerobic digestion process (Abraham, 2015; Feijoo et al., 1995; Minale and Worku, 2014). Both heavy and light metals are crucial for microorganisms in trace amounts to catalyze enzymatic anaerobic reaction processes and transformations (Matheri et al., 2016). They are required to maintain bacterial metabolism and growth, but their deficiency as trace metals negatively affects anaerobic microorganisms' activity in bioreactors. If found above the required level, they also affect anaerobic digestion by stimulating, inhibiting, or blocking the enzyme function (Fermoso et al., 2009). Furthermore, their toxicity is non-specific, reversible, and less frequent if competing with the substrate. An experimental study on the relative toxicity or inhibition of heavy metals on methanogenic activity indicates lead and zinc are the most and least toxicants, respectively. Heavy and light metals in optimal concentrations increase the degree of anaerobic microorganism growth, and almost all the metals analyzed in this study were found below the tolerable limit for the AD system, as metal concentrations beyond the tolerable limits were not anticipated in the SHWW due to the nature of inputs in the slaughtering process (Matheri et al., 2016). In general, both heavy and light metals do not cause significant problems in most anaerobic digestion process, as their concentrations are kept low as a result of sulfide and carbonate precipitation (Boe, 2006).

Table 3: Slaughterhouse wastewater characteristics

Parameter	**Unit**	**This study** Mean value ± SD	**Values indicated in literature**	**Reference**
pH	-	7.06 ± 0.30	6.95-7.30	Abdullahi et al., 2015; Abraham, 2015; Adou et al., 2020; Anyango et al., 2022; Budiyono, Widiasa, Johari, 2011; Bustillo-Lecompte & Mehrvar, 2015b; Feijoo *et al.*, 1995; González-Fernández et al., 2016; Kundu et al., 2013; Minale and Worku, 2014; Padilla-Gasca et al., 2011; Ren et al., 2014; Zemene Worku and Seyom Leta, 2017
Salinity	mg/L	1208.98 ± 43.48		
EC	μS/cm	1346.00 ± 46.88	1696-2000	
Resistivity	Ω	458.46 ± 16.75		
TDS	mg/L	1170.74 ± 40.84	2705-3110	
ORP	mV	-80.50 ± 18.13		
TVFA	mg/L	816.60 ± 38.67		
TCOD	mg/L	5366.43 ± 83.80	500-16000	
SCOD	mg/L	4842.21 ± 83.81	5610-7630	
TN	mg/L	1198.45 ± 12	565 -1536	
NH_4^+-N	mg/L	338.40 ± 58.13	223-242	
SO_4^{-2}	mg/L	410.33 ± 15.61	275-612	
Sulfide	mg/L	2.04±0.24	0.1-3.6	
Fe	mg/L	2.33 + 1.25	14.55 ± 0.49	
Cu	mg/L	0.03 ± 0.02	0.15	
Zn	mg/L	0.40 ± 0.31	0.28 ± 0.02	
Mg	mg/L	47 ± 22	74.13-96.8	
Ni	mg/L	0.12 ± 0.07	ND	
Mn	mg/L	0.43 ± 0.25		
Na	mg/L	459.33 ± 135.50	107-1287	
K	mg/L	0.03 ± 0.02		
Ca	mg/L	657.67 ± 163.88	727-820	
FC	MPN/100ml	$1.53 * 10^6 \pm 0.85 * 10^6$	$1.70 * 10^6$-$2.1* 10^7$	
TC	MPN/100ml	$7.73 * 10^4 \pm 7.12 * 10^4$	$2.2 * 10^5$-$3.6*10^6$	

In general, the high decomposable organic matter content (BOD:COD ratio), TN, TP, high ions for buffering the system, and micronutrient (metals) content, as well as the pH near neutral of SHWW, show its suitability for the AD process or system.

4.2. Effect of Operational Parameter on Hydrolytic-Acidogenic Reactor

4.2.1. Effect of HRT and OLR on reactor stability and performance

The digestion of the anaerobic process begins with the microbial hydrolysis of the feedstock material in order to breakdown polymers such as carbohydrates, proteins, and fats and make them available for the microorganisms. The organic polymers undergo microbial degradation, which results in the production of soluble sugars. According to Wang et al. (2015), under optimal wastewater treatment conditions, anaerobic digestion is much more susceptible to changes in reactor operating conditions than aerobic digestion for the same degree of factor devotion. The average steady state of the stability and performance of the system is shown in Table 5 and discussed in subsequent sub-sections in detail.

4.2.1.1. Effect of OLR and HRT on salinity, EC, TDS and resistivity

In the anaerobic digestion process, salinity and TDS are the buffering capacity enhancers. The average salinity and TDS values of HR effluent are shown in Table 5 and the variations during the experimental period are indicated in Figure 9, 12, and 14 for the parameters under study at different HRTs. The average (mean ± SD) of salinity, TDS, EC, and resistivity of the HR ranged from 1785.27 ± 18.54 mg/L at HRT 4 days to 1538.33 ± 16.04 mg/L at HRT 2 days, 1726.93 ± 53.74 mg/L at HRT 5 days to 1469.80 ± 17.75 mg/L at HRT 2 days, 1964.80 ± 80.26 S/cm at HRT of 5 days to 1674.07 ± 174.68 μS/cm at HRT of 2 days, and 341.66 ± 41.01 Ω at HRT of 2 days to 289.43 ± 12.49 Ω at HRT of 5 days, respectively. The correlation statistical analysis of pH, ORP, EC, TDS, salinity, and resistivity was also computed. Table 4 indicates the correlation matrix of some optimized parameters in the present study. Accordingly, pH, ORP, salinity, EC, and TDS have strong positive and negative correlations with each other and resistivity, respectively (Table 4).

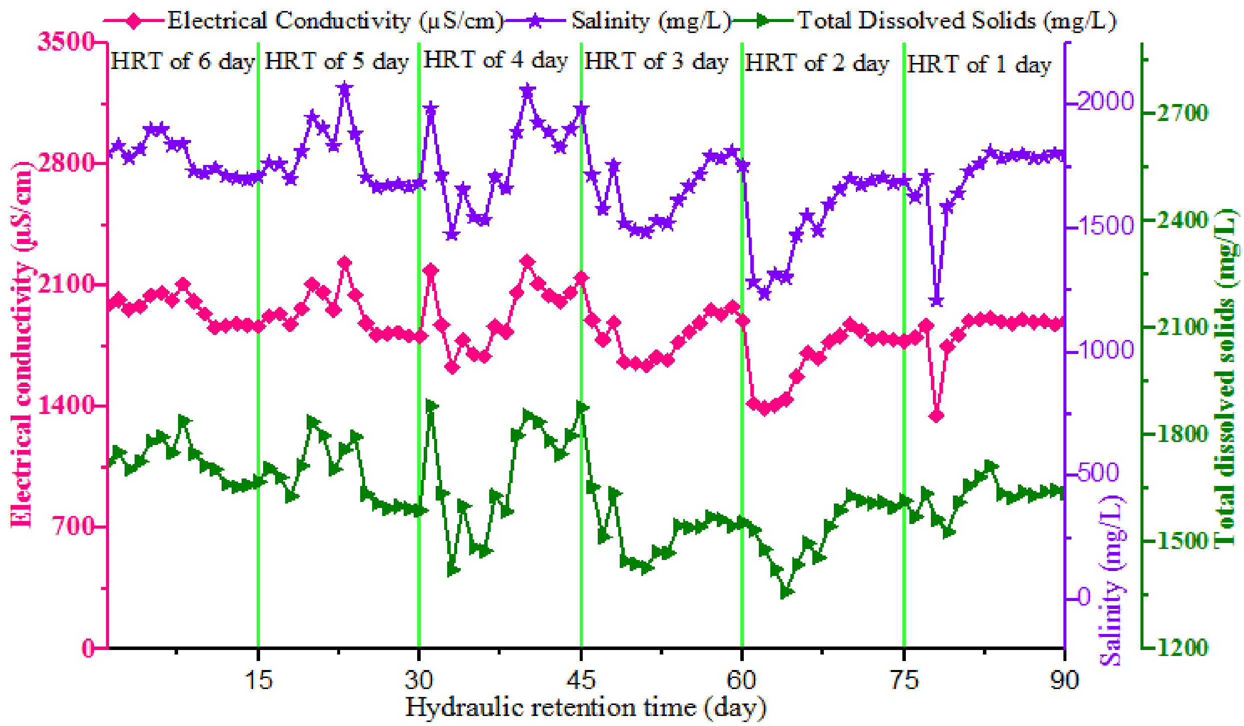


Figure 9: Variation in TDS, salinity and EC of hydrolytic-acidogenic reactor at different HRT

Table 4: Correlation matrix of some parameters of hydrolysis-acidogenesis reactor

Parameter	pH	ORP	EC	TDS	Salinity	Resistivity
pH	1					
ORP	0.99	1				
EC	0.9	0.94	1			
TDS	0.86	0.91	1	1		
Salinity	0.9	0.93	0.99	0.98	1	
Resistivity	-0.87	-0.9	-1	-0.99	-0.98	1

Table 5: The average stability and performance of hydrolytic-acidogenic reactor at steady state for different HRT and OLR

	HRT (days)					
Parameter	**6**	**5**	**4**	**3**	**2**	**1**
pH	6.81 ± 0.14	6.34 ± 0.17	6.00 ± 0.18	5.78 ± 0.32	5.77 ± 0.25	5.60 ± 0. 27
Salinity (mg/L)	1784.67 ± 12.26	1784.27 ± 71.04	1785.27 ± 18.54	1650.40 ± 12.22	1538.33 ± 16.04	1710.00 ± 16.65
EC (μS/cm)	1934.47 ± 13.43	1964.80 ± 80.26	1950.20 ± 19.85	1809.73 ± 12.07	1674.07 ± 17.68	1835.87 ± 14.18
Resistivity (Ω)	292.23 ± 16.00	289.43 ± 12.49	296.43 ± 29.83	318.01 ± 22.49	341.66 ± 41.01	313.17 ± 32.41
TDS (mg/L)	1697.27 ±11.48	1726.93 ± 53.74	1702.00 ± 17.20	1576.47 ± 11.60	1469.80 ± 17.75	1602.33 ± 13.24
ORP (mV)	-81.21 ± 3.36	-82.58 ± 3.49	-81.69 ± 5.25	-82.25 ± 6.54	-78.59 ± 3.21	-79.03 ± 3.15
TVFA (mg/L)	1084.83 ± 14.37	1006.42 ± 30.35	1155.92 ± 16.20	1176.50 ± 81.66	1006.42 ± 30.35	996.75 ± 14.60
TCOD (mg/L)	4924.47 ± 25.79	4799.33 ± 37.49	4913.67 ± 22.79	4934.6 ± 25.24	4721.07 ± 67.35	4821.73 ± 30.37
SCOD (mg/L)	2324.80 ± 25. 16	2359.00 ± 40.79	2483.73 ± 47.72	3430.2 ± 80.44	3106.87 ± 72.65	2084.4 ± 71.00
NH_4^+–N (mg/L)	278.67 ± 47.25	281.20 ± 8.79	319.08 ± 40.21	369.46 ± 11.28	369.33 ± 51.75	346.42 ± 40.67

4.2.1.2. Effect of OLR and HRT on pH

The average values and trends in pH variation of the HR during the study period at different OLRs and HRTs are shown in Table 5, Figure 9 and 10, respectively. As indicated in Figure 9 and Table 5, the pH was influenced by the reactor's operational conditions: OLR and HRT. Accordingly, the average pH values decrease as HRT decreases from 6-1 day at the respective OLR, and the variation in mean pH was significant ($p \leq 0.05$), but there was no significant difference (p-value = 0.84) between the mean pH of 2 and 3 days of HRT. Furthermore, during the HR optimization process, an overall pH range of 7.06-5.15 was observed, with OLR and HRT ranging from 894.41-5366.43 mg COD/L*day and 6-1 day, respectively. Specifically, the pH ranges for HRT at 6, 5, 4, 3, 2, and 1 day were 7.06-6.67, 6.79-6.24, 6.49-5.68, 6.49-5.34, 6.57-5.47, and 6.36-5.15, respectively. Furthermore, as indicated in Figure 10, pH values above six were recorded during the startup of the reaction period, decreased, and gradually increased until the system attained a relative steady state for all the OLR and corresponding HRT. As it is revealed from Figure 9 and Table 5, the mean pH values at HRT of 5-1 were in the range of 6.34 ± 0.17-5.60 ± 0.27 which is within the optimum range (5.2-6.3) for the hydrolytic-acidogenic consortium of bacteria except at HRT of 6 days (Dobre, 2014). A pH range of 5.25–6.11 in a hydrolytic-acidogenic reactor with high TVFA and SCOD production was reported (Funk, 2021; Yu, 2002), which is consistent with the findings of this study, and an HR operating at a constant pH value of 5.7 can potentially support the maintenance and growth of hydrolytic-acidogenic bacteria (García-Depraect et al., 2021), which will enhance the production of TVFA. In the hydrolytic-acidogenic phase, pH values between 5.0 and 6.0 are reported as the optimal range, though it depends on the target products and feedstock (Chatterjee and Mazumder, 2019; Menzel et al., 2020).

The decrease in pH during the start or acidification phase was likely due to the VFA intermediates, lactate and ethanol, produced from organic matter in slaughterhouse effluent used as feedstock degradation. Alkaya and Demirer (2011), and Eylem Dogan Subasi (2017) also reported similar trends in pH during the acidification of sugar beet processing wastes. Furthermore, Shifare and Seyoum (2017) reported pH values ranging from 7.98 to 4.90 during the optimization of HR operating conditions for the anaerobic co-digestion of dairy and tannery wastewater at different HRTs and OLRs.

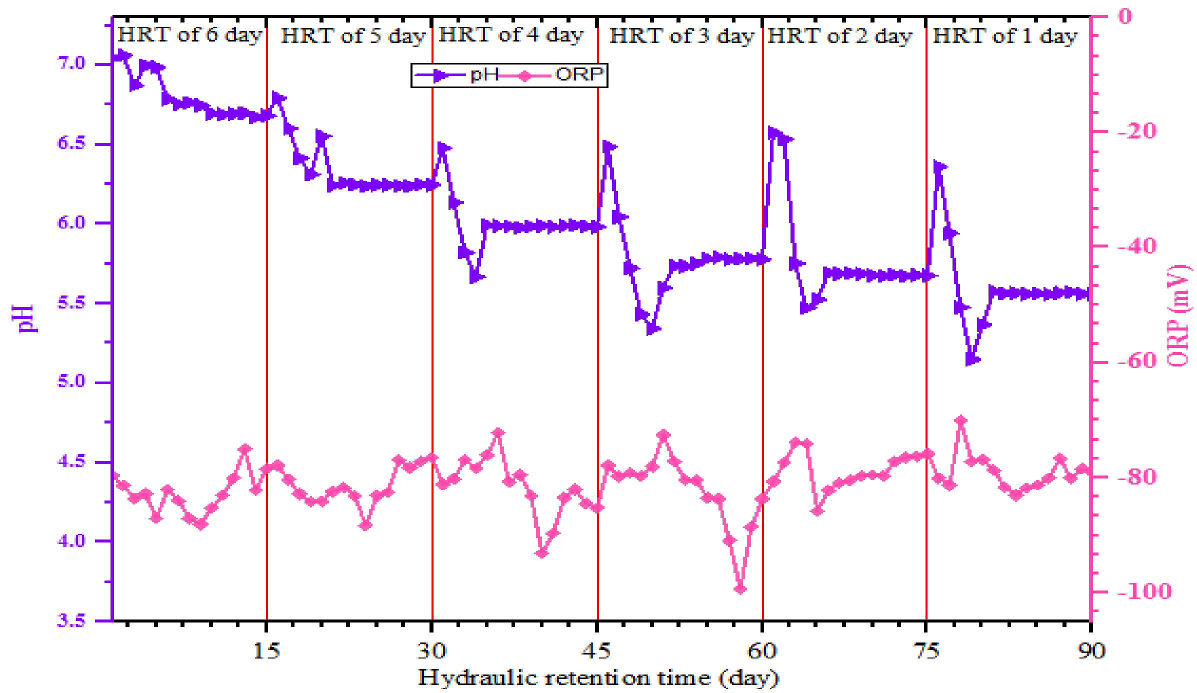


Figure 10: Variations in pH and ORP of hydrolytic-acidogenic reactor at different HRT

The optimal ORP value for methane-reducing bacteria is above -230 mV, and though no clear mechanism of inhibitions were disclosed, an ORP value above -280 mV is inhibitory to sulfate-reducing bacteria (Duangmanee, 2009). The optimal ORP for a hydrolytic-acidogenic reactor, which is a function of pH, is between -75 mV and -250 mV (Sukphun et al., 2021). Hence, the present study result shows the ORP values are within the range suitable for optimum methane production at all HRTs (Table 5).

4.2.1.3. Effect of HRT and OLR on TVFA

The average TVFA concentrations at steady-state conditions for the HR during all HRT are shown in Table 5. The average concentrations of TVFA produced during the optimization of the system were 1084.83 ± 14.37, 1006.42 ± 30.35, 1155.92 ± 16.20, 1176.50 ± 81.66, 1006.42 ± 30.35, and 996.75 ± 14.60 mg/L at HRT of 6, 5, 4, 3, 2, and 1 days; OLR of 894.41, 1073.27, 1341.61, 1788.81, 2683.22, and 5366.43 mg/L of COD, respectively. As shown in Table 5 and Figure 11, the OLR and HRT have an effect on the TVFA concentration in the reactor. The TVFA concentration increased with the increase in OLR from 894.4 to 1788.81 mg of COD/L*day and as HRT decreased from 6 to 3 days, as the increased OLR resulted in the fast production of a high-quality intermediate product like TFVA by a consortium of bacteria in the HR. This may be attributed to the large amount of biodegradable organic matter in the slaughterhouse wastewater. The decrease in TVFA concentration as HRT decreases from 3 to 1 day and OLR increases from 1788.81 to 5366.43 mg COD/L*day may be attributed to the washout of consortiums of bacteria in the HR, which is also consistent with the previous report (Jiang et al., 2013).

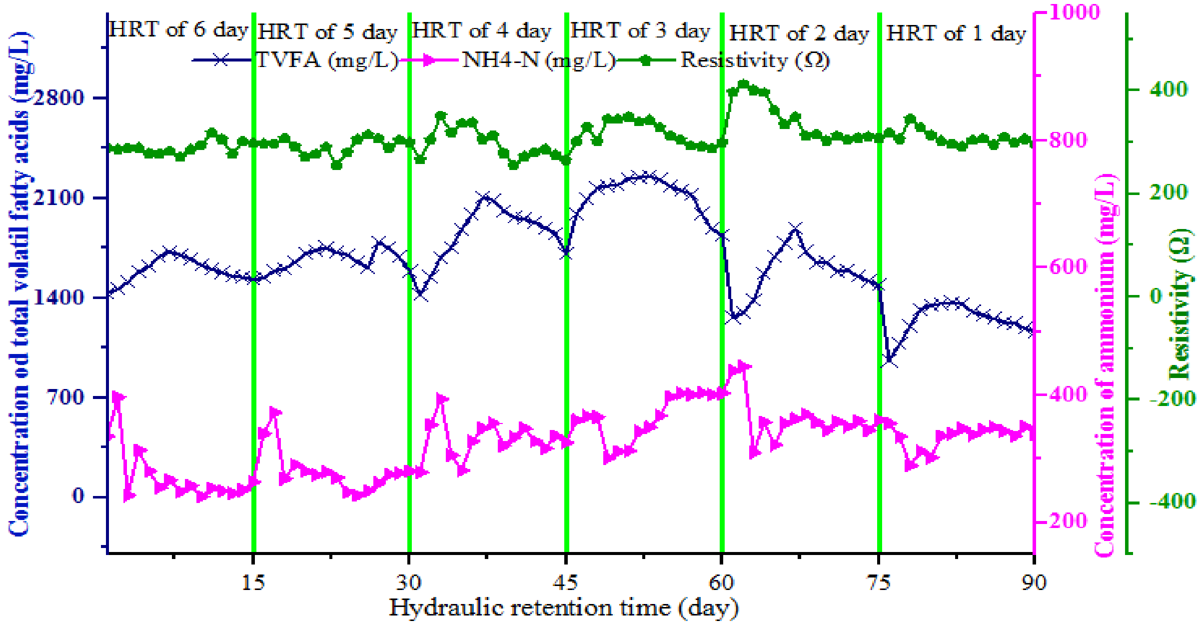


Figure 11: Variation of resistivity, ammonium and TVFA of hydrolytic-acidogenic reactor at different HRT

The trends of the VFA produced at all HRTs and corresponding OLRs during the experimental period are presented in Figure 11. As indicated in Figure 11, the TVFA concentration during the reaction course shows an increasing trend at startup and becomes nearly stable after the 6th day of the reaction period for each HRT under study. This may be due to the fact that microorganism consortiums usually take time to start their metabolic activity before becoming fully efficient.

Scholars such as Berhe Shifare and Seyoum (2017), Demirel et al. (2008), and Lim et al. (2008) also reported that the concentration of TVFA decreased as HRT increased but that further increases in HRT would not increase the production of TVFA. Moreover, for the HR operating at optimum condition, TVFA values ranging from 2800 to 4453 mg/L as $CaCO_3$ were reported during the co-digestion of effluent from agro-processing industry in two phase AD system (Shifare and Seyoum, 2017), slaughterhouse waste, and municipal vegetable and fruit waste (Hailu et al., 2020). The acidic pH (5.57) of the reactor favors the high TVFA production in the HR reactor in the two-phase AD process over alkaline conditions (Al-sulaimi et al., 2022; Castilla-Archilla et al., 2021; Cheah et al., 2019). Other researchers reported that the high TVFA produced in HR is critical for methane production as well as organic pollutant removal and speeds up the methanogenesis phase because methanogens use it immediately (Wang et al., 2016). In the two-phase AD process, the high TVFA produced in the HR is the main methanogenic bacteria carbon source and precursor of the biogas produced (Hailu et al., 2020), which at the same time is an indicator for the better performance of the HR (Tsegaye and Seyoum, 2023). It was also reported that increasing the temperature increased the constant hydrolysis rate, with the maximum found at 37.5 ± 0.1 ℃, while increasing the temperature to 45 ℃ decreased the constant rate (Donoso-Bravo et al., 2009; Parajuli et al., 2022).

4.2.1.4. Effect of HRT and OLR on TCOD and SCOD

TCOD and SCOD were also among the parameters taken into consideration in the present study in order to optimize the performance of the HR. As presented in Table 5, the average HR effluent TCOD was 4924.47 ± 25.79, 4799.33 ± 37.49, 4913.67 ± 22.79, 4934.60 ± 25.24, 4721.07 ± 67.35, and 4821.73 ± 30.38 mg/L at HRT of 6, 5, 4, 3, 2, and 1 days and OLR of 894.41, 1073.27, 1341.61, 1788.81, 2683.22, and 5366.43 mg/L of COD, respectively. As presented in Table 5, the average SCOD was 2324.80 ± 25. 16, 2359.00 ± 40.79, 2483.73 ± 47.72, 3430.20 ± 80.44, 3106.87 ± 72.65, and 2084.40 ± 71.00 mg/L at HRT of 6, 5, 4, 3, 2, and 1 and OLR of 894.41, 1073.27, 1341.61, 1788.81, 2683.22, and 5366.43 mg/L of COD, respectively. The ANOVA followed by a post hoc test revealed that the variation in mean SCOD was significant at $p \leq 0.05$, with the exception of HRT of 5 and 6 days ($p = 0.33$). The variations in mean of the TCOD concentration was not significant between HRT of 1 day and 2, 3, 4, 5, 6 days, and respective OLR at $p \leq 0.05$ but the variation of 2 day HRT TCOD mean from 3,4, and 6 days is a significant.

The trend of TCOD and SCOD concentrations during the experimental period at each HRT and OLR is presented in Figure 12. The TCOD concentrations for all HRT fluctuated at the start of the operation period and stabilized after the 7th day (Figure 12). As depicted in Figure 12, at each OLR and HRT, the SCOD concentration shows a steady increase with operation time. This may be due to the fact that the microbial consortia had acclimatized and were acting at their optimal condition; increasing the fermentation performance also increases fast solubilization, as observed at an HRT and an OLR of 3 days and 1788.81 mg of COD/L. With a three-day HRT and an OLR of 1788.81 mg COD/L*day, the highest TCOD (mg/L) and SCOD (mg/L) levels were obtained. As a result, a HRT of 3 days at an OLR of 1788.81 mg COD/L was chosen as the optimal HRT and OLR for HR. Earlier findings also indicate that feedstock with a high SCOD concentration is the precursor for high biogas yield and an indicator of HR performance (Zhang et al., 2011; Zhang and Banks, 2012) when used as a feedstock for a methanogenesis reactor. Furthermore, HR serves as a buffer in the two-phase AD process, as does high SCOD production, which is easily converted to VFA and then to methane in the methanogenesis phase (Cheah et al., 2019; Duque, 2021; Mahmod et al., 2020; Sukphun et al., 2021; Tesfaye, 2014).

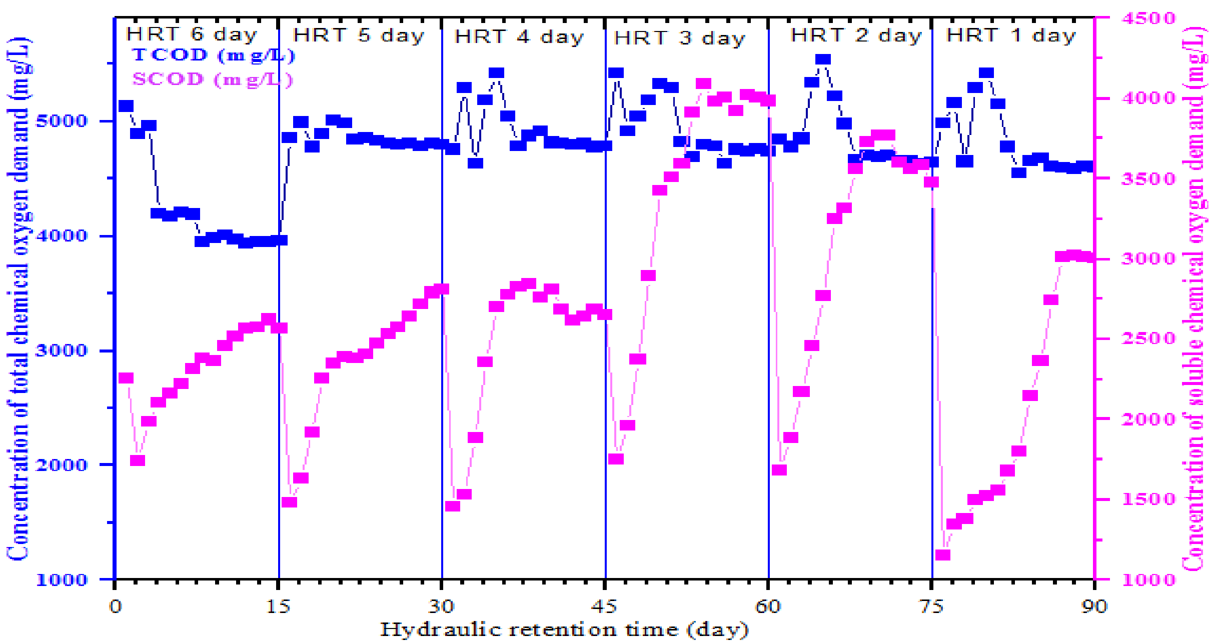


Figure 12: Variation trends of TCOD and SCOD of hydrolytic-acidogenic reactor at different HRT

4.2.1.5. Degree of acidification

In this study, the extent of acidification was assessed using the degree of acidification, and their acidification performances were also compared and depicted in Figure 13. Increases in OLR from 894.41 to 1788.78 mg/L of COD increased the DA from 17.17 to 57.26%, and then increasing beyond this resulted in a decrease in DA (Figure 13). The minimum and maximum acidification were achieved for the TCOD loading rates of 894.41 mg/L and 1342.61 mg/L, respectively, and the influent SCOD of 2354.71 mg/L. In general, DA results ranging from 17.17 to 57.26% obtained in this study are within the range of previously studied research. The DA value of the current study was in the range of the value reported (20-60%) (Demirel and Yenigun, 2004) in their study on the anaerobic digestion of dairy wastewater but higher than the assumed optimum DA value reported (40-50%) for anaerobic digester process stability (Karlsson et al., 2014). Bouallagui et al. (2005) reported a DA range of 38.9- 4.4% in HR at an HRT of 3 days in their study on two-phase AD of a vegetable and fruit biowaste mixture. The maximum DA value (57.26%) obtained in the present study is consistent with the value reported (Shifare and Seyoum, 2017), which is 55.5% at optimal conditions in their study of two-phase anaerobic co-digestion of agro-processing industry wastewaters, focusing on the effect of operational parameters on the efficiency of the first or initial phase.

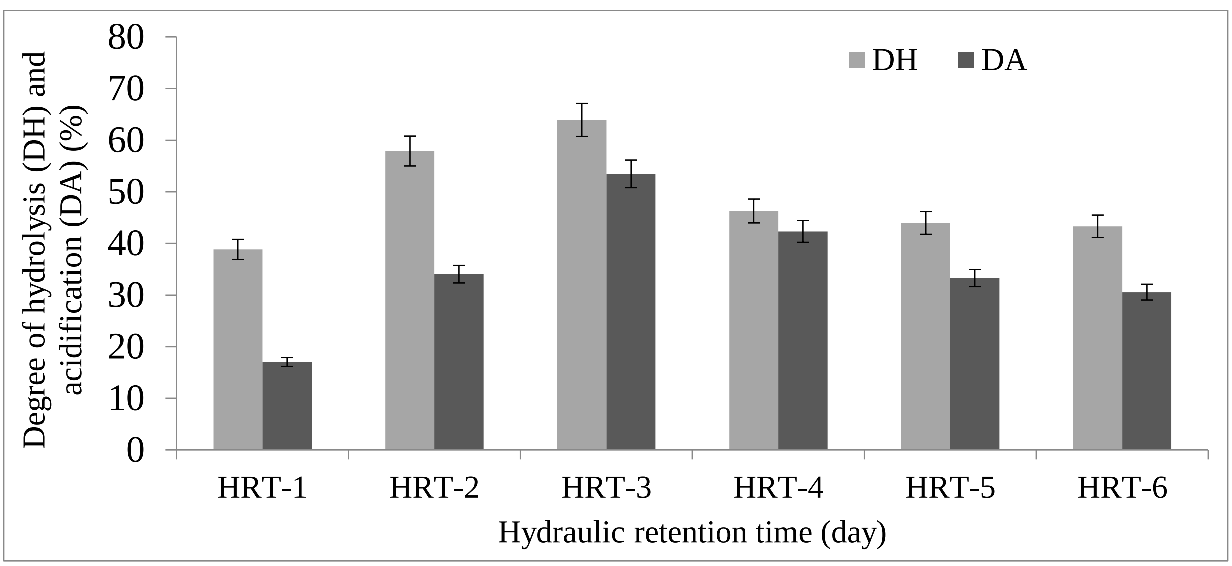


Figure 13: DH and DA of the hydrolytic-acidogenic reactor at different HRT

4.2.1.6. Ammonium production at different HRT and OLR

In reality, AD feedstock like slaughterhouse wastewater containing high nitrogen levels can frequently pose problems for the process stability of anaerobic digesters. The average NH_4^+ concentration of the effluent of the HR during the optimization of the two-phase anaerobic digestion of the slaughterhouse wastewater is presented in Table 5. The NH_4^+ produced during the hydrolytic-acidogenic phase of AD is mostly in the form of nitrogenous compounds, mostly proteins that were hydrolyzed into amino acids and further degraded into ammonia. The NH_4^+–N produced during hydrolysis has a significant role in buffering the digester, stimulating microbial growth, and stabilizing the hydrolysis process (Garcia-Peña et al., 2011). It is also a preferred nitrogen nutrient for methane-forming bacteria, but when present in high values, it will cause reticence in the anaerobic process (Mehta et al., 2015; Nielsen and Ahring, 2007). The concentrations of NH_4^+–N produced were high at the start of the operation periods, gradually decreased, and reached a steady state almost after the 4th day of the operation period (Figure 11) at all HRTs. As indicated in Table 5, the highest and lowest mean NH_4^+–N values were observed at HRT of 3 days (369.46 11.28 mg/L) and 6 days (278.67 47.25 mg/L), while the highest and lowest NH_4^+–N values were observed at HRT of 2 and 6 days, on the 2nd (501 mg/L) and 10th (241 mg/L) days of the reaction course period, respectively (Figure 11). Sossa et al. (2004) investigated ammonium inhibition on an anaerobic film enriched by methylaminotrohic methane-producing Archaea and reported that 848.8 mg/L were the maximum inhibitory ammonia values on the activity of methanogenic bacteria. Different scholars reported different NH_4^+–N inhibition levels. Angelidaki and Ahring (1994), Braun et al. (1981), and Speece (1983) reported the minimum inhibitory NH_4^+–N concentration in the anaerobic digester as 5000, 8500, 14000, and 400 mg/L, respectively. The results of the present study indicate that the concentration of ammonia reported during the optimization process does not adversely disturb the reactor process stability as well as performance.

Table 6: Summary of the mean values for the parameters indicating stability of the reactor at optimum working condition (HRT and OLR) of hydrolytic-acidogenic reactor

Stability and performance indicator parameter	Concentration
pH	5.78 ± 0.32
SCOD (mg/L)	3430.20 ± 80.44
NH_4^+-N (mg/L)	369.46 ± 11.28
TVFA (mg/L)	1176.50 ± 81.66
DH (%)	63.92
DA (%)	57.26
HRT (day)	Three
OLR (mg COD/ L*day)	1788.81
Flow rate (L/day)	12

Table 6 shows the selected values for the parameter indicating the digester stability at the optimal operating conditions of HR during the two-phase AD of slaughterhouse wastewater. Therefore, as indicated in Table 6, the optimum values for most of the stability-indicating parameters were obtained at an HRT of 3 days and an OLRT of 1788.81 mg of COD/L*day.

4.3. Performance of Methanogenesis Reactor

This section presents the operating parameter optimization effects on the process stability using indicator parameters such as pH, volatile fatty acids, alkalinity, and the VFA/Alkalinity, as well as process performance parameters such as TCOD, SCOD, TN, and TP removal efficiency, biogas production and composition, and methane yield of the MR receiving HR effluent of 3-day HRT (previously optimized) (Tsegaye and Leta, 2022a). The average values of HR effluent at HRT of 3 days used as the feedstock or influent of the methanogenesis phase are provided in Table 7, whereas the evaluation results for the stability and process performances of MR are presented in Table 8.

Table 7: MR influent (HR effluent at HRT of 3 days)/feedstock characteristics

Parameter	HR effluent/MR influent concentration
pH	5.78 ± 0.04
Salinity (mg/L)	1650 ± 12
Electrical Conductivity (µS/cm)	1810 ± 12
Resistivity (Ω)	318 ± 22
TDS (mg/L)	1576 ± 107
ORP (mV)	-82 ± 7
TVFA (mg/L)	1177 ± 12
BOD (mg COD/L)	1175 ± 19
TCOD (mg/L)	4945 ± 24
SCOD (mg/L)	3430 ± 83
NH_4^+–N (mg/L)	369 ± 11

4.3.1. Methanogenic stability in the reactor

The methanogenic reactor stability was evaluated based on parameters such as TVFA, TotA, TVFA/TotA ratio, NH_4^+–N, pH, and ORP. Table 8 and Figure 15 indicate the mean ± SD and variation of the reactor stability parameters for the methanogenic reactor, respectively.

Table 8: Methanogenesis reactor effluent stability indicator parameters values at different HRT and OLR

	HRT (days)			
Parameter	**12**	**9**	**6**	**3**
OLR (mg COD/L)	149	199	298	596
TVFA (mg/L)	541 ± 18	526 ± 45	520 ± 19	604 ±26
TotA (mg/L)	1174 ± 45	1424 ± 63	1534 ± 11	1537 ± 78
TVFA: TotA ratio	0.46	0.35	0.36	0.39
Salinity (mg/L)	1172 ± 17	1311 ± 16	1172 ± 17	1224 ± 15
NH_4^+–N (mg/L)	362 ± 10	372 ± 53	382 ± 53	400 ± 55
Effluent pH	6.91 ± 0.2	6.90 ± 0.2	6.92 ± 0.04	6.53 ± 0.1
ORP (mV)	-82 ± 4	-67 ± 3	-80 ± 3	-73 ± 6

As indicated in Table 8, the TVFA, TotA, TVFA/TotA ratio, pH, and ORP values of MR varies from 604- 541 mg/L, 1537-1173 mg/L, 0.46-0.35, 6.92-6.53 and -82-(-67) mV, respectively.

4.3.1.1. TVFA profile of the reactor

The mean TVFA for all methanogenic reactors at different OLRs and HRTs is presented in Table 8. As revealed in Table 8 and Figure 15, the concentration of TVFA decreased at all HRTs, and its concentration increased as the HRT decreased from 12 to 3 days. In addition, the variation of TVFA goes along with the variation of the reactor pH (Figure 15 and Figure 16). In stable MR, the TVFA decreased as it was used as a carbon source for the growth of methanogens in the two-phase AD systems (Michael et al. 2020). Also, Zemene Worku and Seyom Leta (2017) reported similar TVFA trends in their AD of slaughterhouse wastewater study. Furthermore, the present study finding is also in agreement with the finding by Padilla-Gasca et al. (2011), which showed a maximum TVFA concentration of 448 mg CH_3COOH/L without altering system stability in their study of anaerobic treatment of slaughterhouse wastewater. But the present study's finding regarding TVFA concentration in methanogenic reactors is lower than the values reported by Shifere and Seyoum (2018) and Padilla-Gasca et al. (2011), ranging from 790-980 mg

CH_3CHOOH/L for methanogenesis reactors in their study of two-phase anaerobic co-digestion of agro-processing industry wastewaters in different mixing ratios. The lower TVFA value of the present study may be due to the mono-digestion of the feedstock (slaughterhouse wastewater effluent alone).

The TVFA production rate was determined as the TVFA concentration result and VS reduced and decomposed. Accordingly, as shown in

Figure 14, the TVFA production rate was 5 mg/mg of VS removed. The TVFA concentration during the methanogenesis phase tended to be directly proportional to the organic matter (VS) removed (Padilla-Gasca et al. 2011). Similarly, the concentration of the TVFA concentration (production rate) is highly positively correlated (R^2 = 0.99) to the VS reduction in the methanogenesis phase (

Figure 14). Moreover, the high linear correlation between TVFA production rate and VS removed from the MR shows that there is no high consumption of the intermediate (TVFA) or accumulation of TVFA, but better reactor stability and performance during the two-phase AD process (Singharat et al. 2017).

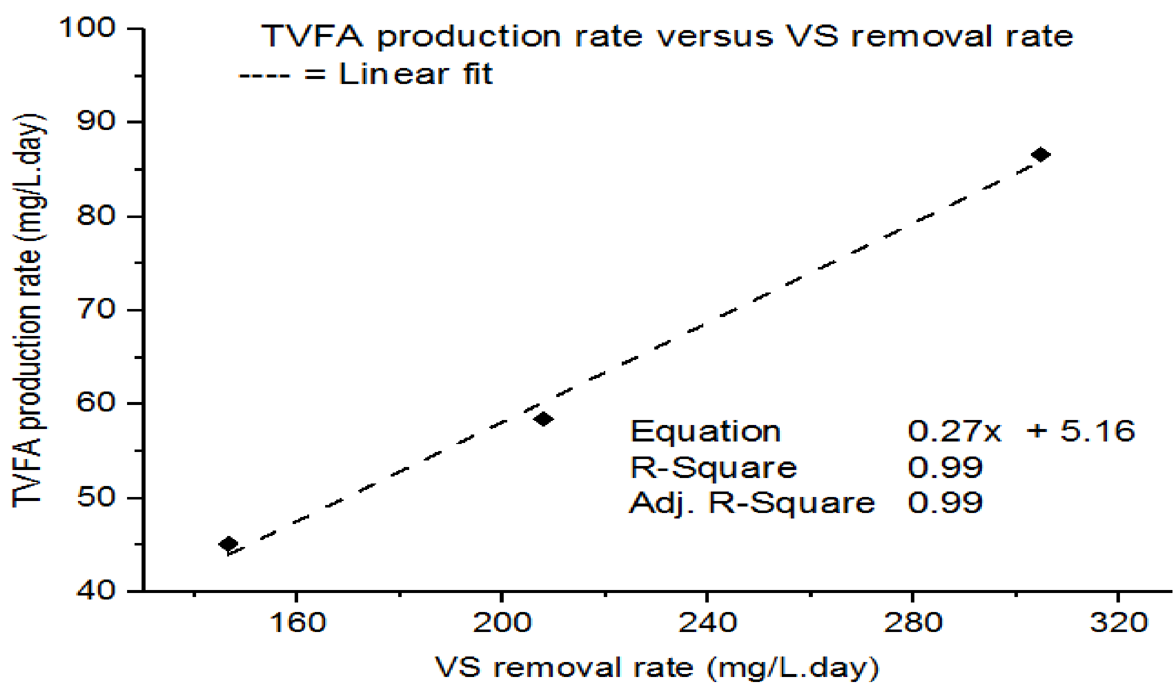


Figure 14: TVFA production rate verses VS removal rate

4.3.1.2. Total alkalinity level

The average influent and effluent TotA results of the methanogenic reactor or phase are shown in Table 8 and Figure 15. The TotA of the reactor was gradually increased and stabilized with reaction time at all HRT, indicating the reactor stability of the system, as is also indicated in previous studies (Shifere and Seyoum 2018; Padilla-Gasca et al. 2011). As HRT decreased from 12-3 days or OLR increased from 149-596 mg of COD/L, the average alkalinity value of the reactor increased (Figure 15), which is in line with the result reported earlier by Rocamora et al. (2020).

In well-performing or stable reactors, TotA values ranging from 1000-5000 mg $CaCO_3$/L were reported (Shifere and Seyoum, 2018). In the present study, the TotA values are in the range that favors reactor stability and enhances biogas production. This buffering capacity of the reactor recovers the hydrolytic-acidogenic reactor effluent pH of 5.8 and that of the startup reaction period to almost neutral, which suggests the utilization of H^+ by microbial in the reactor like hydrogenotrophic methanogens, chemolithotrophic sulfur oxidizing bacteria, or/and oxidizing homoacetogens (Padilla-Gasca et al., 2011). Cao et al. (2019) reported that the TotA value within the acceptable range favors the production of biogas through buffering the reactors by maintaining the pH.

4.3.1.3. TVFA/ TotA ratio profile of the reactor

Previous studies showed that the TVFA/TotA ratio is a parameter that is used to evaluate anaerobic reactor stability at an early stage (Padilla-Gasca et al., 2011; Rincón et al., 2008). Accordingly, the present study also examined the TVFA/TotA ratio of the methanogenesis reactor at different HRTs, and the result showed that the ratio of the acidity to that of TotA varies between 0.46-0.34 (Table 8; Figure 15). This can be due to the consumption of organic matter by microorganisms for the production of biogas (Padilla-Gasca et al., 2011). The TVFA/TotA ratio that falls within 0.10-0.30 Barampouti et al. (2005), Padilla-Gasca et al. (2011), 0.30-0.40 Chen et al. (2015), Fonoll et al. (2015), Rincón et al. (2009), Sindhu and Meera (2012), and above 0.40 Padilla-Gasca et al. (2011) indicates the avoidance of acidification, stability, and instability of the process in the methanogenesis reactor, respectively. However, the ratio values of the present study are in the stable methanogenesis reactor range of 0.3-0.4 and show high self-buffering capacity of MR except at HRT of 12 days (Table 8 and Figure 15). The optimum TVA/TotA ratio of stable and best-performing MR lies in the range of 0.3-0.4 (Chen et al., 2015; Sindhu and Meera, 2012; Fonoll et al., 2015; Rincón et al., 2009). Furthermore, elsewhere it was reported that the values of pH and ratio of TVFA/TotA of 6.9 ± 0.04 and 0.35 ± 0.02, respectively, have high buffer capacity and less acidification risk, hence leading to the high process stability of the methanogenesis reactor as the environmental conditions in the AD process can control the system (Meesap et al., 2012; Grau et al., 1975).

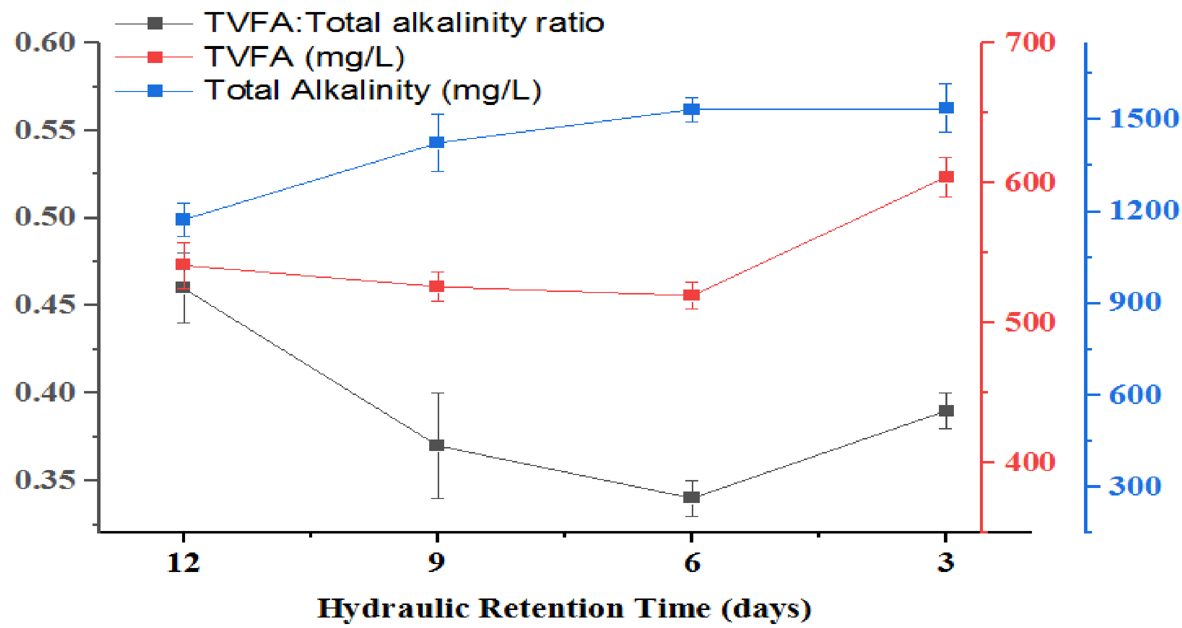


Figure 15: Average TVFA, TotA and TVFA/TotA ratio of MR at different HRT & OLR

4.3.1.4. pH profile

The mean pH values of the methanogenesis reactor were 6.91, 6.90, 6.92, and 6.53 at HRTs of 12, 9, 6, and 3 days, respectively (Figure 16). This indicates that the mean pH value for the present study is near neutral and in the peak pH stability range of a methanogenic reactor, though it drops in the first few days of the experiment. Significant variations of pH were observed for HRTs of 12, 9, 6, and 3 days at $p \leq 0.05$ (Fegade et al., 2013). In addition, a similar trend was observed for methanogenic reactors operating at HRTs 12, 9, 6, and 3 days, i.e., a small decrement during the startup period due to the accumulation of VFA and a gradual rise due to the better self-buffering capacity of the reactor as it receives partially treated effluent from HR and comes to steady state (Figure 16). Methanogenesis or AD reactors operating at optimal conditions have a pH range of 6.5-8.5 though the peak is near 7 (Rajakumar et al., 2012; Mao et al., 2017).

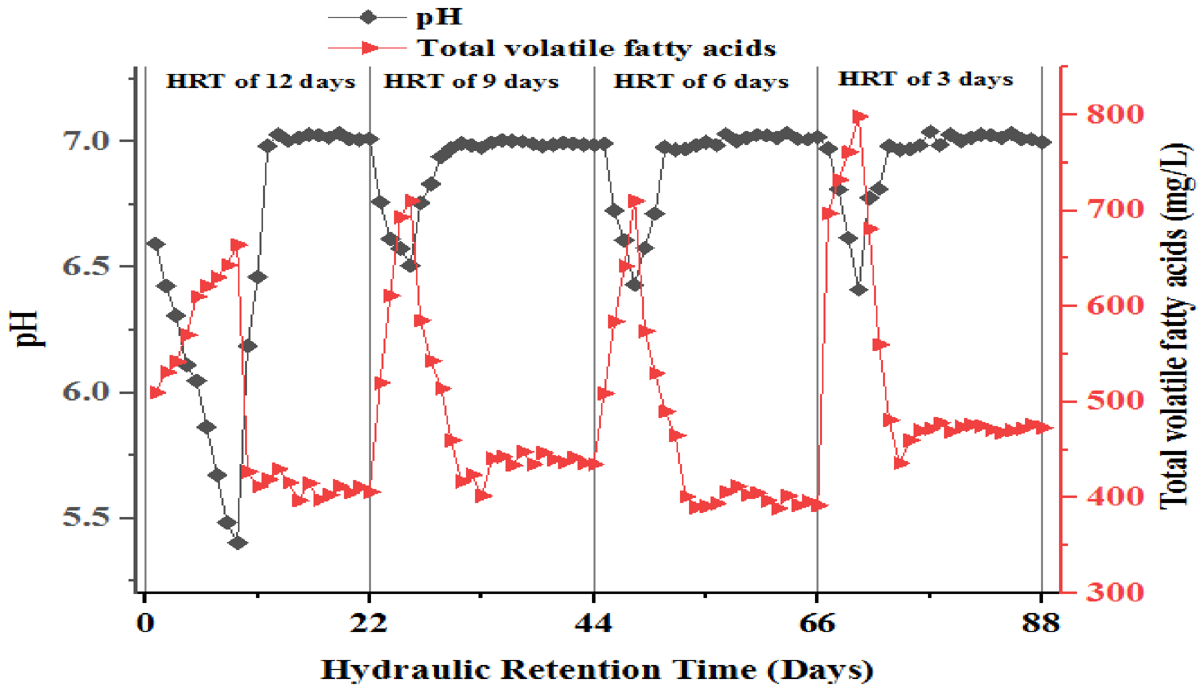


Figure 16: Variation of pH and TVFA in MR at different HRT and OLR

4.3.1.5. Ammonium profile in the reactor

NH_4^+–N concentration was investigated for the methanogenesis phase at different HRT and OLR, and the mean value is provided in Table 8**.** As it is indicated in Table 8, the NH_4^+–N concentration ranges from 362-400 mg/L, which is not in the range of inhibitory concentration levels for the bacteria in the methanogenesis phase. Moreover, the present study findings also showed a decrease in NH_4^+ concentration as OLR decreases or HRT increases, which coincides with the argument narrated by Nakakubo et al. (2008) and Rocamora et al. (2020) regarding the concentration of NH_4^+–N in MR decreasing with decreases in OLR or increases in HRT. A methanogenesis reactor NH_4^+–N concentration of less than 200 mg/L is used as a nutrient source for the microorganism, while a high level may cause a reduction in methanogen activity, which in turn increases TVFA concentration and reduces methane production (Chen et al., 2008; Appels et al., 2008; Rajagopal et al., 2013).

4.3.1.6. Oxidation reduction potential

ORP was also evaluated in the present study for the methanogenic phase, and the mean ± SD for each OLR/HRT is presented in Table 8. ORP is used to define the environment of biochemical reactions, and the ORP obtained favors methane-reducing bacteria and is inhibitory to sulfate-reducing bacteria, which is in agreement with the finding reported by Duangmanee (2009). The negative ORP values indicate that reduced substances like methane and ammonia are produced from the degradation of the wastewater (Hailu et al., 2020). The negative value of ORP in the present study also shows the working conditions, i.e., anaerobic type, and is an indicator of methane production possibility, as also demonstrated by Vongvichiankul et al. (2017).

4.3.2. Effect of HRT on reactor performance

The methanogenesis phase performance evaluation was conducted for pollutant reduction or removal efficiencies (organic matter and nutrient), biogas production, and methane yield.

4.3.2.1. Organic matter removal efficiencies

4.3.2.1.1. Chemical oxygen demand and soluble chemical oxygen demand removal efficiencies

TCOD and SCOD reduction and removal efficiencies were used to evaluate the methanogenesis phase performance at different HRTs and corresponding OLRs. The TCOD and SCOD reductions at each HRT are indicated in Table 9. TCOD consumed was 3663 ± 13, 3852 ± 45, 4026 ± 36, and 2886 ± 38 mg/L at HRTs of 12, 9, 6, and 3 days, respectively. The result showed that TCOD removal efficiency increases as HRT decreases from twelve to six days and decreases as HRT decreases from six to three days, and high TCOD removal efficiency (81%) was observed for the methanogenesis reactor operated at HRT of six days and OLR of 298 mg/L of COD (Table 9; Figure 18). In addition, TCOD reduction was significantly varied among MR operated at different HRT and OLR ($p \leq 0.05$) (Fegade et al. 2013). The variation of TCOD with reaction period (in days) for each HRT is indicated in Figure 17. TCOD values were high during the startup of the experiment, sharply decreased with the reaction period, and came to a steady state after twelve days of reaction time at an HRT of twelve, six, and three days. Though higher values of TCOD were observed at an HRT of nine days, they dropped sharply and came to a steady state after a thirteen-day reaction period (Figure 17**)**.

Table 9: Mean values of MR organic matter at all HRT and OLR

	HRT (days)			
Parameter	**12**	**9**	**6**	**3**
Influent TCOD (mg COD/L)	4945 ± 24	4945 ± 24	4945 ± 24	4945 ± 24
Influent SCOD (mg COD/L)	3430 ± 83	3430 ± 83	3430 ± 83	3430 ± 83
Influent BOD (mg COD/L)	1175 ± 20	1175 ± 20	1175 ± 20	1175 ± 20
TS (mg/L)	232 ± 18	260 ± 46	209 ± 31	439 ± 102
TSS (mg/L)	88 ± 56	155 ± 102	35 ± 20	153 ± 112
VS (mg/L)	140 ± 45	131 ± 37	89 ± 23	200 ± 18
Effluent TCOD (mg/L)	1281 ± 12	1093 ± 29	919 ± 21	2059 ± 46
Effluent SCOD (mg/L)	991 ± 65	679± 16	555 ± 11	1219 ± 17
Effluent BOD (mg/L)	111 ± 45	137 ± 34	166 ± 51	200 ± 36
TCOD removed (mg/L)	3663	3852	4026	2886
VS removed (mg/L)	1759	1870	1828	1819
RE TS (%)	82	80	84	66
RE TSS (%)	84	73	94	73
RE VS (%)	93	93	95	90
RE TCOD (%)	74	78	81	66
RE SCOD (%)	71	80	84	64
RE BOD (%)	91	90	87	85

Another parameter used in the present study for the performance investigation of MR at different HRTs was SCOD. The mean values of SCOD and trends or variations with reaction period for different HRT times are presented in Table 9 and Figure 17, respectively. As HRT decreased from twelve to six days, the SCOD reduction improved, but further decreases in HRT decreased the removal efficiency of SCOD. As seen in Table 9, the highest and lowest SCOD removals were recorded for the MR operated at HRT of six and three days, respectively. From the trend graph of SCOD against the reaction period, the steady-state condition for SCOD was achieved early when compared to TCOD at each HRT under study. The highest and lowest BOD removal efficiency for the methanogenesis phase were achieved at HRT or OLR of 12 and 3 days, or 149 and 596 mg of

COD/L, respectively. In general, the findings of the present study show MR operated at an HRT of six days and an OLR of 298 mg of COD/L, showing high performance in terms of all organic matter removal efficiency except for BOD.

The increase in TCOD and SCOD removal efficiency as HRT decreases from 12-6 days or OLR increases from 149-298 mg of COD/L may be attributed to the optimal microorganism activity of the methanogenesis phase, though longer HRT usually allows enough contact time for the microorganism with the partially treated wastewater in HR so that the decomposition of the organic matter by the system becomes efficient (Utami et al., 2016). Studies also indicate biomass drift-out and microorganism granulation are the drawbacks of the anaerobic reactors operating at short and long HRT, respectively (Demirer and Chen, 2004; Utami et al., 2016; Zemene Worku and Seyom Leta, 2017). Moreover, Zemene Worku and Seyom Leta (2017) reported a similar effect of OLR and HRT on TCOD and SCOD removal efficiency during AD of slaughterhouse wastewater. To this end, the organic matter (TCOD, SCOD, BOD, and VS) was reduced as it was hydrolyzed and degraded to TVFAs by hydrolytic bacteria and acid-forming bacteria, respectively, in the hydrolytic-acidogenic reactor, then converted to biogas by methanogens in the methanogenesis reactor or phase (Demirer and Chen, 2004; Zhang et al., 2014).

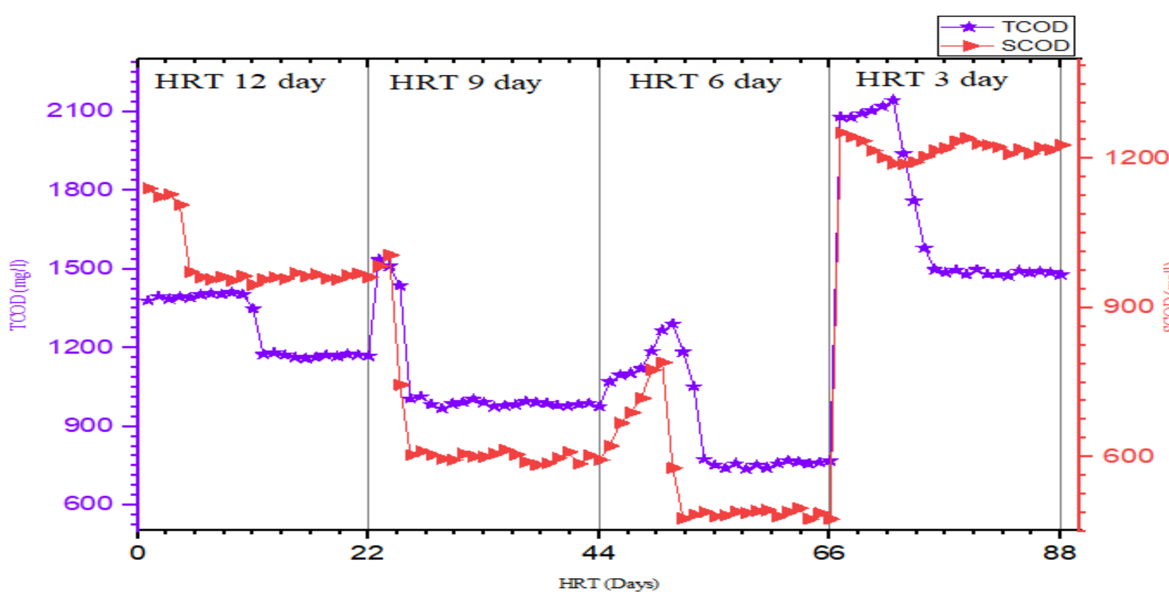


Figure 17: Trends/variation of COD and TCOD in MR at different OLR/HRT

4.3.2.1.2. Total solid and volatile solid removal efficiencies

The average reduction and removal efficiency of organic matter (TS and VS) are shown in Figure 18 and Table 9, respectively. The result shows a significant variation of TS and VS removal efficiencies for HRTs of 12, 9, 6, and 3 days with F-values of 13 and 8, which is greater than the corresponding *p*-values of 0. 00 for both (Fegade et al., 2013). The lowest (66%) and highest (84%) removal efficiencies of TS were observed at HRT of three and six days, respectively (Table 9). The VS removal efficiency of the MR increased from 93-95% as HRT decreased from twelve to six days, and a decrease in removal efficiency from 95-90% was observed for further decreasing HRT. The effect of HRT or OLR on VS RE is comparable with the finding reported by Demirer and Chen (2004) during their study of the effect of HRT and OLR on biogasification. The higher VS removal efficiency than TS in the two-phase AD system at all HRT for methanogenesis reactors in the present study is mainly due to the high uptake of the organic fraction of total solids in the effluent of HR (Singharat et al., 2017; Zhang et al., 2014). Furthermore, VS removal efficiency was negatively correlated with OLR (Figure 21).

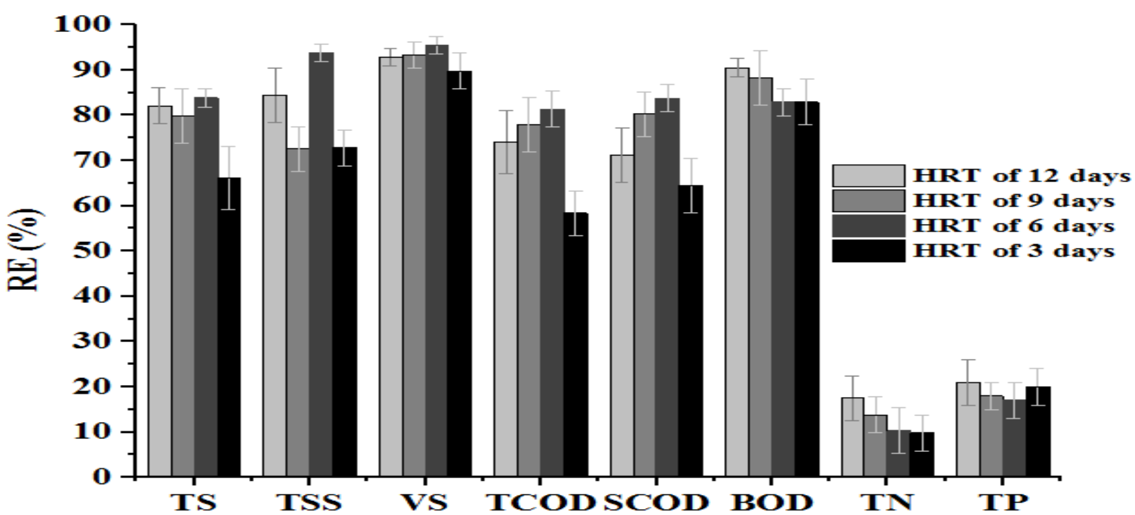


Figure 18: Organic matter, TN, and TP removal efficiency of methanogenesis reactor

4.3.2.3. Total nitrogen, Total phosphorous and sulfate level of MR effluent

The average TN of MR effluent concentration is presented in Table 10. Accordingly, the average concentration of TN was 464 ± 32, 443 ± 35, 461 ± 41, and 464 ± 32 at HRTs of 12, 9, 6, and 3 days, respectively. The removal efficiencies of TN were 10, 17, 14, and 10% at HRTs of 12, 9, 6, and 3 days, respectively (Table 10; Figure 18).

The average methanogenesis reactor effluent concentration of TP at HRTs of 12, 9, 6, and 3 days was 100 ± 17, 103 ± 7, 105 ± 7, and 101 ± 8 mg/L, respectively (Table 10). The highest and lowest TP concentrations (mean ± SD) of the MR effluent were 105 ± 7 and 100 ± 8 at HRT of six and twelve days, respectively. The maximum and minimum removal efficiencies of TP were 22% and 17% at HRT of 12 and 6 days, respectively. The removal efficiencies for TP were 22, 18, 17, and 20% at HRTs of 12, 9, 6, and 3, respectively (Figure 18). The decrease in MR effluent TP concentration is mainly due to the synthesis of biomass in the course of the AD process. In Marcin (2022), it was also stated that the decrease in TP concentration in the AD system was attributed to microbial activity and cell formation. The average PO_4^{-3} concentrations at HRTs of 12, 9, 6, and 3 days were 63 ± 6, 70 ± 6, 75 ± 8, and 86 ± 10 mg/L, respectively (Table 10). The high and low PO_4^{-3} concentrations of 86 ± 10 and 63 ± 6 mg/L were recorded at HRTs of 3 and 12 days, respectively. Likewise, the variation of the PO_4^{-3} levels of MR effluent at different HRT and OLR is significant at $p \leq 0.05$.

Table 10: Average TN, TP, PO_4^{-3}-P, SO_4^{-2} and S^{-2} concentration of methanogenesis reactor effluent at different HRT (mg/L)

	HRT (days)			
Parameter	**12**	**9**	**6**	**3**
TN	464 ± 32	443 ± 35	461 ± 41	464 ± 34
TP	100 ± 17	103 ± 7	105 ± 7	101 ± 8
PO_4^{-3}-P	63 ± 6	70 ± 5	75 ± 8	86 ± 10
SO_4^{-2}	130 ± 30	146 ± 9	166 ± 4	197 ± 14
S^{-2}	0.98 ± 0.04	1.00 ± 0.01	1.01 ± 0. 01	1.02 ± 0.01

The average SO_4^{-2} and S^{-2} concentrations of MR effluent are presented in Table 10. The average SO_4^{-2} and S^{-2} concentrations varied from 130-197 and 0.98-1.02, respectively. The decrease in the concentration of SO_4^{-2} at the methanogenic phase is mainly due to the anaerobic microbial process (sulfate reduction). This sulfate reduction was mainly attributed to the hydrolytic-acidogenic reactor, which acts as the sulfidogenic-acidogenic reactor in phase-separated AD (Janesch et al., 2021; Mburu et al., 2012). Furthermore, a comparable conclusion was drawn with the findings of the present study for the SO_4^{-2} and S^{-2} effluent concentrations of the AD by different scholars in treating slaughterhouse and other agro-industrial wastewater using an anaerobic reactor via biogas production (Alemu et al., 2019; Toledo et al., 2016) due to the low synthesis of bacteria or sulfate reduction process in the methanogenesis phase in particular and in the AD system in general (Sindhu R and Meera V, 2012), recommending further post-AD biological treatment system requirements.

4.3.2.4. Biogas production, methane content and yield

Biogas production

The trends of biogas production at the entire HRT of the methanogenic phase are shown in Figure 20. At all HRT, the biogas production was low in the start of the experiment, gradually increased, and came to a steady state after the 15th day. The low biogas production at start periods was mainly due to the lag phase of microbial growth, as the biogas production in the batch condition is directly equal to the specific growth of the methanogenic bacteria in the reactor. The gradual increase in biogas production for all HRT may be attributed to the exponential growth of the methanogens. The average biogas production was 125 ± 16, 150 ± 10, 185 ± 4, and 154 ± 17 mL at HRTs of 12, 9, 6, and 3 days, respectively. Biogas production increases from 125 to 185 mL as HRT decreases from twelve to six days, but a further decrease in HRT or increase in OLR decreases the biogas production (Figure 20). Significant variation in biogas production with HRT (p-value = 0.01) was observed for the methanogenesis phase (Fegade et al., 2013). The lower biogas production at a highest HRT and lowest OLR was mainly attributed to the high consumption of organic matter by methanogenic microorganisms for growth, which resulted in insufficient organic matter to be converted to biogas. The lowest biogas of 125 ± 16 mL produced at HRT of three days was attributed to the methanogen’s activity due to the washout or overload during the discharge of the reactor effluent that causes process instability and a reduction in biogas production (Vongvichiankul et al., 2017; Wang et al., 2014). The highest biogas production of 185 ± 4 mL at an HRT of six days and an OLR of 298 mg COD/L was mainly due to the maximum substrate utilization by the methanogen and the pH of the methanogenesis phase. The methanogens utilize maximum substrate at nearly neutral pH, which in turn favors high biogas production (Kavitha and Murugesan, 2007; Rocamora et al., 2020). The biogas produced is comparable with the results reported by Demirer & Chen (2004) and Sindhu and Meera (2012) in the treatment and biogas production from the same feedstock. Moreover, increasing OLR can reduce the contact period of methanogenic bacteria consortia and feedstock (Hailu et al., 2020). Increasing the OLR or decreasing HRT up to a certain level increases biogas production, but further increases can decrease or do not affect biogas production (Berhe and Leta, 2018; Demirer and Chen, 2004; Hailu et al., 2020; Kavitha and Murugesan, 2007; Zemene Worku and Seyom Leta, 2017).

The biogas production rate was computed with VS reduced in the process. The biogas production was highly correlated (R^2 = 0.93) with the VS removal, showing that the process gained not only biogas production but also organic matter removal (

Figure 19). This shows the VS were transformed to TVFA and then converted to biogas in the methanogenesis reactor (Lee et al., 2015; Singharat et al., 2017).

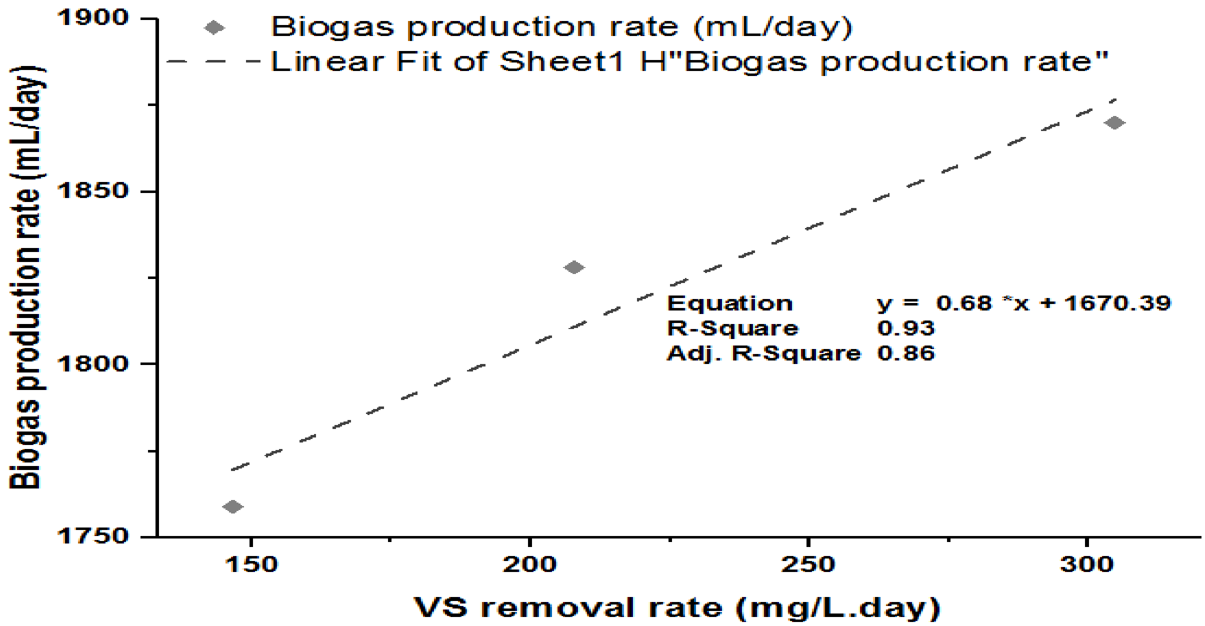


Figure 19: Biogas production rate

Methane and carbon dioxide composition of biogas

The methane content of biogas produced at all HRTs of the methanogenesis phase is illustrated in Figure 20. As it is indicated in Figure 20**,** low methane content or percentage was observed at the beginning of the operation period and gradually increased to a steady state condition at all HRT. The MR operated at HRT for six days showed good performance of 67% and 123 mL/day methane content and methane production rate, respectively. However, MR operated at HRT of three and twelve days showed low performance (55% and 70 mL/day) in terms of the methane content of the biogas produced and methane production rate, respectively. The decrease in HRT from twelve to six days increases the methane production rate from 70 to 123 mL/day. The ANOVA test for the variation of methane content at HRTs of 12, 9, and 6 days was significant ($p \leq 0.05$).

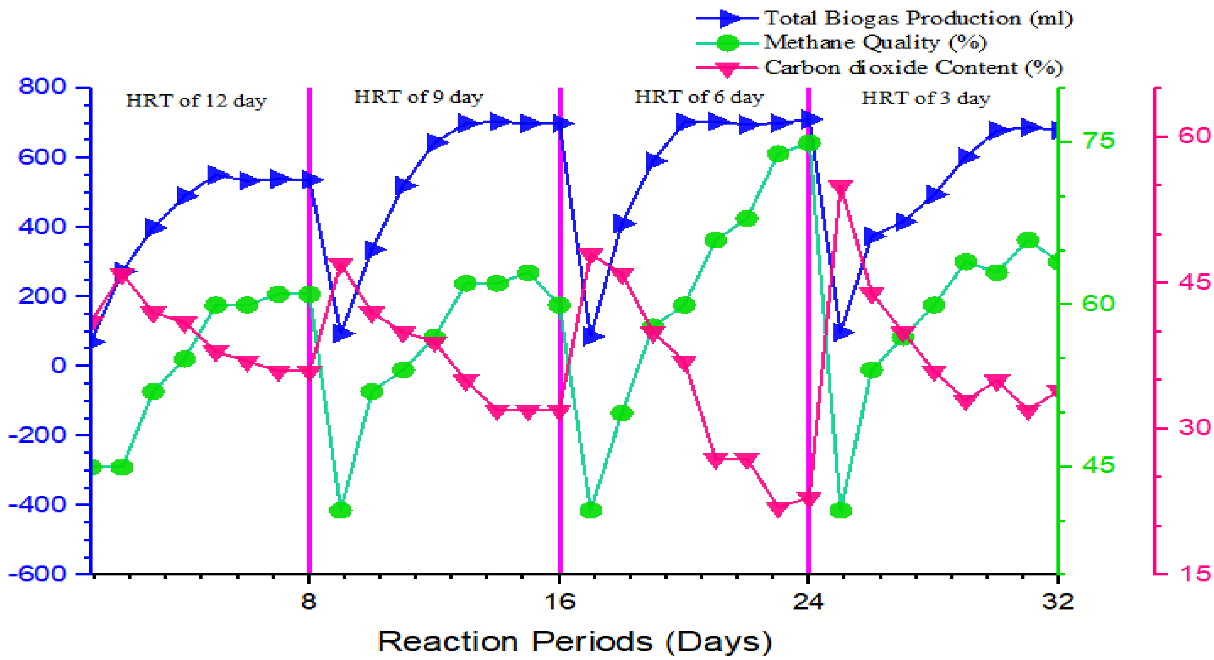


Figure 20: Variations of biogas production, methane and carbon dioxide in methanogenesis reactor at different HRT.

As shown in Figure 20, the CO_2 content in percent of biogas produced at the methanogenic phase was high at the start of the experiment and gradually decreased with time. The average CO_2 content of biogas in percentage was 54 ± 4%, 37 ± 7%, 30 ± 12%, and 36 ± 9% at HRTs of 12, 9, 6, and 3 days, respectively. The results show that the CO_2 content (%) of biogas produced decreases as HRT decreases or increases as OLR increases. Sindhu and Meera (2012) and Zemene Worku and Seyom Leta (2017) reported similar trends in biogas composition in their study of the AD of slaughterhouse wastewater at different HRTs and OLRs. A methane content of 43-63% was reported by Yilmaz (2007) in a phased AD system, which is comparable to the findings of this study. Furthermore, Demirer and Chen (2004), Michael et al. (2020), and Ortner et al. (2015) also reported equivalent methane content of 66-70% from slaughterhouse wastewater. The substantial methane and lower carbon dioxide content of the present study at HRT of 6 days may be attributed to the operating conditions, OLR and HRT, and feedstock type in relation to the earlier findings reported. The methane and carbon dioxide content of biogas obtained from the AD of organically rich feedstock varies from 50-75% and 25-45%, respectively (Michael et al., 2020). The same scholars also stated that the composition of the biogas depends on the substrate used for the anaerobic digestion and the methanogenesis bacteria consortium activity in the process, which is the main reason for the lower methane content and higher carbon dioxide content of the biogas produced.

Methane yield

The average methane yield at all HRTs of the methanogenesis phase is presented in Figure 21. As shown in Figure 21, methane yield was 0.019, 0.021, 0.027, and 0.026 mL per mg of COD removed at HRTs of 12, 9, 6, and 3 days, respectively.

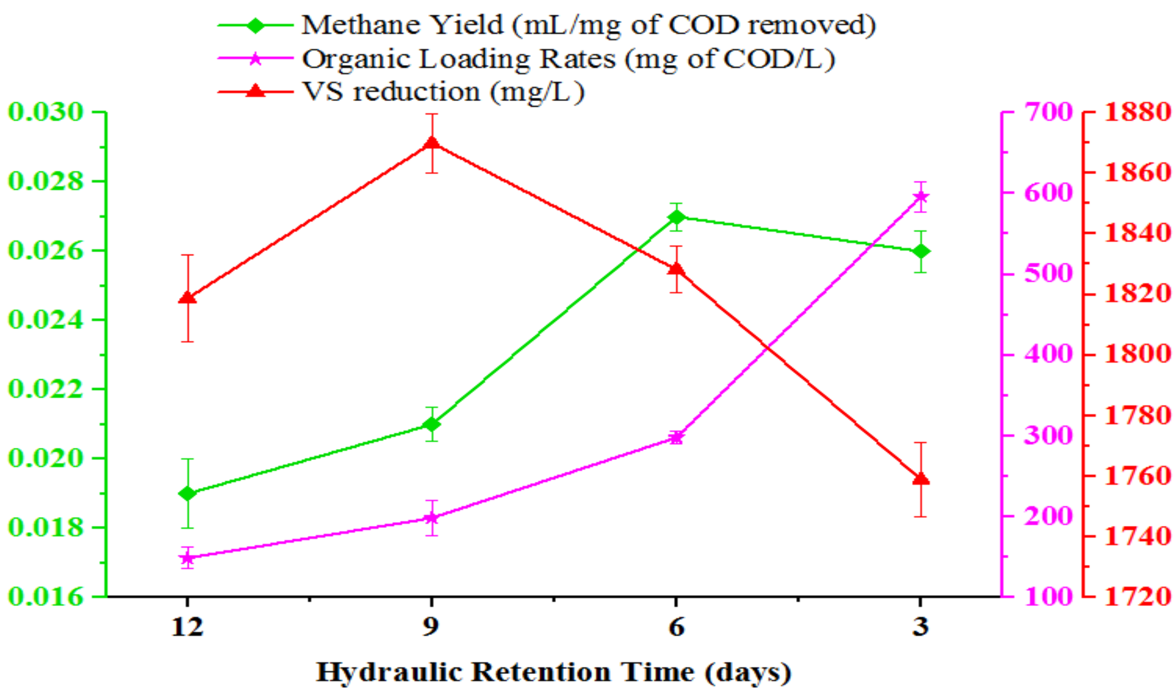


Figure 21: Variations of methane yield with HRT and OLR in methanogenesis reactor.

Furthermore, the decrease in HRTs from twelve to six days increases the methane production rate from 0.019 to 0.027 mL per mg COD removed (Figure 21). The variation of methane content at different HRTs was significant at $p < 0.05$ (Fegade et al., 2013). The highest methane yield was recorded for a methanogenic reactor operated at an HRT of six days with a corresponding OLR of 298 mg COD/L (Figure 21). The decrease of HRT or increase of OLR increases the methane yield, which is attributed to the maximal microorganism consortia activity of the methanogenesis phase and their contact time with substrate or feedstock (Ahmad, 2013; Demirer and Chen, 2004; Sindhu and Meera, 2012; Zemene Worku and Seyom Leta, 2017).

The minimum and maximum HRT (OLR) the methanogenesis phase accommodated were 3 days (596 mg/L) and 12 days (149 mg/L), respectively. In general, the two-phase anaerobic digestion of slaughterhouse wastewater, MR, operated at an HRT of six days and an OLR of 298 mg of COD/L, showed high performance in terms of pollutant removal efficiency, except for BOD, biogas production, and methane yield. The system might be further improved by natural nanoparticle employment, effluent recirculation, and co-digestion.

4.4. Performance of Two-phase Anaerobic Reactor

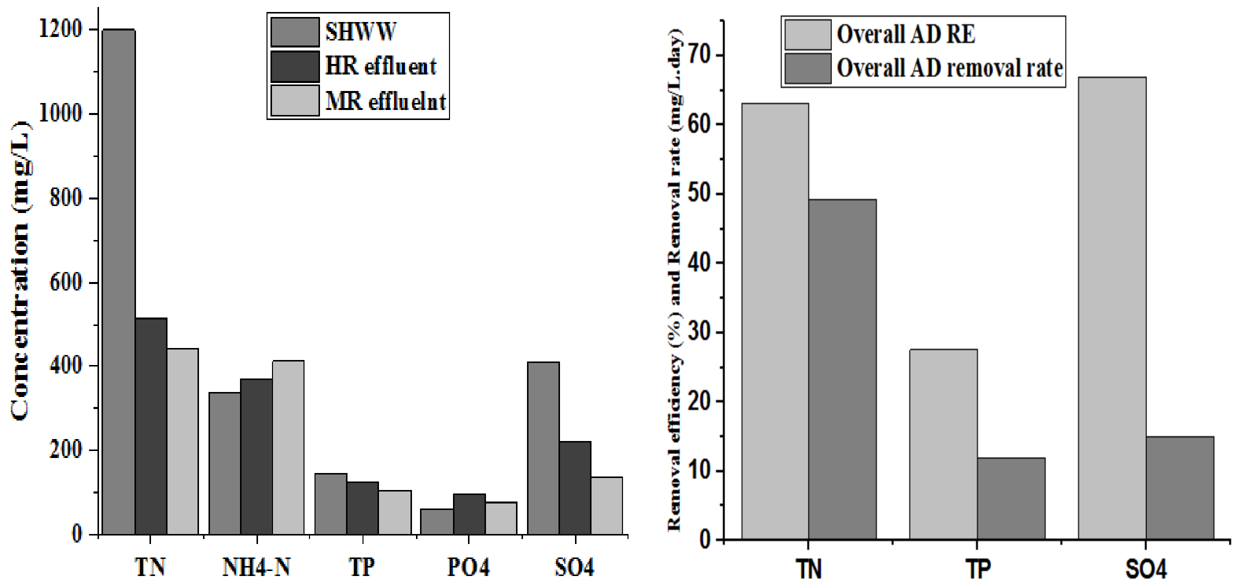
This section mainly focuses on the overall two-phase AD performance efficiencies in terms of TCOD, SCOD, BOD, TDS, and nutrients such as TN, NH_4^+–N, and TP reduction, biogas production and compositions, as well as the methane yield. The two-phase AD system was run at optimized HR and MR operating conditions.

4.4.1. Total nitrogen and total phosphorus and sulfate removal efficiencies

The hydrolytic-acidogenic and methanogenic phases of the SHWW anaerobic digestion process were both optimized separately (Tsegaye and Leta, 2022a, 2022b). This section evaluates the overall process of the two-phase AD system. The average (mean ± SD) concentration and RE of TN, TP, and SO_4^{-2} of the HR and MR are shown in Table 11 and Figure 22, respectively. The average two-phase effluent concentrations of TN and SO_4^{-2} were 430.44 ± 40.57 and 135.50 ± 44.31 mg/L, respectively (Table 11). The REs of TN, TP, and SO_4^{-2} in the two-phase AD system were 57-63, 27.57, and 59.54%, respectively. TN, TP, and SO_4^{-2} levels decreased as it moved from HR to MR, while NH_4^+-N, and S_2^{-2} concentrations increased (Table 11; Figure 22a). The decrease in the TN concentration in the effluent of the two-phase system is attributed to the conversion of nitrogen-containing compounds in the wastewater to NH_4^+-N in the AD system by the consortium of microorganisms in the system. Similarly, a decrease in concentration and subsequent increase in the removal rate of TP were attributed to its conversion to PO_4^{-3} in the AD process, which the microorganisms use as a nutrient source for their growth. The slight decrease in SO_4^{-2} concentrations might be due to their ability to be used as an electron acceptor by microorganisms to decompose organic matter in an oxygen-free environment or anaerobic systems, which is in line with the previous reports (Jeong et al., 2008; Lim et al., 2008; Mburu et al., 2012; Yilmaz and Demirer, 2008). The increments in S_2^{-2}, and NH_4^+-N concentrations in the two-phase AD effluent were attributed to the reduction of sulfate to sulfide by an anaerobic microbial process, and the decomposition of organic nitrogen to NH_4^+-N by an ammonification process, which is also consistent with previous study findings (Mburu et al., 2012; Mohan et al., 2005). The RE for TN is higher than the previously reported values of 17.1-29.96 (Zemene Worku and Seyom Leta, 2017). The higher TN removal efficiency of this study may be attributed to the phase separation and the optimization of the operating parameters of each reactor (Tsegaye and Leta, 2022b). It can be concluded that the concentration of both TN and TP decreases, but the RE increases as it goes from HR to MR (Figure 22). The ANOVA monitored with a post hoc test result showed that the decrease in mean TN and TP concentration as it went from HR to MR was significant ($p \leq 0.00$).

Table 11: Chemical characteristic of the treated effluent in HR and MR (mg/L)

Parameter	**Slaughterhouse wastewater**	**HR effluent**	**MR effluent**	**Effluent discharge limit of slaughterhouse in Ethiopian discharge limit**
COD	5633	48	919	250
TN	1198	514	430	40
NH_4^+–N	338	369	382	20
TP	145	125	105	5
SO_4^{-2}	410	220	166	
S_2^{-2}	0.87	0.9	1.01	



a) b)

Figure 22: (a) TN, NH_4^+-N, PO_4^{-3}, TP & SO_4^{-2} concentrations of SHWW, HR and MR, (b) TN, TP, and SO_4^{-2} RE and removal rate of overall AD system

4.4.2. Biogas yield and methane production

The biogas production trend of the phased system at optimized conditions is presented in Figure 23. Accordingly, the biogas and methane production rates were 189.45 and 128.40 mL/day, respectively. The methane and carbon dioxide contents of the biogas were 67.69% and 29.9%, respectively. This biogas production and methane yield can be attributed to the better activities of microorganisms resulting from the slaughterhouse wastewater BOD/COD ratio of 0.46, the suitable environment created due to the separation of HR and MR, which again enhances the activities of the microorganisms, and the higher methanogen activity over sulfur-reducing bacteria due to the higher COD/SO_4^{-2} ratio (13.10) of slaughterhouse wastewater. Widiasa (2015) also reported a BOD/COD ratio of 0.50, indicating slaughterhouse wastewater biodegradability, which in turn enhances biogas production. Scholars such as Chen et al. (2008) and Lim et al. (2008) reported high COD removal and production of biogas with a methane content greater than 50% at a COD/SO_4^{-2} ratio higher than 2.70 due to the low sulfur-reducing bacteria activities at this ratio.

Ahmad (2013) reported a methane yield of 370 mL/day from AD slaughterhouse effluent, which is higher than the present study's finding, mainly due to the feeding conditions and reactor type.

The study findings revealed that biogas production was positively and highly correlated ($R^2 = 0.98$) with total VS removed, implying that the process gained not only biogas production but also organic pollutant removal (Figure 23, Figure 24, Figure 25). The overall biogas produced versus mg of COD and VS reduced was 0.96 and 1.37 mL, respectively. This shows the efficient conversion of VS to TVFA in the HR and subsequently to biogas in the MR. Reports also indicate that in an anaerobic digestion system with separate HR and MR, substantial biogas production is achieved due to the conversion of organic matter in the wastewater to VFA in the HR, followed by the conversion of the VFA to biogas in the MR, and effective microorganism activities (Lee et al., 2015; Singharat et al., 2017).

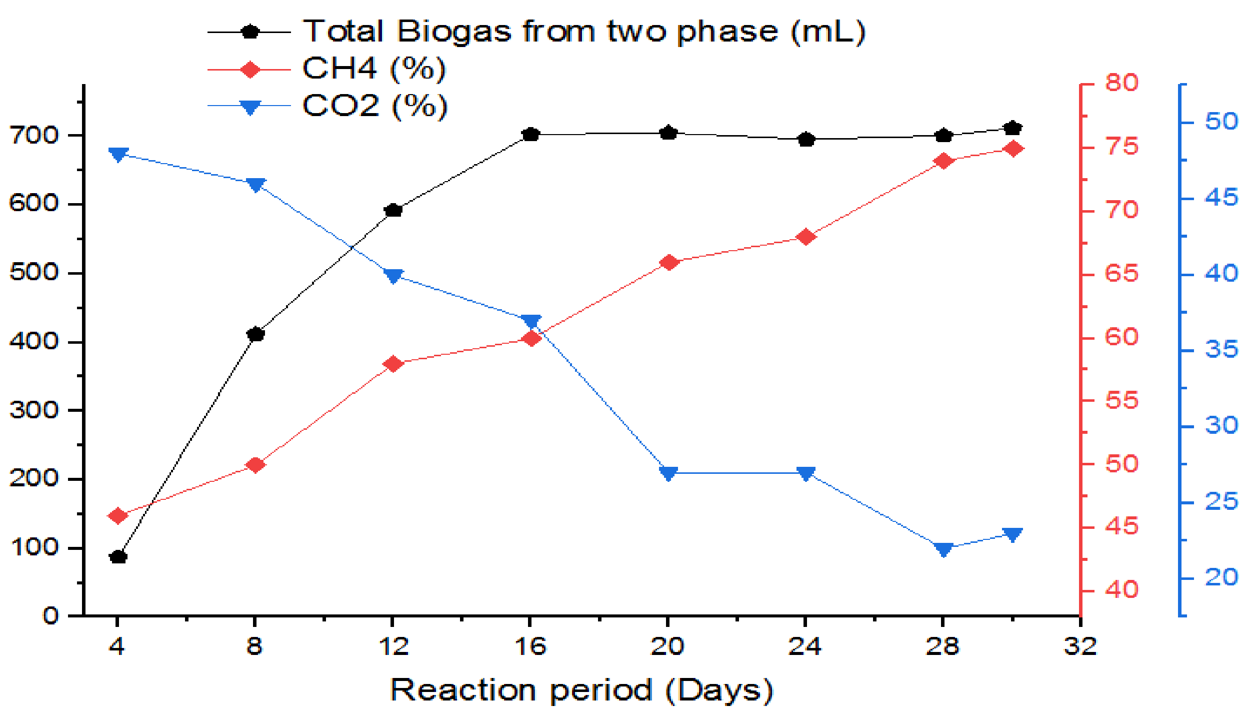


Figure 23: Total biogas and compositions of the two-phase anaerobic digestion

4.4.3. Organic matter removal efficiencies of two-phase AD system

The effluent concentration of the main organic pollutants, namely TCOD, SCOD, BOD, solids, and turbidity, is presented in Figure 24 and Figure 25. The result demonstrates that the RE of the two-phase AD system was 82.87% for TCOD, 88.53% for SCOD, 93.32% for BOD, 75.35% for TDS, 95.55% for TS, 98.95% for TSS, 97.42% for VS, and 98.06% for turbidity (Figure 24 and Figure 25). This revealed the high organic matter removal capacity of the phased AD system treating SHWW. The high organic matter RE may be attributed to the slaughterhouse wastewater BOD/COD ratio of 0.46, which shows the high biodegradability of the wastewater; the separation of the reactors, which enhances the activities of the microorganisms; and the high COD/SO_4^{-2} ratio of 13.10, which in turn favors the methanogen activity over sulfur-reducing bacteria. Chen et al. (2008) and Lim et al. (2008) reported a high conversion of organic matter in the wastewater to biogas at a COD/SO_4^{-2} ratio higher than 2.7. Though a high COD RE was obtained, the phased AD system effluent COD level is higher (919.05 ± 205.84) than the EEPA discharge limit (250 and 80 mg/L for COD and BOD, respectively) (Table 11), indicating the need for further post-AD treatment.

The organic pollutant RE of the HR and MR ranged between 7.9-91.7% and 75.3-99.9%, respectively. For both reactors, the lowest RE was observed for TDS (7.9% for HR and 75.3% for MR), while the highest RE were 92% and 99% for turbidity and TSS in HR and MR, respectively. In general, 4811.23 and 3354.19 mg/L of TCOD and VS were removed by the two-phase AD system, respectively. As inferred from Figure 24 and Figure 25b, organic matter removal efficiencies were found to be high, proving the phased AD system's conducive conditions for the removal of main organic matter. The RE trend for most organic pollutants was similar, i.e., the majority of organic pollutants were removed in the second phase of the system (MR), owing to the conversion of organic pollutants to TVFA in HR, then to biogas, and consumption by methanogens as a carbon source. Hence, a persistent increase in all organic matter RE and a decrease in organic matter final effluent concentration were also observed, and the increase in the RE was significant (p-value = 0.00). In general, the overall AD system RE of the organic matter ranged between 31.55% and 98.95% (Figure 24; Figure 25).

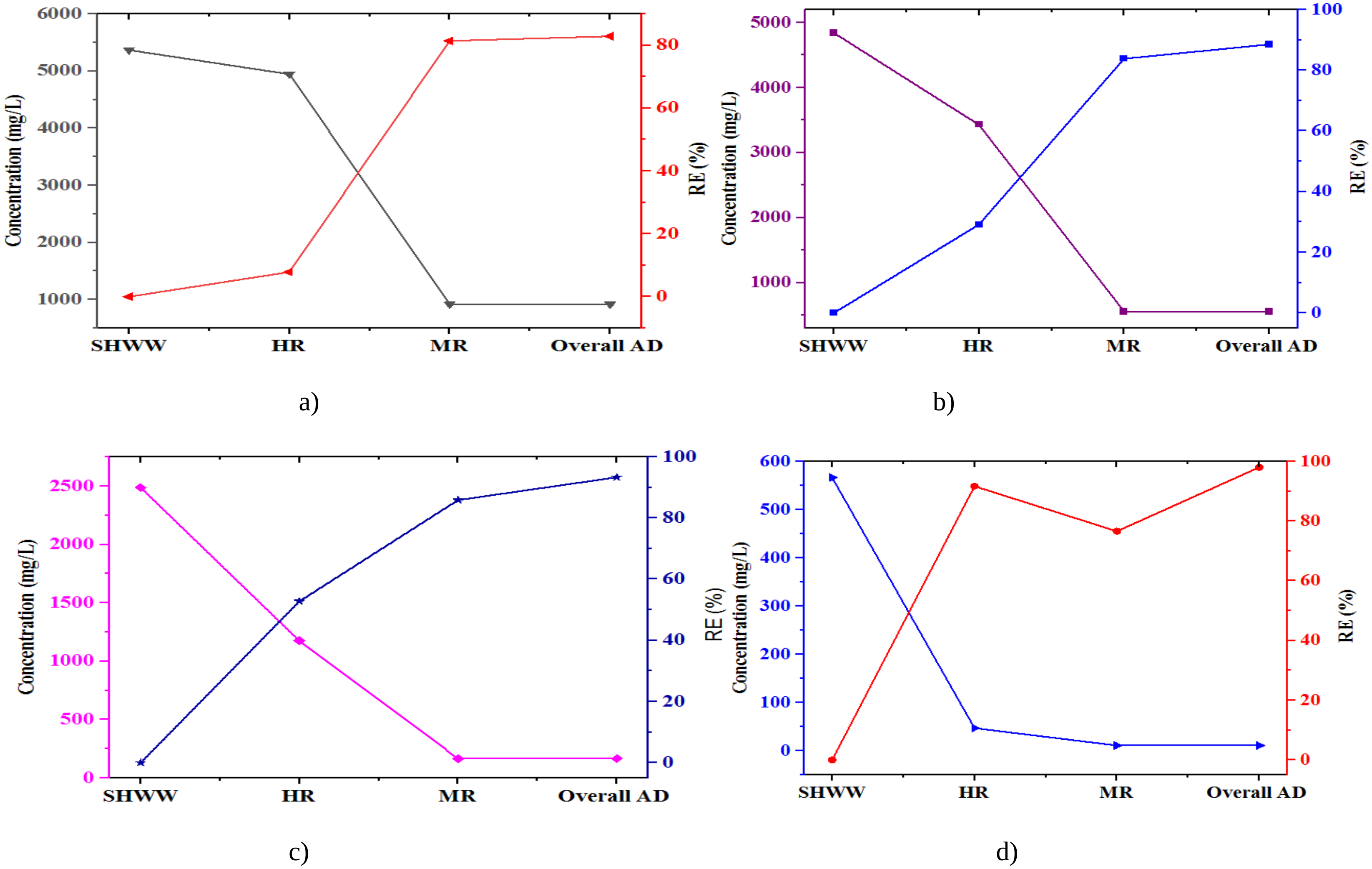
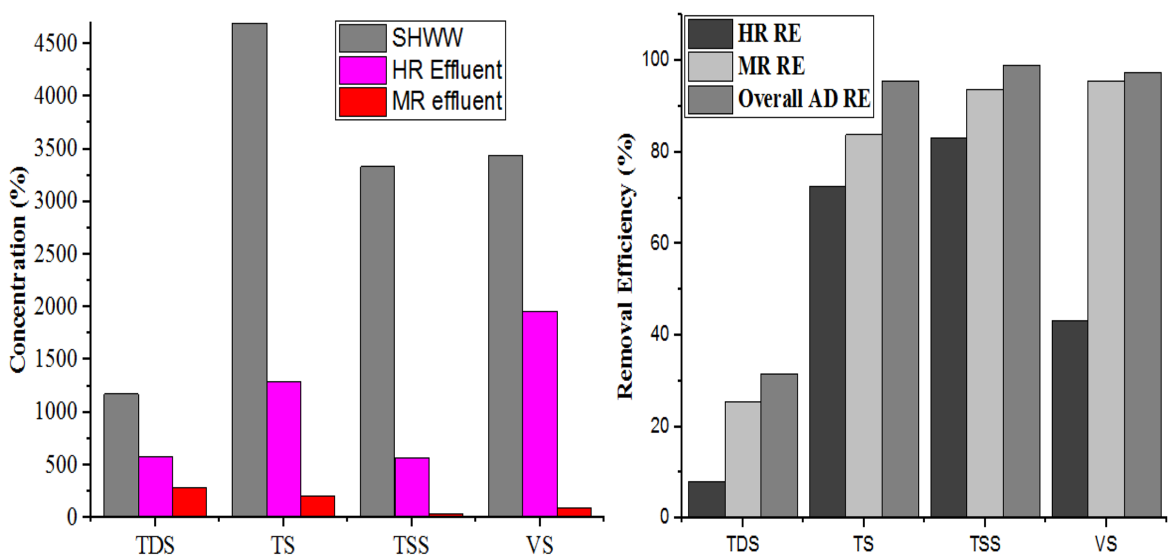


Figure 24: SHWW, HR and MR effluent values and overall, RE for TCOD (a), SCOD (b), BOD (c) and Turbidity (d) of the two-phase AD syste

The organic matter removal efficiency result obtained is comparable to the previous reports by Varga et al. (2013) and Demirer and Chen (2004). In line with this, Liu and co-authors Liu et al. (2006) reported 17.8%, while Scherer et al. (2000) reported a 46% reduction in HR. Regardless of the difference in operational conditions, uniqueness of the reactor configurations, and parameters under study, the reduction in VS observed in this study is within the range reported in the literature (Liu et al., 2006; Scherer et al., 2000).

Table 12: Pearson correlation coefficient of stability and performance indicator parameters for HR

Parameter	Salinity	pH	NH_4^+-N	TVFA	SCOD
Salinity	1				
pH	0.41	1			
NH_4^+-N	0.86	0.40	1		
TVFA	-0.70	-0.39	-0.56	1	
SCOD	0.13	-0.50	0.36	0.03	1



a) b)

Figure 25: TDS, TS, TSS and VS concentration of SHWW, HR & MR effluent (a) and RE (b)

Correlation of Reactors Stability and Performance Indicator Parameters

The Pearson correlation coefficient table of stability and performance indicator parameters for HR and MR is indicated in Table 12 and Table 13, respectively. Salinity is positively correlated to pH (0.41), NH_4^+-N (0.86), and SCOD (0.13), while negatively correlated to TVFA (-0.70). Furthermore, pH is positively correlated with 0.40 NH_4^+-N but negatively correlated with TVFA (-0.39) and SCOD (-0.50). Similarly, NH_4^+-N is negatively correlated to TVFA (-0.56) and positively correlated to SCOD (0.36). TVFA and SCOD are positively correlated (Table 12). The pH of the HR decreases as TVFA and NH_4^+-N concentrations increase. TVFA and SCOD concentrations in HR increased at the same time. Furthermore, the decrease in pH, or in acidic pH, favors the hydrolysis of large compounds into soluble ones, which is attributed to an increase in the SCOD concentration in HR.

The MR stability indicator parameter pH is positively correlated to (0.52) salinity and (0.25) biogas production while being negatively correlated to (0.89) TVFA, (0.61) TVFA:TotA ratio, and (0.96) methane yield. Furthermore, TVFA is positively correlated with the TVFA/TotA ratio and methane yield while being negatively correlated with salinity and biogas production. The TVFA/TotA ratio is negatively correlated with salinity and biogas but positively correlated with methane yield. Salinity, biogas production, and methane yield are positively correlated with each other (Table 13). Therefore, an increase in TVFA and a decrease in salinity decrease the pH of MR. The decrease in the TVFA concentration has a positive effect on biogas production (biogas production increases), indicating the TVFA produced in HR is converted to biogas in MR. The salinity concentration of MR also favors biogas production and methane yield as it supports the self-buffering of the system. This shows that increasing one parameter increases the other, or vice versa (Shigut et al., 2017).

Table 13: Pearson correlation coefficient table of stability and performance indicator parameters of MR

Parameter	pH	TVFA	TVFA:TotA	Salinity	Biogas	Methane yield
NH_3-N						
pH	1					
TVFA	-0.89	1				
TVFA: TotA	-0.87	0.99	1			
Salinity	0.52	-0.61	-0.71	1		
Biogas production	0.25	-0.38	-0.52	0.86	1	
Methane yield	-0.96	0.92	0.91	0.86	0.86	1

4.4.4. Total and faecal coliform removal efficiencies

The mean load of pathogens (TC and FC) in the SHWW was $7.73 * 10^4 \pm 7.12 * 10^4$ and $1.53 * 10^6 \pm 0.85 * 10^6$, respectively, as shown in Table 3, but was not distinguished in the two-phase AD effluents, which is mainly due to the operating temperature. The pathogen content of the two-phase AD effluent is below the level indicated in the WHO guideline for agro-processing industry effluent discharge limits. Studies also indicated the high removal efficiency of AD systems treating agro-processing industry effluent in removing pathogens more than 95% up to complete removal (Alemu et al., 2019; Zemene Worku and Seyom Leta, 2017), consistent with the this study findings.

4.5. Microalgae Co-cultivation for Polishing Nutrients and Residual Organic Matter

The subsequent sub-section, two microalgae, *Chlorella* species and *Scenedesmus* species, and co-cultivation in a photobioreactor with a total volume of 9000 cm^3 to polish the residual organic matter and high nutrient load AD effluent and microalgae biomass production are discussed. In addition, the high-value macromolecule (lipid) content of microalgae from which biodiesel was extracted, to indicate the bioenergy production in addition to the treatment from the polishing unit, is also presented.

4.5.1. Operating conditions for polishing unit (microalgae photobioreactors)

The light intensity of the fluorescent lamp used in this study varied between 150 and 300 μmolem^{-2}s^{-1}. Microalgae species-specific light intensity needed for optimal growth was reported to be between 150 and 400 μmolem^{-2}s^{-1} for *Scenedesmus* species (Mostafa et al., 2012) and 200 to 500 μmolem^{-2}s^{-1} for *Chlorella* species (Maltsev et al., 2021), while optimum biomass production of both species of microalgae was at 150 μmolem^{-2}s^{-1} (Nzayisenga et al., 2020).

In this study, the temperature of the surface above the reactor during the experimental period varied from 28.2 to 32.5 °C. A temperature range between 15 and 30 °C is assumed to be optimal for microalgae photobioreactors, with a critical maximum temperature that depends upon specific species, providing the nutrient concentration, and light supply not being limiting factors (Andersen and Kawachi, 2005; Singh and Singh, 2015).

In this study, the pH of the photobioreactor in which *Chlorella*, *Scenedesmus,* and co-culture were used to treat the two-phase AD effluent varied from 7.53 to 11, 7.31 to 10.6, and 6.7 to 11.5, respectively, which is consistent with the reported pH values (Acevedo et al., 2017; Asmare et al., 2014; Bohutskyi et al., 2016; Chevalier et al., 2000; Kusmayadi et al., 2022).

4.5.2. *Photobioreactor effluent quality*

Two-phase AD effluent COD, TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P concentrations subjected to treatment with *Chlorella* sp., *Scenedesmus* sp., and co-culture varied between 905–919, 359–376, 330–365, 89–102, 93–105, and 61–88 mg/L, respectively. Related study results showed that the COD, TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P concentrations were varying between 97–1100 mg/L, 163-410 mg/L, 21-237 mg/L, 22–265 mg/L, 12-221 mg/L, and 0.6–170 mg/L, respectively, and can be used as a nutrient and carbon source for microalgae growth (Bakraoui et al. 2023), revealing the two-phase AD effluent supports microalgae growth. Scholars also reported that the residual TN, TP, COD, and several micronutrients in the anaerobically treated agro-processing industry effluent can potentially support microalgae cultivation (Elvira E. Ziganshina, Svetlana S. Bulynina 2022; Bauer et al. 2021; L. Zhu, Yan, and Li 2016; Tambone et al. 2017).

4.5.2.1. Nitrogen, nitrate and ammonium removal

The TN concentration of the two-phase AD effluent fed to the photobioreactor in which *Chlorella*, *Scenedesmus*, and the co-culture grown was 367.33 ± 8.50 mg/L. The final photobioreactor effluent TN concentration treated by *Chlorella*, *Scenedesmus*, and co-culture varied between 25 and 13, 6 and 11, and 2 and 9 mg/L, respectively. The lowest TN concentrations of 10, 12, and 9 mg/L were observed for the photobioreactor effluent in which *Chlorella*, *Scenedesmus*, and co-cultures were grown, respectively. The changes in TN concentration and REs during the incubation or experimental period are shown in Figure 26. In all three treatments, the concentration of TN decreased sharply in the 1st eight days but steadily afterwards. The final concentrations of TN in all the treatments were below 15 mg/L. Furthermore, 60% TN removal efficiency was achieved on the 8th day of the incubation or experimental period, and about 95% TN removal efficiency was achieved at the end of the 20 days by all three treatments. Besides, the TN removal rate or uptake by *Chlorella*, *Scenedesmus*, and co-culture was 20.80, 20.82, and 20.95 mg/L*day, respectively (Table 14). As stated in the different literature, the TN removal efficiency of AD effluent treated by microalgae varies between 90 and 100% depends on the operating conditions, microalgae used, reactor type, and other factors (Yirgu et al., 2020). A study conducted by Shayesteh et al. (2021) using *Chlorella* sp. for agro-processing wastewater treatment indicated that the TN nutrient assimilation by microalgae was 75.50% with a final effluent concentration of 84.24 mg/L. Whereas, TN RE of 76–95% (Cho et al., 2011; Kim et al., 2013; Zhu et al., 2022) and 88% (da Fontoura et al., 2017) were attained during the biomass cultivation of *Chlorella* species and *Scenedesmus* species cultivation for biomass production using tannery wastewater. Similarly, Ji et al. (2013) studied the nutrient removal efficiency of *Chlorella* and *Scenedesmus* species and achieved an almost complete removal of TN by both species. Other studies also reported that the microalgae consortium had a TN removal efficiency of 67% (Hu et al., 2019). The TN removal efficiency obtained in this study is consistent with the previous research findings for the partially treated agro-processing industry wastewater feedstock using *Chlorella* species and *Scenedesmus* species (Cai et al., 2013; Darpito et al., 2015; Farooq et al., 2013).

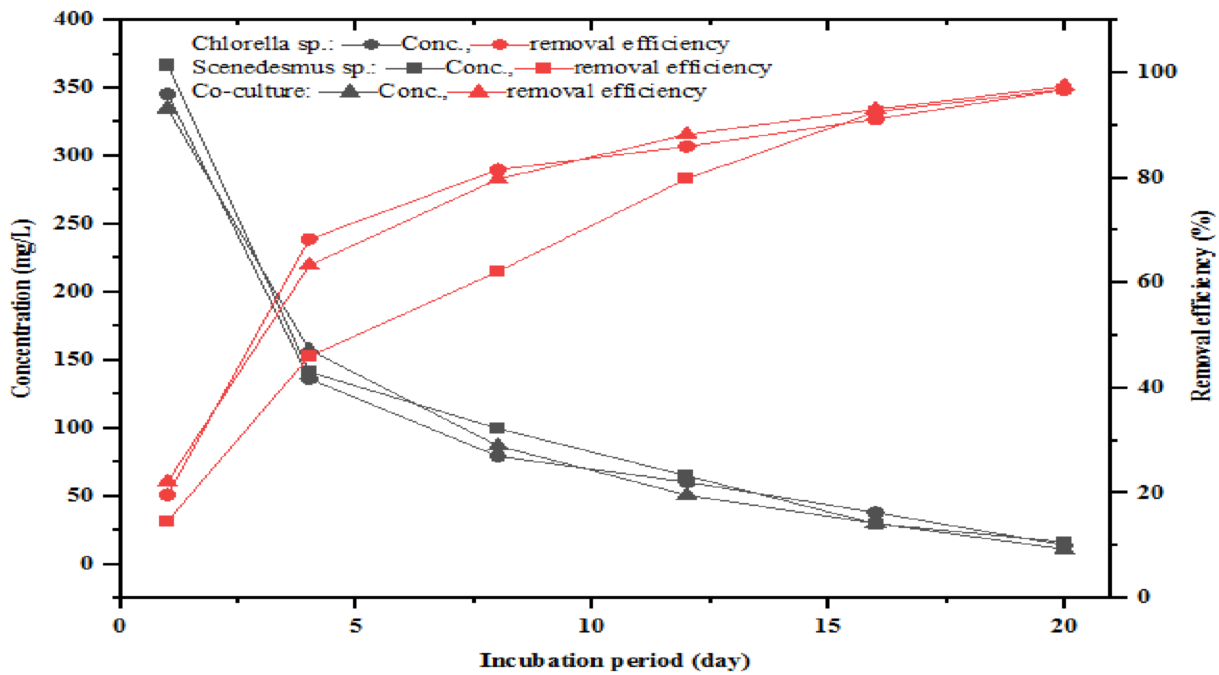


Figure 26: Variation of total nitrogen concentration and removal efficiency

The NO_3^-–N concentrations of two-phase AD effluent treated by *Chlorella*, *Scenedesmus*, and mixture were between 13-25, 6–11, and 2–9 mg/L, respectively. The NO_3^-–N concentrations in all treatments showed a steady and sharp decrease in the first 8 days and afterwards of the treatment period, respectively (Figure 27). Hence, a low RE of NO_3^-–N was observed in the first 8 days of the incubation period. A NO_3^-–N RE of more than 60% was achieved after 12 days of the incubation or experimental period for all the microalgae and their mixture. Furthermore, the final effluent NO_3^-–N concentrations treated with *Chlorella*, *Scenedesmus*, and co-culture were below 10 mg/L with 91, 93, and 93% REs, respectively. The moderate decrease followed by a sharp increase in NO_3^-–N RE in the 1st week of the incubation periods and afterwards, respectively, was attributed to the less preference of NO_3^-–N by microalgae when the NH_4^+–N concentration is enough to support the microalgae's cell growth. The NO_3^-–N removal efficiency result achieved in this study is consistent with the previous study findings, as depicted below. Nitrate is the second inorganic nitrogen that microalgae prefer for growth, next to ammonium (Whitton et al., 2015). Studies have shown low (53%), 90%, and 100% NO_3^-–N removal efficiencies by *Chlorella* sp., from partially treated agro-processing industry wastewater (Godos et al., 2009; D. Hu et al., 2021; Shi et al., 2007).

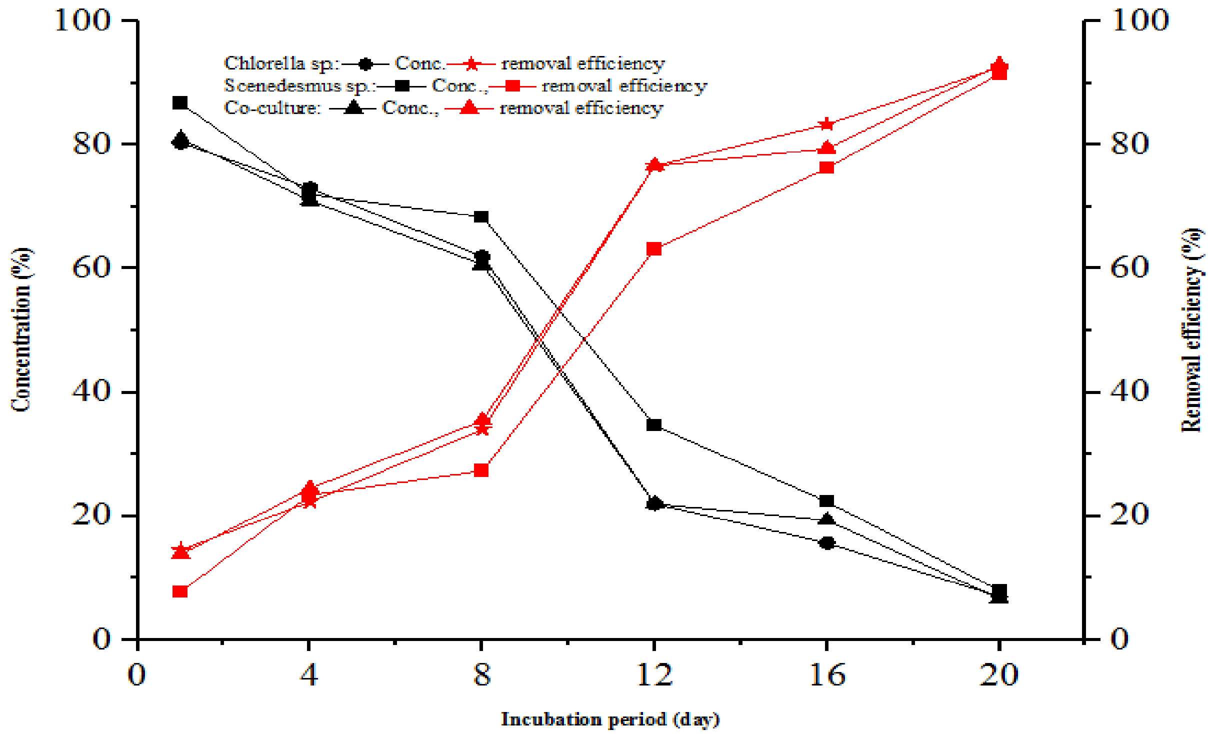


Figure 27: Nitrate concentration and removal efficiency variations

Figure 28 shows the change in NH_4^+–N concentrations and removal efficiency during the experimental period by *Chlorella*, *Scenedesmus*, and co-culture. As indicated in Figure 28, NH_4^+–N concentrations progressively decreased while the RE increased with time in all treatments. The decrease and increase of the concentration and RE of NH_4^+–N were high during the first 8 days of the incubation period and slow afterwards, respectively. Furthermore, the NH_4^+–N RE is higher than other nitrogen forms, which may be attributed to its preference for microalgae over other forms. Accordingly, in all three treatments, more than 50, 75, and 61% removal efficiency of NH_4^+–N were achieved in the 1, 8, and 12 days of the incubation or experimental period, respectively. The final NH_4^+–N concentrations were 3.67 ± 1.53, 7.67 ± 1.53, and 2.00 ± 1 mg/L, with removal efficiencies of 97. 94, 99.01, and 99.46% for *Chlorella*, *Scenedesmus*, and co-culture, respectively. After 20 days of incubation, the lowest NH_4^+–N concentrations of 2, 6, and 1 mg/L were recorded in the photobioreactor with *Chlorella*, *Scenedesmus*, and co-culture, respectively. Simultaneously, after 20 days of incubation, the NH_4^+–N removal rates by *Chlorella*, *Scenedesmus,* and co-culture were 18.42, 17.22, and 18.50 mg/L*day, respectively (Table 14). The higher removal efficiency of NH_4^+–N was achieved by the co-culture of microalgae than monocultures, which is supported by earlier findings by Cai, (2013), though it was reported that *Chlorella* sp. can effectively tolerate NH_4^+–N. The higher removal efficiency and removal rates of NH_4^+-N than NO_3^-–N in all treatments were due to their consumption, preferably by microalgae for the growth of cells. Furthermore, the higher removal efficiency of NH_4^+–N is attributed to a reduction in culture pH, which in turn shifts the equilibrium from NH_3–N toward NH_4^+–N, which the microalgae prefer for their growth as a source of nutrients. Furthermore, this may be due to the lower energy requirement for NH_4^+–N assimilation of glutamine and the release of the hydrogen ion than for nitrite and nitrate. These results (removal efficiency and final effluent concentration of NH_4^+–N) are consistent with the removal efficiency and final effluent concentration of NH_4^+–N reported by Ruiz-Martinez et al. (2012) for a culture of *Scenedesmus* sp. collected from freshwater bodies in a synthetic medium (removal rates of 13.5–4.2 mg/L*day). Studies also show NH_4^+–N removal efficiency of 85.63% (da Fontoura et al., 2017), 92–99% (Gao et al., 2015; Katırcıoğlu Sınmaz et al., 2022; Praveen and Loh, 2015; Su et al., 2016), and NH_4^+-N removal efficiency of 100% (Shayesteh et al., 2021; Zhou et al., 2012) by *Chlorella* sp. used for secondary wastewater treatment. Furthermore, almost complete NH_4^+–N removal efficiency was also achieved by *Scenedesmus* sp. (Ji et al., 2014). *Scenedesmus* sp. microalgae grown using effluent from partially treated breweries by the USAB system showed a progressive increase in removal efficiencies and reached 99% at the end of the experiment. It was also reported that microalgae usually prefer NH_4^+–N as a main source of inorganic nitrogen (Cai et al., 2013; Yirgu et al., 2020; Zhou et al., 2014).

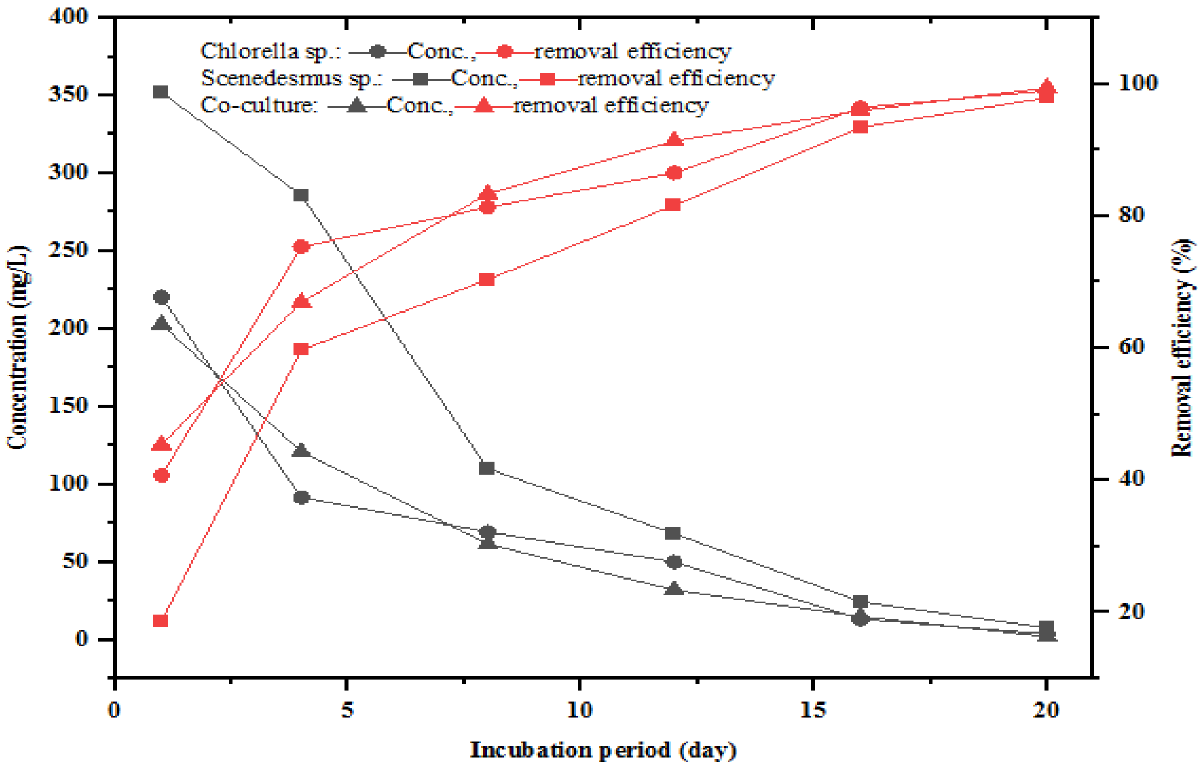


Figure 28: Variation of ammonium concentration and removal efficiency

4.5.2.2. Phosphorous and phosphate removal efficiency and uptake by microalgae

The variation of TP and PO_4^{-3}-P concentrations and RE during the experimental period is shown in Figure 30 a and b, respectively. The concentrations of TP decreased with time, while the RE increased for all the treatments. On the 4th day of the experimental period, about 50, 36, and 62% removal efficiency of TP were achieved for the two-phase AD effluent treated with *Chlorella*, *Scenedesmus*, and co-culture, respectively. The final effluent TP concentration and RE by *Chlorella*, *Scenedesmus*, and co-culture were found to be 5.67 ± 0.58, 19 ± 3.61, 6.67 ± 2.52 mg/L, and 94.28%, 89.22%, and 93.27%, respectively. The final microalgae photobioreactor effluent PO_4^{-3}–P concentrations were 6.33, 4.67, and 3.38 mg/L, with a removal efficiency of 91.08, 93.43, and 95.31% for *Chlorella*, *Scenedesmus*, and their mixture, respectively (Figure 29). The removal rate for both TP and PO_4^{-3}–P was high in the 1st eight days and gradually decreased, as revealed in Figure 30, which is attributed to the uptake of TP and PO_4^{-3}–P by microalgae for biomass formation.

Furthermore, AD effluent phosphorous level reduction by microalgae is mainly due to the phosphate storage in the cytoplasmic presence in the form of polysaccharides, polymers, fatty materials, reproducing biomass, biosorption to the cell wall (Valchev and Ribarova, 2022), and luxury reserves as polyphosphate in suitable circumstances. Similarly, other previous studies also indicated the phosphorous removal by microalgae was through assimilation in growing and duplicating biomass, luxury reserves in aerobic conditions as a source of energy for anaerobic conditions, and biomass used as a bioenergy source (Cormier, 2010; Rybicki, 1997; Solovchenko et al., 2016). Studies have shown different TP removal efficiency ranges between 70 and 90% (Praveen and Loh, 2015; Radin et al., 2017) and 85 and 96.43% (Gao et al., 2015; Katırcıoğlu Sınmaz et al., 2022; Kim et al., 2013; Zheng et al., 2019) for PO_4^{-3}–P by *Chlorella* vulgaris. Lower (31 to 70%) TP removal efficiency by *Scenedesmus* sp. (AlMomani and Örmeci, 2016; Yirgu et al., 2020) and 71.29% of PO_4^{-3}–P (Usha et al., 2016), while higher TP RE of 89–97% (da Fontoura et al., 2017; Shayesteh et al., 2021; Zhou et al., 2012; Zhu et al., 2022) and 100% (Ji et al., 2013) were reported. Likewise, lower PO_4^{-3}–P removal efficiencies between 12–21%, 22–83%, and 57–85% by *Chlorella* sp., *Scenedesmus* sp., and the co-culture of the two, respectively, while TP removal efficiencies of *Chlorella* species were 62.5–74.7%, 75% by *Scenedesmus*, and 86% by the co-culture (Asmare et al., 2014) were also previously indicated. The TP removal efficiency of 100% by *Chlorella* and *Scenedesmus* species noted in Ji et al. (2013) is in line with the findings of this study. Another study by Qin et al. (2016) reported that microalgae co-cultures or consortia showed a better removal efficiency (91–96%) than monocultures of *Chlorella* species (87%). Similarly, in agro-industry processing effluent treated by the two microalgae sp., phosphorus removal efficiency of 20–100% was reported (Cai et al., 2013). Rasala and Mayfield (2015) noted that phosphorus uptake by microalgae from AD effluent is stored in the form of polyphosphate in the microalgae cell. Slaughterhouse wastewater treated by *Chlorella and Scenedesmus* showed a removal efficiency of 69% of PO_4^{-3}–P (Hu et al., 2019). Regarding the removal rates of TP and PO_4^{-3}–P, 0.33 and 0.29 mg/L*day were reported by microalgae used for treating anaerobic digester effluent, respectively. The findings of the study revealed that the TP concentrations attained at the end of the experimental or cultivation period fulfilled the permissible discharge limit for wastewater treatment plant effluent standards suggested by EEPA.

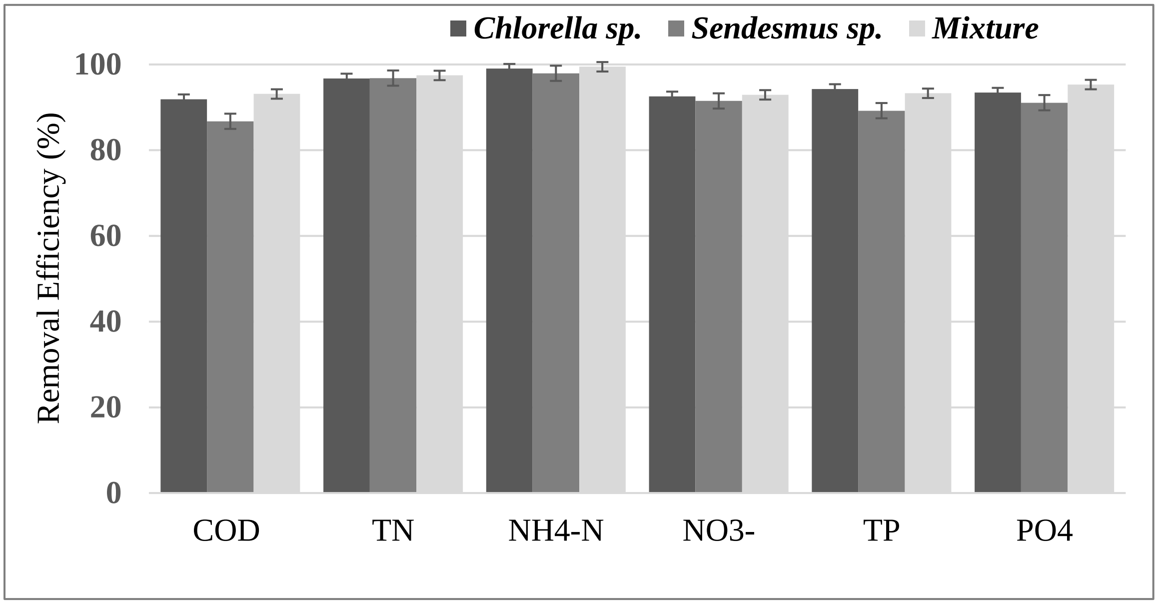
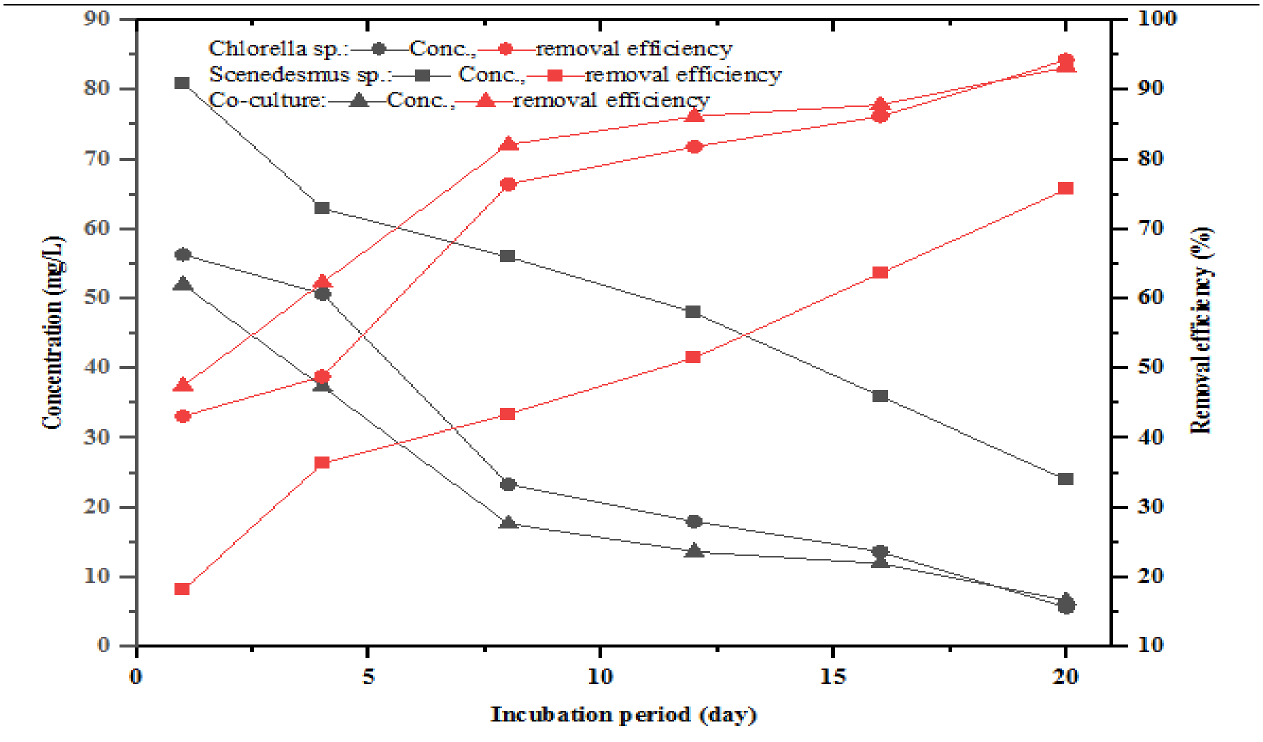
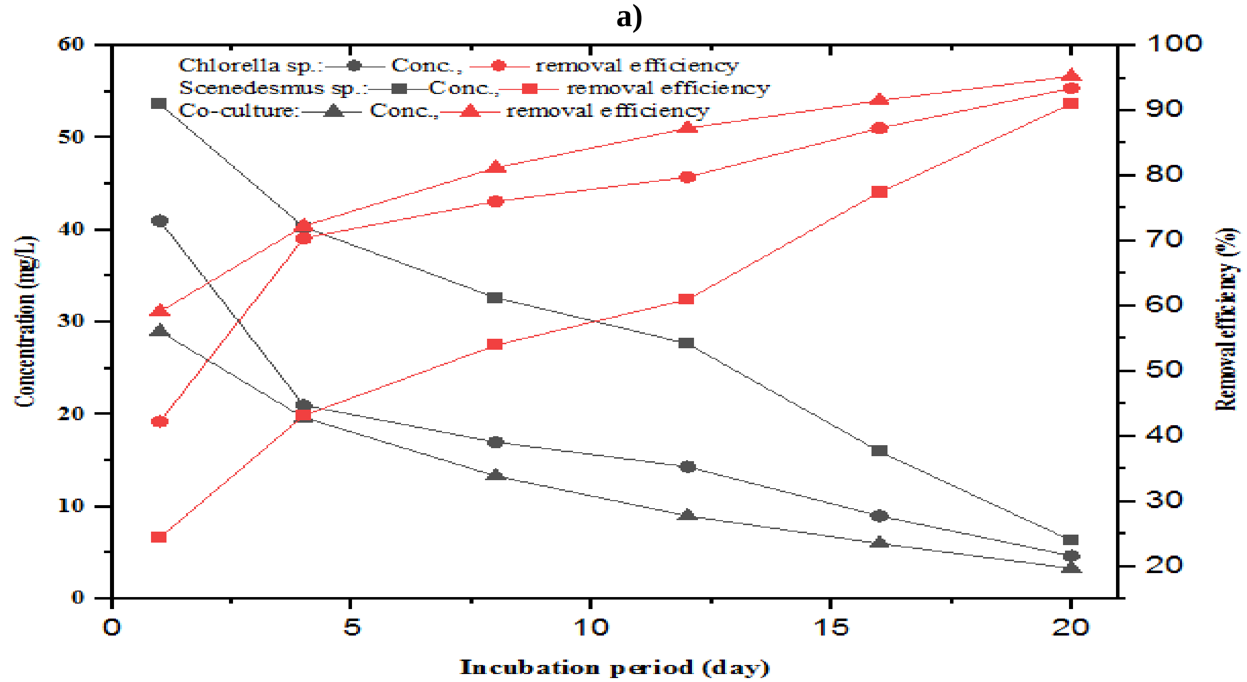


Figure 29: Organic matter and nutrient removal efficiencies by the microalgae & their co-culture



a)



b)

Figure 30: Trends of total phosphorous (a) and ortho-phosphate (b) concentration and removal efficiency

Table 14: Removal rate of COD, TN, NO_3^-–N, NH_4^+–N, TP, and PO_4^{-3}–P

Parameter	*Scenedesmus sp.*	*Chlorella sp.*	Co-culture
	Rr (mg/L*day)	Rr (mg/L*day)	Rr (mg/L*day)
COD	39.47	41.82	42.37
TN	20.82	20.8	20.95
NH_4^+–N	17.22	18.42	18.5
NO_3^-–N	4.3	4.35	4.37
TP	4.42	4.67	4.625
PO_4^{-3}–P	3.23	3.32	3.38

In general, the final values of TN and TP effluent treated with Chlorella, Scenedesmus, and mixture media are lesser than the maximum permissible concentrations level of TN and TP from industrial wastewater treatment plants.

4.5.2.3. Organic matter removal efficiency

Effluent COD concentration variation during the experimental period in which *Chlorella*, *Scenedesmus*, and co-culture microalgae were used to treat the two-phase AD effluent is shown in Figure 31. COD removal efficiency of more than 50 and 80% was achieved after the 4th and 8th days of the incubation or experimental period in all treatments. The COD level in all treatments showed a continuous decrease in the first 15 days of treatment. The final photobioreactor effluent COD concentrations were 73.67 ± 6.51, 120.67 ± 6.51, and 62.67 ± 4.73 mg/L, with removal efficiencies of 87, 92, and 93% for *Chlorella*, *Scenedesmus*, and co-culture, respectively. The variation of mean COD concentrations in the treatments was significant between *Chlorella* and *Scenedesmus* (p-value = 0.01), *Chlorella* and the co-culture (*p*-value = 0.02), but not significant (*p*-value = 0.05) between *Scenedesmus* and the co-culture. The final concentrations of COD was below the Ethiopia effluent discharge limit for slaghterhouse (250 mg/L) in all treatments. The decrease in COD concentrations with the incubation period is attributed to the uptake and usage of COD by microalgae as a source of organic carbon for their cell growth and biomass production, in addition to CO_2. The COD uptake for *Chlorella*, *Scenedesmus*, and co-culture was 41.82, 39.47, and 42.37 mg/L*day, respectively (Figure 31). In general, the concentration of COD in two-phase AD effluent is typically reduced during the incubation of microalgae.

Previous studies also showed that microalgae use organic matter (COD) in partially treated (AD effluent) agro-processing industry effluent for their cell growth (Ding et al., 2015; Wang et al., 2012). The COD RE values of 87–93% noted in this study were consistent with the other study findings using both *Chlorella* and *Scenedesmus* species for agro-processing industry effluent treatment systems, indicating the microalgaes and their co-culture used in this study were capable of effectively growing in partially treated slaughterhouse wastewater (Choi and Lee, 2012; D. Hernández et al., 2016; Otondo et al., 2018). A COD removal efficiency of 89–99% using *Chlorella* sp. was testified in previous research by Zhu et al. (2022); Zheng et al. (2019); Mehta and Chakraborty (2022), which is comparable to this study's finding. A study conducted using *Scenedesmus* sp. for treating agro-processing industry wastewater reported COD and BOD removal efficiencies of 75–80% and 82%, respectively (da Fontoura et al., 2017; Usha et al., 2016). COD removal efficiency of 64.9–76% (Abdel-Raouf et al., 2012; AlMomani and Örmeci, 2016; Liu et al., 2022; Yang et al., 2015) and 83% (Cai et al., 2013) by *Chlorella* sp. from agro-processing industry wastewater, which is lower than this study result. The difference may be due to the reactor type and organic matter sources for microalgae-based wastewater treatment, which is slaughterhouse wastewater effluent treated by two-phase in this study and municipal wastewater in their study. Scholars such as Qin et al. (2016) stated that microalgae co-cultues or consortiums showed better COD removal efficiency (91–96%) than monocultures of *Chlorella* species, and Hena, Fatimah, and Tabassum (2015) reported better growth and stability by conglomerates of microalgae than single culture with 98% nutrients and COD removal efficiency from dairy wastewater.

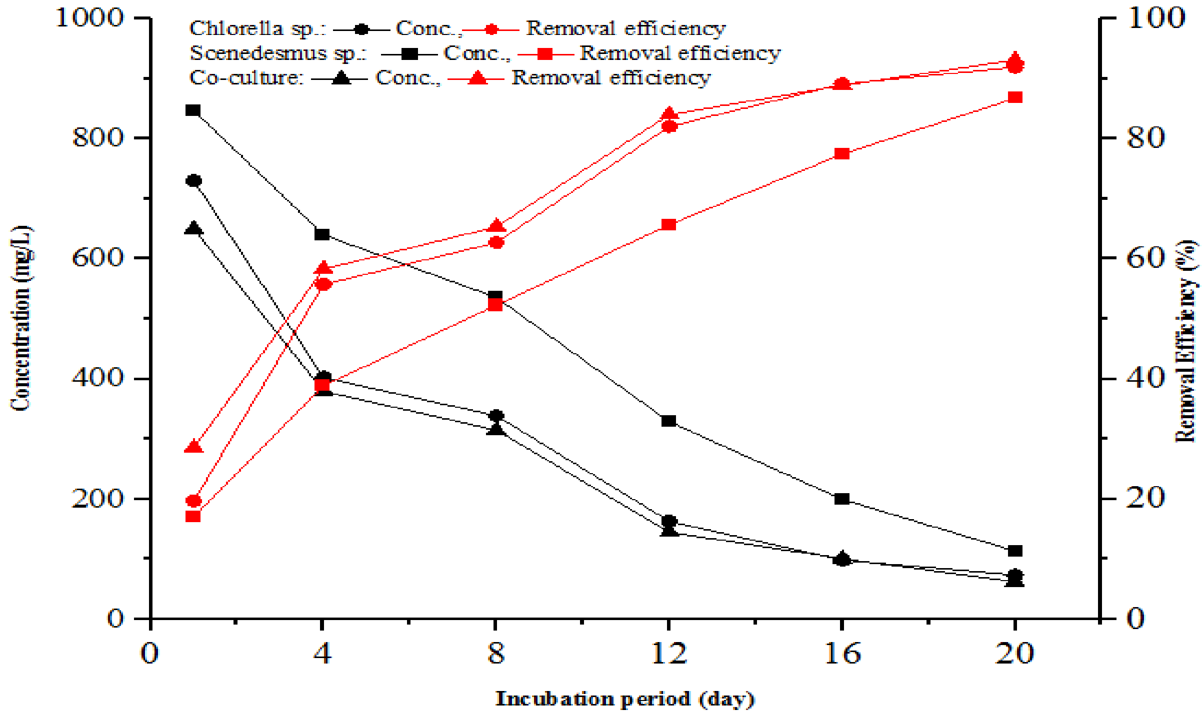


Figure 31: COD concentration and RE variation during the incubation period

In general, studies have demonstrated that the application of *Chlorella* sp. and *Scenedesmus* sp. as well as their co-culture for organic matter removal and nutrient recovery as a tertiary step of agro-processing industry wastewaters is getting due attention, and further advancements are also ongoing in recent days (Hu et al., 2019; Liu et al., 2022; Qin et al., 2016; Wang et al., 2010; Yang et al., 2022; Zhu et al., 2022). Therefore, the implementation of either the individual species microalgae or the consortiums for agro-industry wastewater treatment symbolizes an opportunity to attain more robust treatment techniques or systems that are resilient to antagonistic environments, to minimize the process costs of biomass recovery, and to increase pollutant removal efficiencies.

4.5.3. Microalgae's' biomass for biofuel production

4.5.3.1. Microalgae's biomass yield

The average microalgae biomass produced as well as the productivity of each treatment are indicated in Figure 32. As illustrated in Figure 32, there was a noticeable growth rate of *Chlorella*, *Scenedesmus,* and co-cultures in the two-phase AD effluent. *Chlorella, Scenedesmus, and* co-culture biomass concentrations at the beginning (start) and end of the experiment were 0.11 (1.4 ± 0.1), 0.08 (1.17 ± 0.12), and 0.09 (1.5 ± 0.13) g/L, respectively. The minimum and maximum biomass productivity of *Chlorella*, *Scenedesmus,* and co-culture were 0.095 and 0.26, 0.26 and 0.34, 0.34 and 0.50 g/L*day, respectively. The average biomass yields and growth rate were significantly higher for the co-culture compared to the individual microalgae species ($p \leq 0.05$). The biomass attained for this study may be attributed to the high consumption of the dissolved inorganic and organic N and P in the two-phase AD effluent that are available in PO_4^{-3}–P, NH_4^+–N, and NO_3^-–N, as well as TN and TP forms, which was also inconsistent with the previous finding (García et al., 2002).

Comparing this study's results with the works of other researchers, the following are distinguished: For example, the microalgae's biomass attained in this study was smaller than that reported in Elvira (2022) for both *Chlorella* species (2.13–3.26 g/L) and *Scenedesmus* species (1.46-2.33 g/L). In the research work by F. Fernandes et al. (2022), 0.49 and 0.23 g/L biomass production were attained by *Chlorella vulgaris* and *Scenedesmus* species cultivated in a digestate of pig manure, respectively, using flasks, but a maximum ultimate concentration of 2.49 g/L biomass was reported by Kisielewska et al. (2022) for *Chlorella vulgaris* cultivated in centrifuged agricultural digestate (media-based type) in tubular photobioreactors. Other scholars have shown that the highest microalgae biomass is up to 8.08 g/L for *Chlorella sorokiniana* cultivated in 50% diluted swine wastewater using glass-made vessels (photobioreactors) (Chen et al., 2020). A study conducted in batch mode for the cultivation of *Chlorella vulgaris* in acid-precipitated poultry slaughterhouse wastewater reported comparable biomass production of 1.2 g/L via 83% COD removal efficiency (Hilares et al., 2021). Similarly, biomass productivity reported for microalgae grown on anaerobically treated agro-processing industry effluent varied between 0.1–0.6, 0.2–0.8, and 0.3–1.0 g/L for *Chlorella* species, *Scenedesmus* species, and their co-culture, respectively (Elvira, 2022), which is comparable to this study results. To this end, researchers also noted that *Chlorella* and *Scenedesmus* species can grow competently in anaerobically digested agro-processing industry effluent (Bohutskyi et al., 2015; Kobayashi et al., 2013; Zuliani et al., 2016), though *Spirulina platensis* has also been stated to own the maximum growth and nutrient removal rates, than other microalgae, when cultivated in AD effluent of swine wastewater (Ayre et al., 2017; Kuo et al., 2015; Wang et al., 2010; Xu et al., 2015). Elsewhere, a growth rate of 0.232–0.263, 0.063–0.234, and 0.250–0.289 g/day of *Chlorella*, *Scenedesmus*, and their co-culture cultivated in 10–25% diluted dairy manure, respectively, was also addressed (Asmare et al., 2014). Nevertheless, the variation in the attained microalgae biomass and productivity yields with the results of other research findings would be due to the difference in parameters such as CO_2 supply, light intensity, temperature, bioreactor type, experiment duration, origin, as well as anaerobic digester effluent characteristics during the experiment, which can directly affect the microalgae growth.

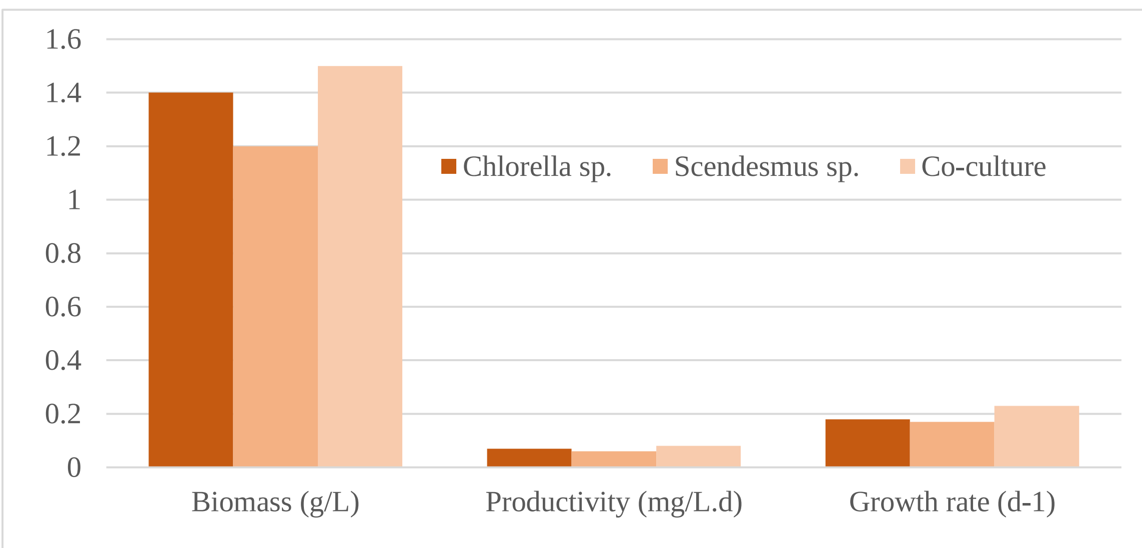


Figure 32: Average biomass yield and productivity of *Chlorella*, *Scenedesmus*, and co-culture

4.5.3.2. Crude lipid content of the microalgae biomass

In this study, the crude lipid content obtained from *Chlorella*, *Scenedesmus*, and co-culture microalgae biomass varied between 13 and 16, 16.7 and 19, 21 and 23, respectively. The total crude lipid content obtained for the co-culture (21.65 ± 1.03%) was higher than that of *Chlorella (*15.70 ± 1.27%) and *Scenedesmus (*17.75 ± 1.08%), respectively. Lipid productivities per dry weight of 0.74, 0.89, and 1.08 g/L*day were obtained from the microalgae biomass of *Chlorella*, *Scenedesmus,* and their co-culture, respectively (Table 15). The variation in lipid content and productivity was significant ($p \leq 0.05$) between the treatments. In the literature, different *Chlorella* species microalgae lipid contents were described. For example, Yao et al. (2015) found lipid content as high as 30%, but Chia et al. (2013) found the lipid content in *Chlorella vulgaris* to be around 5% of total lipid content. Table 15 shows the association in crude lipid level stated in previous and this study finding. The crude lipid content found in this study is within the range indicated in Johanna et al. (2022) for *Chlorella* (5 and 28%) and *Scenedesmus* (14–35%) species and Triampo (2017) for co-culture (15.2–23%). Furthermore, a lipid content of the biomass composition varying from 20 to 40% has been reported, although the levels depend on the microalgae species hence, in certain situations, might be as high as 85% (Koyande et al., 2019; Matos, 2017). In Pancha et al. (2019), it was stated that the microalgae's lipid content cultivated in different wastewaters reported varies between 18 and 79% on a w/w basis. In another study, 20% and 70% crude lipid (oil) content based on the dry weight were reported (Spolaore, 2006; Srimongkol et al., 2022). The variation in lipid content of microalgae's biomass is mainly related to the features of nutrients (e.g., nitrogen, phosphorus, and iron), variations in growth circumstances (e.g., temperature, light, and salinity), or the type or mixture of organic solvent employed for extraction (Johanna et al., 2022). The high crude lipid content difference from microalgae biomass stated in the literature may be attributed to lipid degradation during processing and storage.

Table 15: Comparison of biochemical composition and biomass yield of the microalgae in this study with other studies.

Parameter	***Chlorella*** **sp.**	***Scenedesmus*** **sp.**	**Co-culture**	**References**
Biomass yield (g/L)	0.28–4.81	0.2–1.34	1.63–2.95	Anaid López-Sánchez, 2018, Hilares et al., 2021
	1.4 ± 0.1	1.17 ± 0.12	1.5 ± 0.13 g/L	This study
Biodiesel (%)	44-100	44-57		Anaid López-Sánchez, 2019,
	75.25 ± 3.30	73.5 ± 5.07	77.75 ± 4.03	This study
Lipid content (%)	14-22, 25-58	12.3-32.00	-	Yifan Zhang and Xinyu Zhang 2021, Ting Cai, 2012, Zhang et al. (2014), Kim et al., 2017
	15.70 ± 1.27	17.75 ± 1.08	21.65 ± 1.03	This study

4.5.4.2. Biodiesel yield from the microalgae biomass

The average biodiesel yield and range of the microalgae's biomass are indicated in Table 15. The biodiesel produced from the crude microalgae lipid of *Chlorella* species, *Scenedesmus* species, and their co-culture varies between 72-79%, 67-78%, and 71-81%, respectively (Table 15), showing that the percent indicated of crude lipid is reacted with methanol alcohol. In general, from the lipid and biodiesel content analysis of microalgae biomass in this study, all three (*Chlorella* sp., *Scenedesmus* sp., and co-culture) cultivated in two-phase AD effluent can be noted as proper substrate for biodiesel production. Moreover, the biodiesel obtained from *Chlorella* sp., *Scenedesmus* sp., and co-culture microalgae crude lipid in this study is comparable to results found by different scholars (Birhanu Ayalew, 2014; Coelho, 2017; Xiaochen Ma, 2012). However, the present study finding is lower than previously reported biodiesel yield (94%) from *Chlorella vulgaris* (Ahmad et al., 2013). Though lower value of biodiesel was obtained for this study, it was from the integrated system with an approach of zero waste through circular economy concept. Nevertheless, a higher oil conversion result ranging between 93.52 and 98.15% was attained for *Chlorella* sp. (Farooq Ahmad, 2013; Li et al., 2007), which was attributed to the nutrient contents of the media used to grow microalgae (the effect that wastewater in which microalgae are cultivated has on the growth), extraction methods to generate transesterificable lipids, and catalyst used in the transesterification process. Similarly, Ibarra and Ballen-Segura (2020) obtained 178.95 and 262.5 mg/L of biodiesel from microalgae grown in textile and tannery wastewater, respectively, while 920 and 294 mg/L of biodiesel from *Chlorella* and *Scenedesmus* species cultivated using municipal wastewater were found by Chen, et al. (2011) and Sacristán et al. (2013), respectively.

The reduction of the water quality indicators parameters under study concentrations were high during the initial 10 days of the incubation periods. Furthermore, the final values of TN, TP, and COD in the photobioreactor effluent treated with *Chlorella*, *Scenedesmus*, and co-culture are below the maximum permissible concentration levels of industrial wastewater treatment plants. This is mainly due to the microalgae's uptake of nutrients and organic matter for their growth. Hence, this study results also showed that the utilization of microalgae (C*hlorella* and *Scenedesmus*) collected from the local freshwater body in Ethiopia, namely Lake Awassa, for the

TN, TP, and organic matter removal of anaerobically treated slaughterhouse wastewater has dual importance: resource recovery, biodiesel production and wastewater treatment.

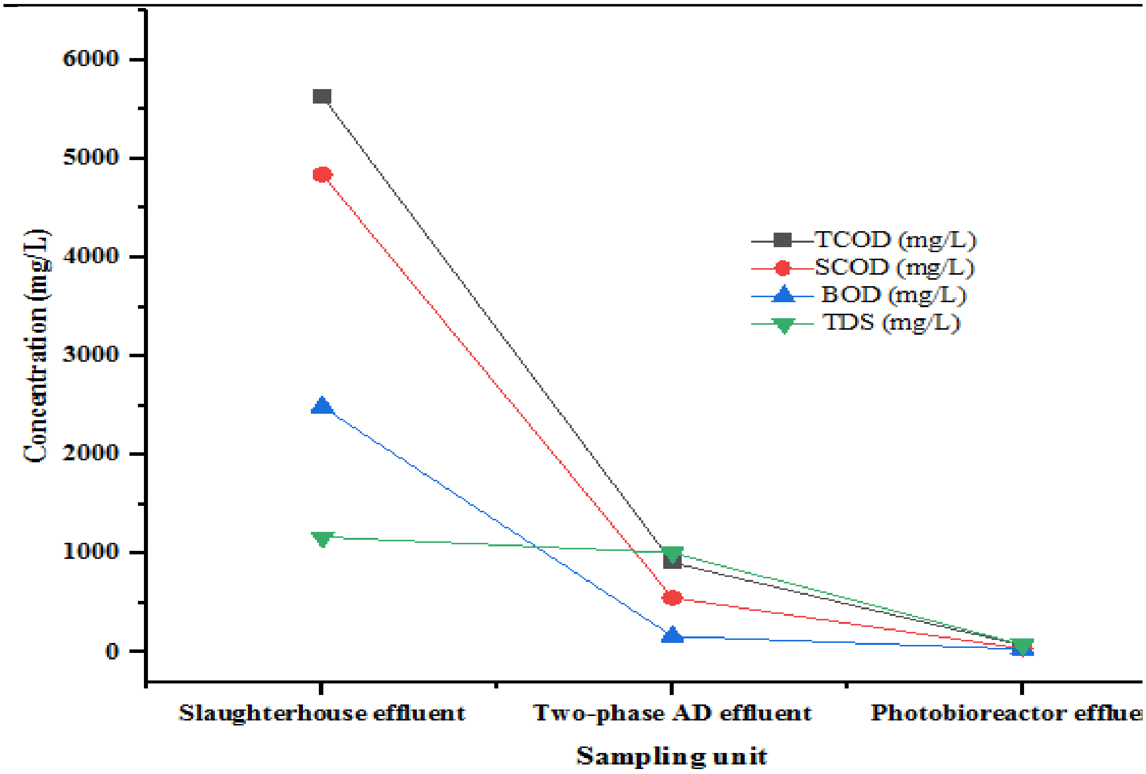
4.6. Comparison of Two-Phase AD and Microalgae Photobioreactor and Performance Evaluation of the Overall system

This section presents the comparison of the components (two-phase and microalgae photobioreactors), as well as the overall removal efficiencies of the integrated biological treatment process for both organic and inorganic pollutants.

4.6.1. Comparison of the two-phase AD and microalgae photobioreactor for pollutant removal efficiency

4.6.1.1. Organic matter removal efficiency

The organic matter such as TCOD, SCOD, BOD, TDS, and turbidity concentration of SHWW, two-phase AD effluent, and photobioreactor effluent in which *Chlorella* species was employed as a polishing unit were 5366.43 ± 83.80, 910 ± 7.82, 73.67 ± 6.51 mg/L; 4842.21 ± 83.81, 555.23 ± 106.25, 40.40 ± 10.67 mg/L; 2487.48 ± 594.54, 166.10 ± 51.40, 35.15 ± 13.88 mg/L; 1170.74 ± 399.84, 1011.41 ± 138.24, 81.28 ± 11.37 mg/L, and 566.5 ± 18.5 NTU, 11 ± 2.160 NTU, 1.95 ± 0.87 NTU, respectively. The TCOD, SCOD, BOD, TDS, and turbidity concentrations of SHWW, two-phase AD effluent, and photobioreactor effluent treated by *Scenedesmus* sp. were 5366.43 ± 83.80, 910 ± 7.82, 120.67 ± 6.51; 4842.21 ± 83.81, 555.23 ± 106.25, 38.17 ± 7.66 mg/L; 2487.48 ± 594.54, 166.10 ± 51.40, 32.74 ± 42.03 mg/L; 1170.74 ± 399.84, 1011.41 ± 138.24, 83.01 ± 16.79 mg/L, and 566.5 ± 18.5 NTU, 11 ± 2.160 NTU, 2.77 ± 1.31 NTU, respectively. Similarly, the average TCOD, SCOD, BOD, TDS, and turbidity concentrations of SHWW, two-phase AD effluent, and photobioreactor effluent in which co-culture was used for the treatment were 5366.43 ± 83.80, 910 ± 7.82, 62.67 ± 4.73 mg/L; 4842.21 ± 83.81, 555.23 ± 106.25, 33.59 ± 10.53 ± 7.66 mg/L; 2487.48 ± 594.54, 166.10 ± 51.40, 31.00 ± 8.78 mg/L; 1170.74 ± 399.84, 1011.41 ± 138.24, 73.00 ± 10.15 mg/L, and 566.5 ± 18.5 NTU, 11 ± 2.160 NTU, 1.72 ± 0.67 NTU, respectively (Table 16). TCOD, SCOD, BOD, TDS, and turbidity removal efficiency of 82.87%, 88.53%, 93.32%, 73.35%, and 98.06%; 97.86-98.89%, 99.17-99.31%, 98.59-98.75%, 92.91-93.76%, and 99.51-99.70% were achieved at the two-phase AD and polishing steps by microalgae's photobioreactor, respectively (Figure 34). In all cases, the TCOD, SCOD, BOD, TDS, and turbidity concentration decreased as it moved from the AD to the microalgae-photobioreactor unit (Figure 33; Figure 34). As denoted in Figure 33 and Figure 34, most of the organic matter (TCOD, SCOD, BOD, TDS, and turbidity) was removed in the two-phase AD, while their residues were removed at the polishing step in the microalgae photobioreactor. The variations of TCOD, SCOD, BOD, TDS, and turbidity removal efficiency in the two-phase AD system and microalgae's photobioreactor were significant ($p \leq 0.05$).



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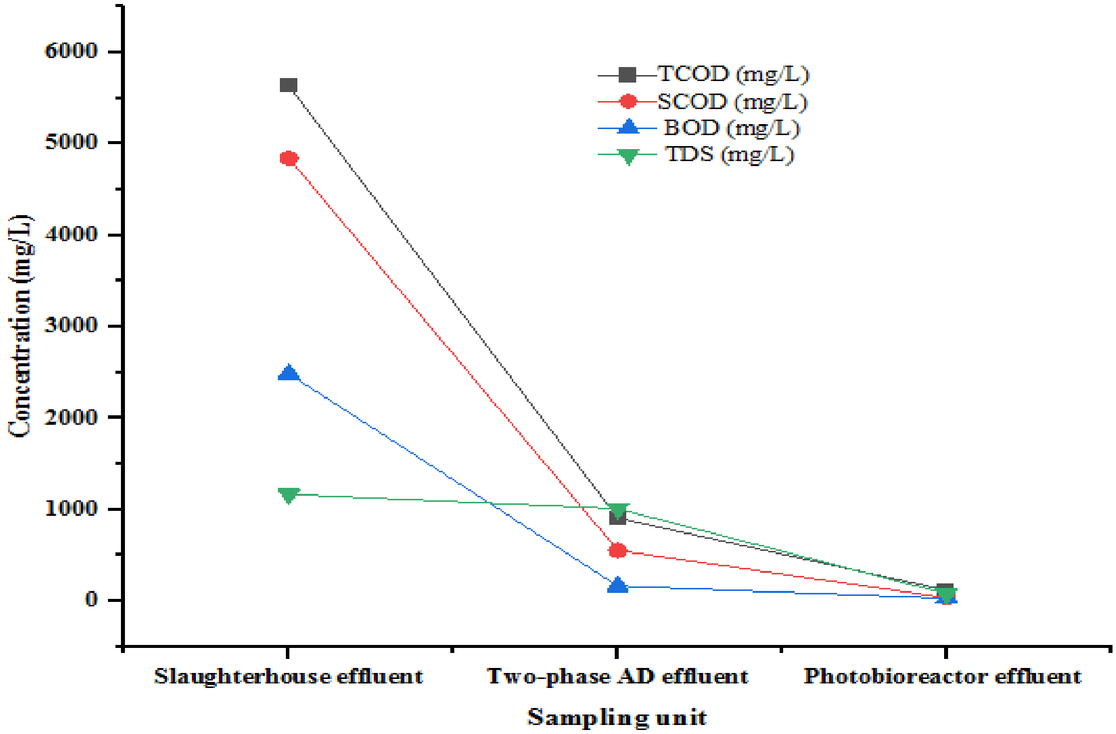

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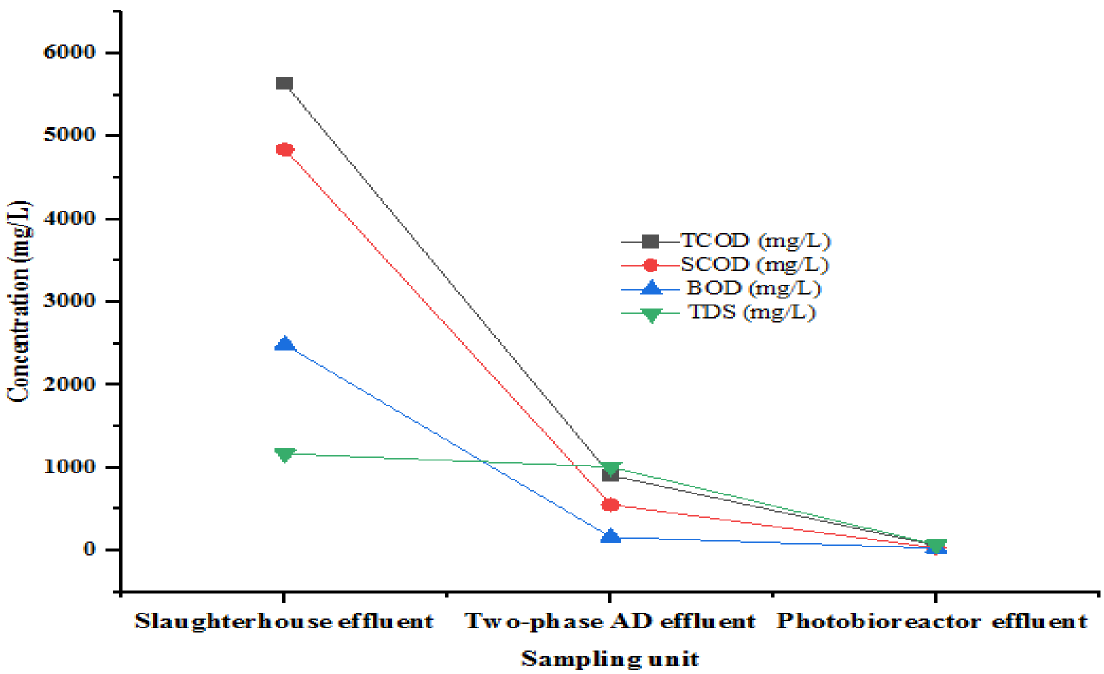

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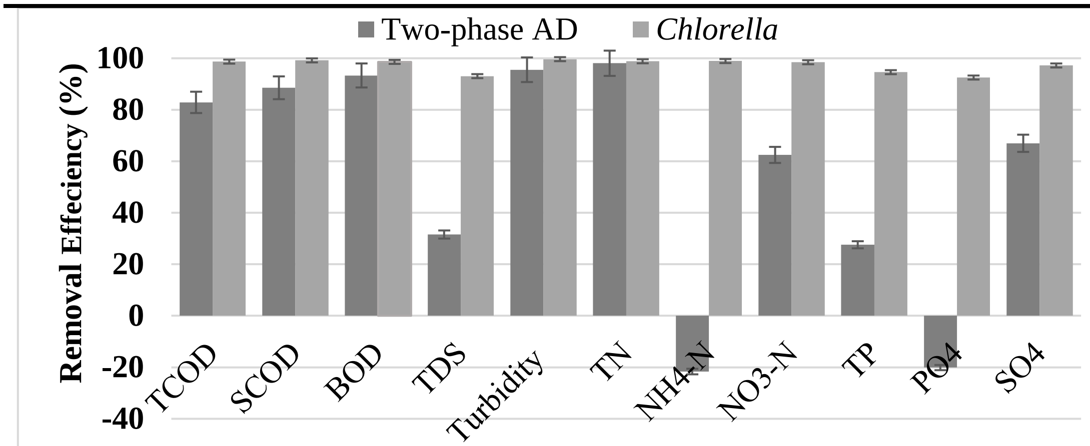
Figure 33: Comparisons of two-phase-AD and photobioreactors organic matter effluent concentration (a) *Chlorella* sp., (b) *Scenedesmus* sp. and (c) co-culture



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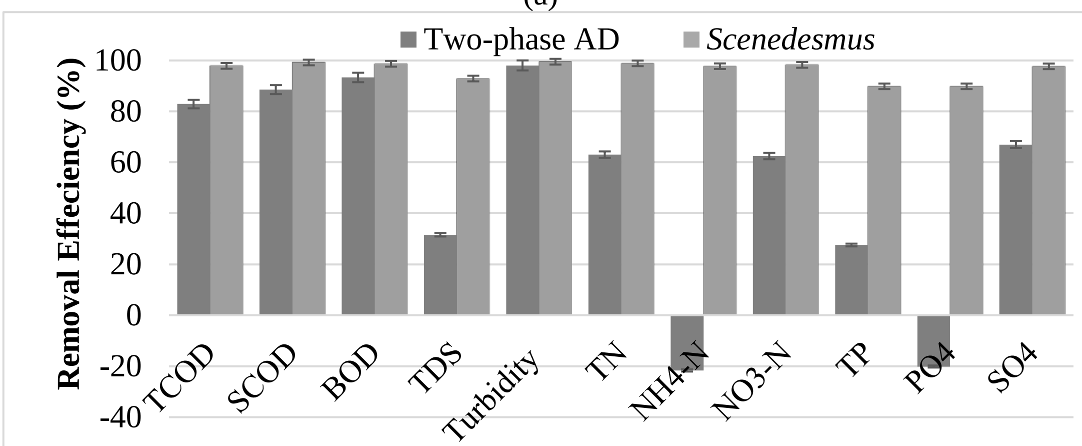

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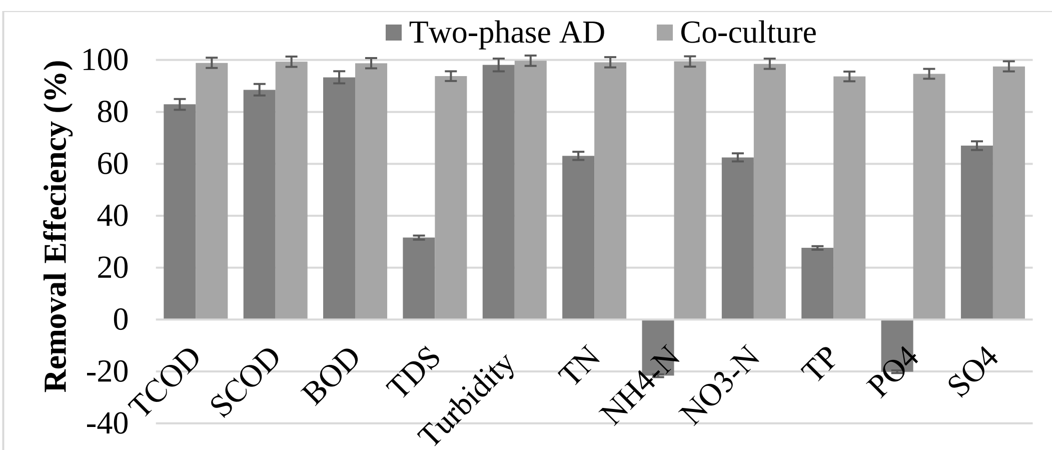
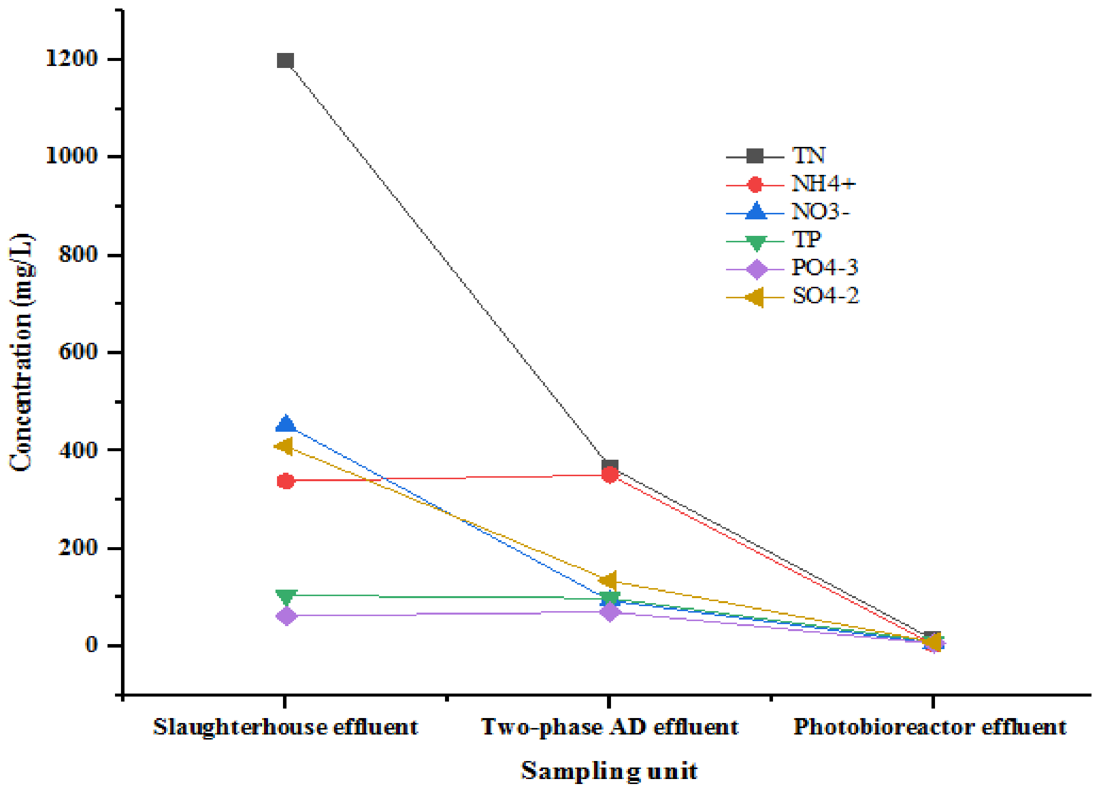


(c)

Figure 34: Comparisons of two-phase-AD and photobioreactors pollutant removal efficiency (a) Chlorella sp., (b) Scenedesmus sp. and (c) co-culture

4.6.1.2. Nutrient removal efficiency

The TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P concentrations of SHWW, two-phase AD effluent, and photobioreactor effluent with *Chlorella* sp. were 1198.45 ± 145.29, 367.33 ± 8.5, 14 ± 3.61 mg/L; 338.4 ± 58.13, 351.33 ± 18.72, 3.67 ± 1.53 mg/L; 453.58 ± 81.47, 94 ± 7, 7 ± 1 mg/L; 105 ± 12.15, 99 ± 6, 5.67 ± 0.58 mg/L and 62.333 ± 15.948, 71.33 ± 14.57, 4.67 ± 1.53 mg/L, respectively. In the same way, for the integrated system in which *Scenedesmus* sp. microalgae and the co-culture were used as the polishing step, TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P photobioreactor effluent concentrations were 13.67 ± 1.53, 11 ± 2.65 mg/L; 7.67 ± 1.53, 2 ± 1 mg/L; 8 ± 2, 6.67 ± 1.53 mg/L; 10.67 ± 4.16, 6.67 ± 2.52 mg/L and 6.33 ± 3.21, 3.33 ± 1.53 mg/L, respectively (Table 16; Figure 35). Removal efficiencies of 64.11%, -13.01%, 27.59%, -20.97%, and 59.51% for TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P^2, respectively, were attained in two-phase AD system. Likewise, at the polishing phase by microalgae photobioreactors, a higher removal efficiency between 98.86-99.10, 97.73-99.41%, 98.24-98.53%, and 89.84-94.60% were obtained for TN, NH_4^+–N, TP, and PO_4^{-3}–P, respectively (Figure 34). The concentrations of TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P decreased as they moved from the two-phase AD to the microalgae's photobioreactor (Table 16; Figure 34; Figure 35). As denoted in Figure 34, TN, NO_3^-–N, and TP removal efficiencies were low in the two-phase AD, and most of the TN, NO_3^-–N, and TP were removed at the polishing step by the microalgae photobioreactor. On the contrary, two-phase AD system showed negative removal efficiencies for NH_4^+-N & PO_4^{-3}–P. The variations of TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P removal efficiency in a two-phase AD system and a microalgae photobioreactor in which *Chlorella* sp., *Scenedesmus* sp., and co-culture cultivated were significant ($p \leq 0.05$). Furthermore, as low reductions of TN, NO_3^-–N, TP, and PO_4^{-3}–P are usually expected in AD systems, higher reductions in TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P were attained in the polishing (microalgae photobioreactor) step (Table 16; Figure 34; Tsegaye and Leta, 2023). The decrease in the TN concentration in the effluent of the two-phase system is attributed to the conversion of nitrogen-containing compounds in the wastewater to NH_4^+-N in the AD system by the consortium of microorganisms in the system, and a decrease in concentration and subsequent increase in the TP concentration were attributed to conversion to PO_4^{-3}–P, which is consistent with the conclusion made by scholars (Mburu et al., 2012; Mohan et al., 2005). But the integration of microalgae's photobioreactor into the two-phase AD system removed the higher TN, NH_4^+–N, NO_3^-–N, TP, and PO_4^{-3}–P levels in AD to the level acceptable by the EPA of the country (Ethiopia) for slaughterhouse industry effluent before releasing it to the environment (Table 16; Figure 34; Tsegaye and Leta, 2023).



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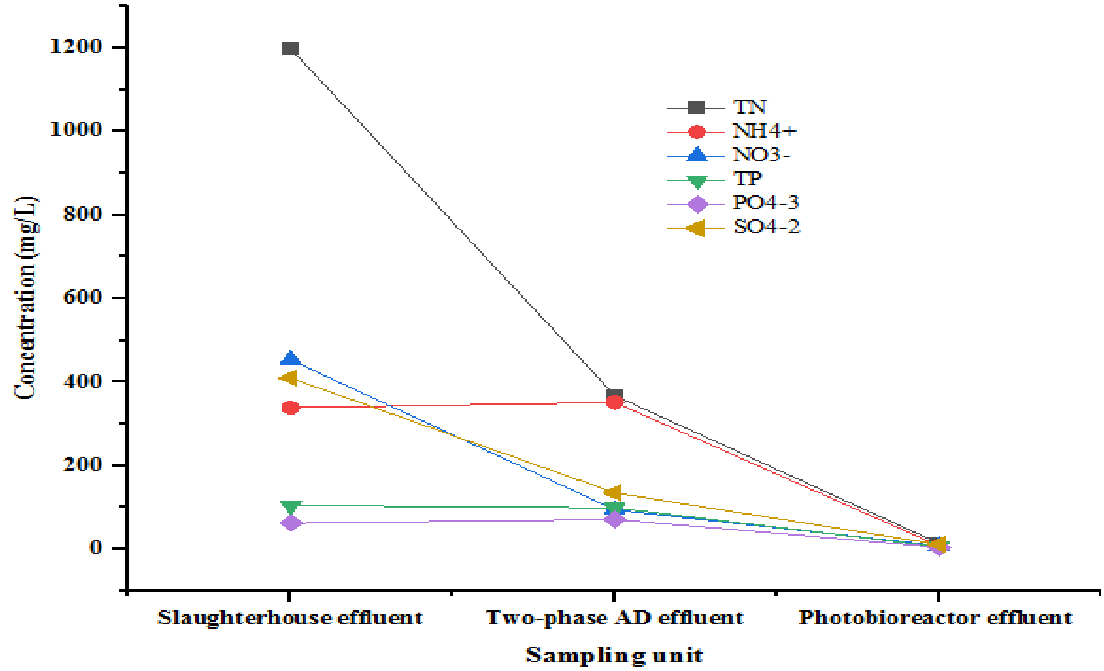

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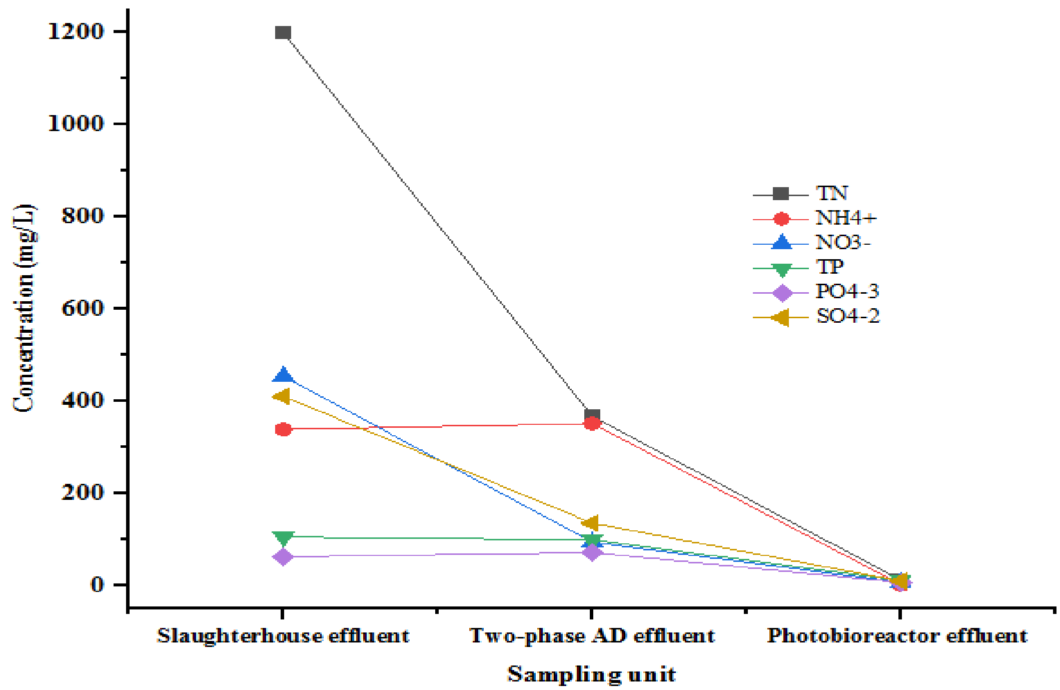

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Figure 35: Comparisons of two-phase AD and photobioreactors nutrient effluent concentration (a) *Chlorella*, (b) *Scenedesmus* and (c) co-culture

4.6.1.3. Sulfate removal efficiency

The SO_4^{-2} concentrations of SHWW, two-phase AD effluent, and photobioreactor effluent with *Chlorella* sp. were 410.33 ± 12.72, 135.50 ± 44.31, and 11.49 ± 7.38 mg/L, respectively. In the same way, for the integrated system in which *Scenedesmus* sp. microalgae and the co-culture were used as the polishing step, SO_4^{-2} photobioreactor effluent concentrations were 9.47 ± 6.51, 10.09 ± 4.74 mg/L, respectively (Table 16; Figure 35). At the polishing step by microalgae photobioreactors, a higher removal efficiency between 97.20-97.69% were obtained for SO_4^{-2} (Figure 34). The concentrations of SO_4^{-2} decreased as it moved from the two-phase AD to the microalgae's photobioreactor (Table 16; Figure 34; Figure 35). As denoted in Figure 34, SO_4^{-2} removal efficiency were low in the two-phase AD, and most of the SO_4^{-2} was removed at the polishing step by the microalgae photobioreactor. The variation of SO_4^{-2} removal efficiency in a two-phase AD system and a microalgae photobioreactor in which *Chlorella* sp., *Scenedesmus* sp., and co-culture cultivated was significant ($p \leq 0.05$). A little or no reduction of nitrate and sulfate was observed in a two-phase AD system, which can be attributed to a sulfate reduction and ammonification of the anaerobic microbial process in an oxygen-free environment.

4.6.2. Performance of the integrated system for pollutant removal efficiencies

4.6.2.1. Organic matter removal efficiency

The organic matter removal efficiency of the bench-scale integrated biological treatment system composed of the two-phase AD and a microalgae photobioreactor as a polishing step was above 90%. The final effluent BOD, SCOD, and TCOD concentrations found for the photobioreactor in which *Chlorella* sp., *Scenedesmus* sp., and their co-culture were cultivated were 73.67 ± 6.51, 120.67 ± 6.51, and 62.67 ± 4.73, respectively. The average overall BOD, SCOD, and TCOD removal efficiency found in this study for *Chlorella* species, *Scenedesmus* species, and the co-culture was 98.59, 98.68, and 98.75%; 99.17, 99.21, and 99.31%; 98.69, 97.86, and 98.89%, respectively. Similarly, the overall removal efficiency of TDS and turbidity of an integrated two-phase AD and microalgae photobioreactor system was 93.06, 92.91, 93.76%, and 99.66, 99.51, 99.7% by *Chlorella* species, *Scenedesmus* species, and co-culture, respectively (Figure 36). The results showed that the organic pollutant removal efficacy of the integrated treatment system was found to be very high, demonstrating that the integrated biological system significantly reduced the organic matter to a level that meets the required environmental quality standards of the country (Ethiopia) (Environmental Protection Agency (EEPA), 2002).

Higher organic matter removal efficiency is typically indicated for AD effluent subjected to microalgae-based treatment systems in the literature. For example, Ding et al. (2015), Gentili (2014), Hena et al. (2015), Huo et al. (2012), Jia and Yuan (2018), Qin et al. (2016), Sacristán de Alva et al. (2013), Travieso et al. (2008), Yirgu et al. (2020), and Zhu et al. (2022) reported comparable BOD, SCOD, and COD removal efficiency ranging between 90 and 98% by *Chlorella* species microalgae cultivated in anaerobically treated agro-processing industry effluent for safe discharge into the environment through substantial microalgae biomass production. In addition, many studies have demonstrated that microalgae can reduce nitrogen by up to 80% (Kusmayadi et al., 2022; Qian et al., 2022; Zhu et al., 2013). Moreover, several studies have also reported a higher COD removal efficiency of 80.0% to complete removal (100.0%) by *Chlorella* species (Huo et al., 2012). For example, *Chlorella vulgaris* microalgae cultivated in swine manure digestate loaded with high COD and a microalgae cultivated in unsterilized dairy wastewater showed a COD removal efficiency between 84.18 and 89.70% in an eight-day incubation period at a lab-scale (Chen et al., 2020; Ding et al., 2020; Kusmayadi et al., 2022; Tao et al., 2017). Hende et al. (2016) and Lu et al. (2015) also found a higher COD removal rate of 65 and 88.38 mg/L*day, respectively, for the microalgae cultivated in an anaerobically treated effluent. On the other hand, a lower COD removal efficiency of 34% was reported for AD microalgae-based agro-processing industrial wastewater treatment facilities (Lu et al., 2015). In general, it was concluded in many studies that *Chlorella* species microalgae are capable of instantaneous utilization of nutrients and COD removal when used in wastewater treatment (Kumar et al., 2019; Silveira et al., 2017).

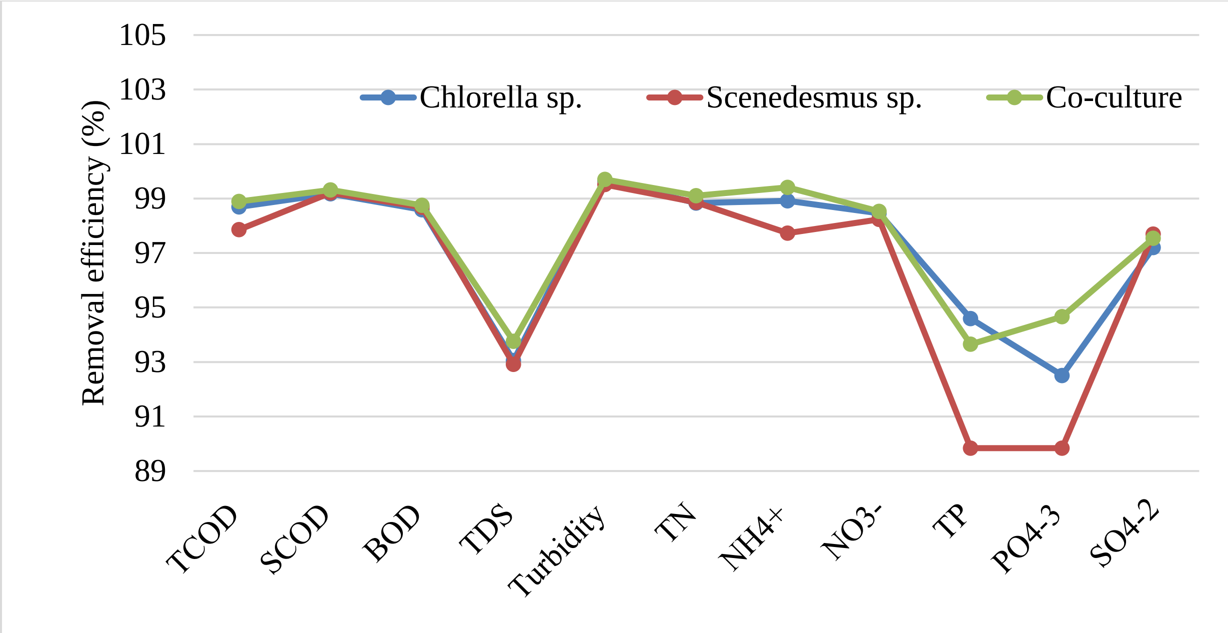

Figure 36: Overall pollutant removal efficiency of integrated system

4.6.2.2. Nutrient removal efficiency

The mean final TN, NH_4^+-N, NO_3^-–N, TP, and PO_4^{-3}–P effluent concentrations were 14 ± 3.61, 13.67 ± 1.53, 11 ± 2.65 mg/L; 3.67 ± 1.53, 7.67 ± 1.53, 2 ± 1 mg/L; 7 ± 1, 8 ± 2, 6.67 ± 1.53 mg/L; 5.67 ± 0.58, 10.67 ± 4.16, 6.67 ± 2.52 mg/L, and 4.67 ± 1.53, 6.33 ± 3.21, 3.33 ± 1.53 mg/L, by *Chlorella*, *Scenedesmus*, and co-culture, respectively (Table 16).

The nutrient RE (%) in terms of TN, NH_4^+-N, NO_3^-–N, TP, and PO_4^{-3}–P by the integrated system was greater than 90%. More specifically, the TN, NH_4^+-N, NO_3^-–N, TP, and PO_4^{-3}–P removal efficiencies by *Chlorella*, *Scenedesmus*, and Co-culture were 98.83, 98.86, 99.1%; 98.92, 97.73, 99.41%; 98.46, 98.24, 98.53%; 94.6, 89.84, 93.65%; 92.51, 89.84, and 94.66%, respectively (Figure 36). The corresponding effluent concentrations obtained by the integrated system for TN, NH_4^+-N, NO_3^-–N, TP, and PO_4^{-3}–P were below the country's slaughterhouse or meat processing effluent discharge limit (EPA, 2003).

The TN removal efficiency found in this study is in line with previous research results by different scholars, Yirgu et al. (2020) and Musa et al. (2018), who reported 94.36-96.08%, 99.20%, 91.90%

of TN by *Scenedesmus* sp. microalgae cultivated in AD effluent agro-processing industry wastewater. Literature also indicated that the removal efficiency of more than 90% to complete removal of NH_4^+-N attained by *Chlorella* species, *Scenedesmus* species, or the co-culture used or applied in tertiary agro-processing industry effluent treatment (Marchão et al., 2018; Yirgu et al., 2020). Subramaniyam et al. (2016) and Lu et al. (2015) found a complete removal efficiency of PO_4^{-3}–P, while A. A. Ansari et al. (2017), F. A. Ansari et al. (2017), and Luo et al. (2019) reported TP removal efficiency between 85-88% by *Scenedesmus* sp. microalgae. Furthermore, different researchers, Alemu et al. (2018), Mekonnen et al. (2017), and Yirgu et al. (2020), also pinpointed the effectiveness of integrated biological treatment systems compared to conventional treatment schemes for agro-processing industrial wastewater. In several studies, it has been shown that microalgae can reduce nitrogen by up to 85% (Li, Zhou, et al., 2011; Rasoul-Amini et al., 2014). Similarly, Cai et al. (2013), Huo et al. (2012), Jia and Yuan (2018), Wang et al. (2012), Wang et al. (2010), and Zhu et al. (2013) have also reported 80–100% nitrogen and 83.2–100.0% phosphorous removal, which discloses the high nutrient removal efficiency of *Chlorella* sp. cultivated on anaerobic piggery digestate. In line with this, *Chlorella zofingiensis* microalgae from freshwater sources employed to treat piggery wastewater removed 90-100% of TP (Chen et al., 2020; Kusmayadi et al., 2022; Zhu et al., 2013). Furthermore, for dairy wastewater in which an integrated AD-microalgae phycoremediation was employed, a 93.0 ± 2.0% removal efficiency of both TP and TN was noted (Kusmayadi et al., 2022). Similarly, Jia and Yuan (2018), Luo et al. (2019), and Praveen and Loh (2015) found higher removal efficiency, which is comparable with this study's results of greater than 97% for both NH_4^+-N and PO_4^{-3}-P using microalgae as a polishing step for AD effluent. Similarly, *Chlorella vulgaris* microalgae cultivated in swine manure digestate showed 97.2% and 99.7% removal efficiency for TN and TP, respectively (Tao et al., 2017). *Chlorella sorokiniana* cultivated in AD digestate achieved TN removal efficiencies of 95.3 ± 1% in 18 days of incubation (Sayedin et al., 2020). Cultivation of microalgae at a lab-scale in unsterilized dairy wastewater showed 83.20–99.26% and 89.92–91.97% removal efficiency of NH_4^+-N and TP, respectively, in eight-days of incubation (Ding et al., 2015). However, studies have also reported removal efficiency as low as 50.2 and 65 for TN and TP in anaerobically treated agro-processing industry wastewater coupled with microalgae-based treatment techniques (Kumar et al., 2019; Qian et al., 2022). Literature also concludes that the microalgae *Chlorella* species are capable of concurrent use of nutrients and organic carbon during wastewater treatment (Kumar et al., 2019; Silveira et al., 2017). In Qian et al. (2022), maximum RE of 58, 88, and 65% for TN, NH_4^+−N, and TP in the partially (anaerobically) treated piggery effluent were reported, respectively, at an initial pH of 9.0. The uptake of phosphate by microalgae is an active process that requires energy, which is stockpiled in the cells and is assimilated in the form of polyphosphate granules by microalgae (Larsdotter, 2006). Huo et al. (2012) evaluated the feasibility of cultivating *Chlorella zofingiensis* using DW in outdoor ponds with pH regulation by CO_2 and acetic acid and achieved a maximum TN removal percentage of 51.7% in 5 days. The maximum TN and TP removal rates acquired in indoor cultivation circumstances were 9.8-38.34 and 2.03 mg/L*day, respectively, by Van Den Hende et al. (2016). The variations in removal efficiencies for TN, NO_3^-–N, NH_4^+−N, PO_4^{-3}-P, and TP by microalgae in the anaerobically treated agro-processing industry may be attributed to the following experimental conditions: media type, photoperiod, temperature, pH, microalgae type, and bioreactor type.

4.6.2.3. Sulfate removal efficiency

The mean final effluent SO_4^{-2} concentrations was 11.49 ± 7.38, 9.47 ± 6.51, and 10.09 ± 4.74 mg/L for photobioreactor in which *Chlorella*, *Scenedesmus*, and co-culture grown, respectively (Table 16). The SO_4^{-2} RE (%) by *Chlorella*, *Scenedesmus*, and Co-culture were 97.20%, 97.69%, and 97.54%, respectively (Figure 36). The integration of microalgae's photobioreactor into the two-phase AD system removed the higher SO_4^{-2} level in AD to the level acceptable by the EPA of the country (Ethiopia) for slaughterhouse industry effluent before releasing it to the environment (Table 16; Figure 34; Tsegaye and Leta, 2023).

Table 16: The average effluent pollutant concentration or values of the integrated treatment parts

Parameter	Slaughterhouse wastewater	HR effluent	MR effluent	Photobioreactor effluent		
				***Chlorella* sp.**	***Scenedesmus* sp.**	**Co-culture**
TCOD (mg/L)	5366.43 ± 83.80	4944.75 ± 241.75	910 ± 7.82	73.67 ± 6.51	120.67 ± 6.51	62.67 ± 4.73
SCOD (mg/L)	4842.21 ± 83.81	3430.2 ± 800.44	555.23 ± 106.25	40.40 ± 10.67	38.17 ± 7.66	33.59 ± 10.53
BOD (mg/L)	2487.48 ± 594.54	1174.68 ± 195.36	166.10 ± 51.40	35.15 ± 13.88	32.74 ± 42.03	31.00 ± 8.78
TDS (mg/L)	1170.74 ± 399.84	1576.47 ± 106.60	1011.41 ± 138.24	81.28 ± 11.37	83.01 ± 16.79	73.00 ± 10.15
Turbidity	566.5 ± 18.5	47.00 ± 10.20	11 ± 2.160	1.95 ± 0.87	2.77 ± 1.31	1.72 ± 0.67
TN (mg/L)	1198.45 ± 15.29	514.0 ± 105.1	367.33 ± 8.5	14 ± 3.61	13.67 ± 1.53	11 ± 2.65
NH_4^+ (mg/L)	338.4 ± 58.13	369.46 ± 11.28	351.33 ± 18.72	3.67 ± 1.53	7.67 ± 1.53	2 ± 1
NO_3^- (mg/L)	453.58 ± 81.47	187.3 ± 40.4	94 ± 7	7 ± 1	8 ± 2	6.67 ± 1.53
TP (mg/L)	105 ± 1215	145.25 ± 17.29	99 ± 6	5.67 ± 0.58	10.67 ± 4.16	6.67 ± 2.52
PO_4^{-3} (mg/L)	62.33 ± 15.95	98.25 ± 14.59	71.33 ± 14.57	4.67 ± 1.53	6.33 ± 3.21	3.33 ± 1.53
SO_4^{-2} (mg/L)	410.33 ± 15.61	220. 0 ± 63.8	135.5 ± 44.311	11.49 ± 7.38	9.47 ± 6.51	10.09 ± 4.74

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

In this study, a bench-scale integrated system composed of two-phase anaerobic digestion coupled with microalgae photobioreactors can be considered as greener management options for slaughterhouse wastewater through conversion to bioenergy, and effluent quality fulfilling the country (Ethiopia) discharge limit.

HR enhanced performance indicator parameters such as DH, DA, SCOD, and TVFA values of 63.92%, 53.26%, 3430.20 ± 80.44 mg/L, and 1176.50 ± 81.66 mg/L, respectively, as well as NH_4^+-N level in the reactor that does not disturb the microorganisms were found at HRT of three days and OLR of 1788 of COD/L*day. Operating parameters HRTs and OLRs affected the results of the performance parameters at steady state ($p \leq 0.05$).

For the methanogenesis phase, a pH of nearly neutral (6.92) that favors the methanogens, a TVFA:TotA ratio of 0.36, which is in the optimum range, the highest alkalinity that maintains the buffering capacity of the reactor, and a non-inhabiting concentration of NH_4^+–N (382 mg/L) obtained at an HRT of six days and an OLR of 298 mg COD/L. The TCOD and SCOD removal efficiency increases as HRT decreases from 12-6 days, but a further decrease in HRT decreases the removal efficiency of both. The highest BOD removal efficiency was achieved at HRTs or OLRs of 12 days or 149 mg of COD/L. The highest biogas production of 185 mL, methane content (67%), and methane production rate (123 mL/day) were also found at an HRT of six days and an OLR of 298 mg COD/L*day. The result of the overall two-phase AD system operated at stable and better performance of the two-phase system was HR and MR the concentration organic matter as well as nutrient were above the standard set for slaughterhouse industry.

The polishing phase, microalgae photobioreactor in which *Chlorella* and *Scenedesmus* species as well as the co-culture used for the treatment of anaerobically treated slaughterhouse effluent, have shown encouraging nutrients and organic matter removal efficiency. Accordingly, removal efficiency varied between 86.74–93.11%, 96.74–97.47%, 91.49–92.91%, 97.94–99.46%, 89.22–

94.28%, and 91.08–95.31% were attained for chemical oxygen demand, total nitrogen, nitrate, ammonium, total phosphorous, and orthophosphate by *Chlorella* sp., *Scenedesmus* sp., and their co-culture, respectively.

The corresponding *Chlorella* species, *Scenedesmus* species, and co-culture biomass yield and productivity were 1.4 ± 0.1, 1.17 ± 0.12, 1.5 ± 0.13 g/L, and 0.18, 0.21, 0.23 g/L*day, respectively. Though it is not significant ($p \leq 0.05$), higher biomass and productivity via higher removal efficiencies of the pollutants were realized by the microalgae co-culture than monocultures. The total crude lipid content obtained for the co-culture (21.65 ± 1.03%) was higher than that of *Chlorella (*15.70 ± 1.27%) and *Scenedesmus (*17.75 ± 1.08%), respectively. In general, from the lipid and biodiesel content analysis of microalgae biomass in this study, all three; *Chlorella* species, *Scenedesmus* species, and co-culture cultivated in two-phase AD effluent can be noted as suitable feedstock for biodiesel.

The average overall pollutant removal efficiency of the integrated treatment system for BOD, SCOD, and TCOD by *Chlorella*, *Scenedesmus,* and their co-culture attained was 98.59, 98.68, 98.75%; 99.17, 99.21, 99.31%; 98.69, 97.86, 98.89%, respectively. Similarly, TN, NH_4^+-N, PO_4^{-3}–P, TP, and SO_4^{-2} removal efficiency of 98.83, 98.86, 99.1%; 98.92, 97.73, 99.41%; 98.46, 98.24, 98.53%; 94.6, 89.84, 93.65%; 92.51, 89.84, 94.66%, and 97.20, 97.69, 97.54% were attained by *Chlorella*, *Scenedesmus*, and Co-culture, respectively. The final BOD, SCOD, and TCOD TN, NO_3^-–N, NH_4^+-N, SO_4^{-2}, TP, and PO_4^{-3}–P effluent concentrations of the integrated system were below the country's slaughterhouse or meat processing effluent discharge limit.

Thus, the implementation of either the individual species microalgae or the consortiums for agro-industry wastewater treatment symbolizes an opportunity to attain more robust treatment techniques or systems that are resilient to antagonistic environments, to minimize the process costs of biomass recovery, and to increase pollutant removal efficiencies. In general, although further supportive research is required, the integrated biological slaughterhouse wastewater treatment composed of an AD-microalgae photobioreactor is considered as a path used for CE as biogas, a renewable energy source is generated and resource (nutrient) is recovered as microalgae biomass again for biodiesel production as well as low pollutant load effluent that can be used for at least non-potable purposes (non-human consumptions) such as floor cleaning, irrigation, and greenery purposes, which in turn improve sustainable practices.

5.2. Recommendations

The following recommendations were drawn from the study:

- The structure, function and microbial diversity of both hydrolytic-acidogenic and methanogenesis reactors at optimal stability and performance conditions should be investigated.
- Pilot and large-scale operation of the AD-photobioreactor for the treatment of abattoir wastewater, bioenergy production and effluent reuse should be carried out in partnership with the abattoir industries.
- Suitability of the effluent for reuse for agricultural production should tested.
- Techno-economic feasibility studies must be carried out for large-scale application.
- The contribution of the system for the initiation and application of circular bioeconomy concept in waste sector should be premeditated.
- The quality of the microalgae biodiesel needs further investigation.

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APPENDICES

i. Abstracts of Published Papers

a. Paper I

sustainability

MDPI

Article

Optimization of Operating Parameters for Two-Phase Anaerobic Digestion Treating Slaughterhouse Wastewater for Biogas Production: Focus on Hydrolytic–Acidogenic Phase

Dejene Tsegaye *, Mohammed Mazharuddin Khan and Seyoum Leta

Center for Environmental Science, College of Natural and Computational Science, Addis Ababa University, Addis Ababa P.O. Box 1176, Ethiopia

* Correspondence: dejene.tsegaye@aau.edu.et

Abstract: In a two-phase anaerobic digestion process, enhanced biogas production and organic pollutant removal depend on the stability and performance of the hydrolytic–acidogenic and methanogenic phases. Additionally, the hydrolytic–acidogenic phase is a rate-limiting step, which calls for the further optimization of operating parameters. The objective of this study was to optimize the operating parameters of the hydrolytic–acidogenic reactor (HR) in the two-phase anaerobic digestion treating slaughterhouse wastewater. The experiment was carried using bench-scale sequential bioreactors. The hydrolytic–acidogenic reactor operating parameters were optimized for six different hydraulic retention times (HRTs) (6–1 day) and organic loading rates (OLRs) (894.41 ± 32.56–5366.43 ± 83.80 mg COD/L*day). The degree of hydrolysis and acidification were mainly influenced by lower HRT (higher OLR), and the highest values of hydrolysis and acidification were 63.92% and 53.26% at an HRT of 3 days, respectively. The findings indicated that, at steady state, the concentrations of soluble chemical oxygen demand (SCOD) and total volatile fatty acids (TVFAs) decrease as HRT decreases and OLR increases from HRTs of 3 to 1 day and 894.41–1788.81 mg COD/L*day, respectively, and increase as the HRT decreases from 6 to 4 days. The concentration of NH_4^+-N ranges from 278.67 to 369.46 mg/L, which is not in the range that disturbs the performance and stability of the hydrolytic acidogenic reactor. It was concluded that an HRT of 3 days and an ORL of 1788.81 mg COD/L*day were selected as optimal operating conditions for the high performance and stability of the two-phase anaerobic digestion of slaughterhouse wastewater in the hydrolytic–acidogenic reactor at a mesophilic temperature. The findings of this study can be applicable for other agro-process industry wastewater types with similar characteristics and biowaste for value addition and sustainable biowaste management and safe discharge.

Keywords: organic loading rate; optimal condition; hydraulic-acidogenic phase; slaughterhouse wastewater; two-phase anaerobic digestion

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1. Introduction

The diverse ecosystems that support human life have deteriorated recently due to industrialization, urbanization, and population growth throughout the world. The mismanagement of agro-industrial wastewater and overuse of water create maximum stress on freshwater bodies such as rivers, lakes (lotic and lentic), seas, and oceans, as well as a decrease in the quality of aquatic ecosystem services [1–4].

Agro-processing industries such as slaughterhouses produce typical wastewater with high values of biochemical oxygen demand (BOD), chemical oxygen demand (COD), biological organic nutrients (nitrogen and phosphate), which are insoluble, slowly biodegradable solids, pathogenic and non-pathogenic viruses and bacteria, and parasite eggs [5–7]. Furthermore, it is high in protein and quickly putrefies, causing an environmental pollution problem. This revealed that slaughterhouses are among the most environmentally polluting agro-processing industries [4,8–11]. The sources of the wastewater are mainly from

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b. Paper II

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Bioresources and Bioprocessing

RESEARCH Open Access

Optimization of operating parameters for biogas production using two-phase bench-scale anaerobic digestion of slaughterhouse wastewater: Focus on methanogenic step

Dejene Tsegaye* and Seyoum Leta

Abstract

The objective of the present study was an optimization of operating parameters and the performance of the methanogenesis reactor in phased anaerobic digestion (AD) of slaughterhouse wastewater at 37.5°C. Accordingly, the feedstock of the methanogenic reactor was effluent from the hydrolytic-acidogenic reactor operating at HRT of 3-days and OLR of 1789 mg/L. The methanogenesis phase was also investigated at different hydraulic retention time (HRT) values ranging from 12 to 3 days at 3-day intervals, and organic loading rates (OLR) of 149, 199, 298, and 596 mg of COD/L. The methanogenesis reactor effluent concentrations of TN, TP, PO_4^{-3}, SO_4^{-2}, and S_2^{-2} were ranging between 424–464, 83–117, 63–86, 130–197, and 0.98–1.02 mg/L, respectively. The removal efficiencies of TN and TP were vary from 10–17% to 17–21%, respectively. The average biogas production was 125 ± 16, 150 ± 10, 185 ± 4, and 154 ± 17 mL at HRT of 12, 9, 6, and 3 days, respectively. Methane quality (%) and yield (mg/L of COD) were 55–67% and 0.02–0.03, respectively. Furthermore, the average stability indicator parameter values of (total volatile fatty acid (TVFA) = 520 ± 19 mg/L, total alkalinity (TotA) = 1424 ± 10 mg/L, TVFA:TotA. Ratio = 0.36, salinity = 1172 mg/L, pH = 6.92) and performance indicator parameters removal efficiency (RE) for (chemical oxygen demand (COD) = 81%, volatile solid (VS) RE = 95%, biogas production = 185 ± 4 mL, methane yield = 0.03 per mg COD consumed) were achieved at HRT of 6 days and OLR of 298 mg of COD/L. Low removal efficiencies of TP and TN at all HRT/OLR were observed for the methanogenic reactor signifying further treatment system.

Keywords: Methanogenesis phase, AD reactor stability and performance, Volatile solid reduction, Biogas production rate, Methane yield

*Correspondence: dejene.tsegaye@aau.edu.et; seyoum.leta@aau.edu.et
Center for Environmental Science, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia

c. Paper III

Biofuels

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Evaluation of biogas production and pollutant removal efficiency of two-phase anaerobic digestion treating slaughterhouse effluent

Dejene Tsegaye & Seyoum Leta

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Evaluation of biogas production and pollutant removal efficiency of two-phase anaerobic digestion treating slaughterhouse effluent

Dejene Tsegaye and Seyoum Leta

Center for Environmental Science, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia

ABSTRACT

Treatment of slaughterhouse and other agro-processing industry effluent has become important due to continuous global population growth and meat demand, particularly in developing countries, for sustainable biowaste management as well as value additions such as biofuel. Therefore, the objective of the study was to evaluate the performance of two-phase anaerobic digestion of slaughterhouse wastewater in terms of biogas production, methane yield, and removal efficiencies of organic matter, total nitrogen, and total phosphorus. Two consecutively connected 40 L galvanized metal anaerobic batch bioreactors were used to conduct the experiment. The total phosphorus removal efficiencies of hydrolytic-acidogenic reactor and methanogenic reactor were 13.34% and 16.58%, respectively. Total chemical oxygen demand, soluble chemical oxygen demand, biological oxygen demand, total dissolved solids, total solids, total suspended solids, volatile solids, and turbidity had overall removal efficiencies of 82.87, 88.53, 93.32, 75.35, 95.55, 98.95, 97.42 and 98.06%, respectively. Biogas production of 189.45 mL/day with methane and carbon dioxide compositions of 67.69% and 29.9%, respectively, was also achieved. It was concluded that the two-phase anaerobic digestion of slaughterhouse wastewater shows substantial organic matter removal efficiencies and biogas production.

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KEYWORDS
Agro-industrial wastewater treatment; bioenergy; reactor stability parameters; methane yield; pollutant removal efficiency

Introduction

Treatment of slaughterhouse and other agro-processing industry effluent has become important due to continuous global population growth and meat demand, particularly in developing countries, for sustainable biowaste management as well as value additions such as biofuel [1–3]. As the availability of fresh water decreases, agro-processing industry effluent in general and slaughterhouse effluent in particular require extraordinary management to reduce or avoid water-body pollution caused by direct discharge of untreated or partially treated effluent to the receiving water body [4–6]. This, in turn, redirected the intentions in the area of agro-processing industry effluent treatment toward reprocessing and reclaiming [2, 7, 8]. Currently, popular and commercial technologies for agro-processing industry effluent in developing countries based on aerobic and physical treatment techniques require complicated machines with high installation and operating costs [1].

Furthermore, the global energy demand is increasing, mainly due to the incessant growth of the world population, human civilization, the exhaustion of fossil fuels, and the potential risk of nuclear energy to the environment and to human health. Concerns have been raised about the use of renewable energy resources, such as agro-processing industry effluent rich in biodegradable organic matter, using an anaerobic digestion (AD) system [1, 3, 9, 10]. Bioenergy technologies have received global attention due to the benefits of renewable energy-based technologies: they enhance energy security and provide other environmental benefits, such as the handling of a wide range of agro-processing industry effluent to reduce pollution and greenhouse gases. Furthermore, it contributes to poverty reduction, primarily in low-income countries, because the digestate from AD used as a soil amendment increases crop yield and reduces the use and concern of agrochemicals (chemical fertilizer) [8, 11–15]. As a result, the environmental effects of improperly managed agro-processing industry effluent on the nearby physical environment are minimized or avoided [16–18].

Separating hydrolytic-acidogenic reactor (HR) and methanogenesis reactor (MR) has numerous advantages, such as maintaining an appropriate bacteria density and optimizing operating conditions, which are not possible in a single AD system. As a result, the phased AD technique has been recognized in recent decades for treating slaughterhouse effluent and reducing numerous environmental problems [8]. Furthermore, it accelerates acidification and methanogenesis by removing hazardous and toxic compounds for methanogenic bacteria (such as oxygen, sulfate, and formaldehyde) [19–24], as well as effectively removing organic matter in effluent from high-fat agro-processing facilities like slaughterhouses [8].

To further enhance the performance of the phased system, the importance of operating parameter optimization based on the wastewater characteristics for both the hydrolytic-acidogenic and methanogenesis phases was highlighted [25, 26]. Though the operational parameter optimization of AD has been broadly studied, pertinent literature on the hydrolytic-acidogenic and methanogenesis phases, operating parameter optimization for biogas

CONTACT Dejene Tsegaye dejene.tsegaye@aau.edu.et Center for Environmental Science, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia

d. Paper IV

Evaluation of microalgae and co-culture for polishing pollutants of anaerobically treated agro-processing industry wastewater: The case of slaughterhouse

Dejene Tsegaye Bedane* and Seyoum Leta Asfaw

Center for Environmental Science, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, P.O. Box 1176, Ethiopia

Address: *dejene.tsegaye@aau.edu.et, seyoum.leta@aau.edu.et

Abstract

Anaerobically treated slaughterhouse effluent is rich in nutrients, organic matter, and cause eutrophication if discharged to the environment without proper further treatment. Moreover, phosphorus and nitrogen in agro-processing industry wastewaters are mainly removed in the tertiary treatment phase. The objective of this study is to evaluate the pollutant removal efficiency of Chlorella and Scenedesmus species as well as their co-culture treating two-phase anaerobic digester effluent through microalgae biomass production. The dimensions of the rectangular photobioreactor used to conduct the experiment are 15 cm in height, 20 cm in width, and 30 cm in length. Removal efficiencies between 86.74–93.11%, 96.74–97.47%, 91.49–92.91%, 97.94–99.46%, 89.22–94.28%, and 91.08–95.31% were attained for chemical oxygen demand, total nitrogen, nitrate, ammonium, total phosphorous, and orthophosphate by Chlorella species, Scenedesmus species, and their co-culture, respectively. The average biomass productivity and biomass yield of Chlorella species, Scenedesmus species, and their co-culture were 1.4 ± 0.1, 1.17 ± 0.12, 1.5 ± 0.13 g/L, and 0.18, 0.21, and 0.23 g/L*day, respectively. The final effluent quality in terms of chemical oxygen demand, total nitrogen, and total phosphorous attained by Chlorella species and the co-culture were below the permissible discharge limit for slaughterhouse effluent standards in the country (Ethiopia). The results of the study showed that the use of microalgae as well as their co-culture for polishing the nutrients and residual organic matter in the anaerobically treated agro-processing industry effluent offers a promising result for wastewater remediation and biomass production. In general, Chlorella and Scenedesmus species microalgae and their co-culture can be applied as an alternative for nutrient removal from anaerobically treated slaughterhouse wastewater as well as biomass production that can be used for bioenergy.

Keywords: Biomass, microalgae; nutrient removal-efficiency, photobioreactor; resource-recovery; wastewater

e. Paper V (manuscript I)

Performance evaluation of a bench-scale two-phase anaerobic reactor coupled with microalgae photobioreactor for slaughterhouse wastewater treatment in Ethiopia.

Dejene Tsegaye Bedane and Seyoum Leta Asfaw
Center for Environmental Science, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, P.O. Box 1176, Ethiopia,

Email address: dejene.tsegaye@aau.edu.et, seyoum.leta@aau.edu.et,

Abstract

Wastewater from the slaughterhouse industry is characterized by high levels of organic matter and nutrients. Due to this, it cannot be handled by a single or standalone treatment technique such as anaerobic digestion, as it does not provide effluent pollutant (organic matter and nutrients) concentrations that meet the desired discharge limits. The objective of this study was to develop an innovative biological wastewater treatment system that would take slaughterhouse wastewater, produce biogas in an anaerobic digestion unit by removing major organic matter, and remove most of the nutrients and organic matter in a microalgae photobioreactor. The performance of an anaerobic reactor and microalgae photobioreactor system was also assessed for selected organic matter and nutrients following standard methods. The integrated bench-scale treatment system indicated perceptible performance for the treatment of slaughterhouse wastewater, with overall removal efficiencies of 99% of TCOD, SCOD, and BOD, 93-99% of TDS, 99% of TN, 98-99% of NH_4^+-N and PO_4^{-3}–P, 90-95% of NO_3^-–N and TP, and 97-98% of SO_4^{-2}. The final effluent of the integrated system meets the minimum acceptable national environmental quality standards of Ethiopia.

Keyword: Biowaste, waste to energy, microalgae based wastewater treatment

ii. Bold's Basal Medium Conistituants

The per liter BBM was composed of KH_2PO_4 (175 mg), K_2HPO_4 (75 mg), $MgSO_4 \cdot 7H_2O$ (75 mg), $CaCl_2 \cdot 2H_2O$ (25 mg), $NaNO_3$ (250 mg), NaCl (25 mg), and H_3BO_3 (11.42 mg), 1 mL microelement stock solution ($ZnSO_4 \cdot 7H_2O$ (8.82 g), $MnCl_2 \cdot 4H_2O$ (1.44 g), MoO_3 (0.71 g), CuSO4 $\cdot 5H_2O$ (1.57 g) and $Co(NO_3)_2 \cdot 6H_2O$ (0.49 g) in one liter), 1 mL of solution-1 of Na_2EDTA (50 g) and KOH (3.1 g) in one liter and 1 mL of ($FeSO_4$ (4.98 g) & concentrated H_2SO_4 per liter) and at pH of 6.8.