ANIMAL BLOOD UTILIZATION FOR BIOMETHANE PRODUCTION THROUGH PRETREATMENTS AT LAB SCALE

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ABSTRACT: High levels of waste produced by slaughterhouses are harmful to the environment and are a possible source of numerous pathogens dissemination. There is no effective system so far to control the effluent discharge from the slaughtering processing. Daily energy demands rise, but energy sources, particularly fossil fuels, are becoming more and more depleted. Offering alternative energy sources like biogas is one way to solve these issues. The productivity of the industrial sector can be boosted through effective management, which also minimizes environmental issues. The purpose of this study is to generate biomethane using animal blood as a substrate exposed to different pretreatments (NaOH/Sonication, NaOH/Heat, Only Sonication, H_2O_2/ Sonication and blank substrate). Biomethane production was carried out by Anaerobic Digestion in a lab scale digester. Anaerobic digestion can convert organic materials of animal blood into biogas. Results elucidated that NaOH/Sonication pretreatment was the most efficient and showed the highest percentage of methane production (86%) followed by other pretreatments *i.e.*, NaOH/Heat (85%), Only Sonication (83%), H_2O_2/ Sonication (82%) and blank substrate (76%). Chen and Hashimoto model of kinetics was found to be the most feasible model of kinetics for the current study.

Keywords: Animal blood, Anaerobic digestion, Pretreatments, Biomethane production, Kinetic modeling.

(Received 17.10.2022 Accepted 11.12.2022)

INTRODUCTION

One of the industries with the quickest growth is the abattoir, which is fueled by the rising demand for food (Mozhiarasi *et al*., 2022). During the butchering and processing of animals, bodily fluids such as blood and stomach contents produce very potent organic waste. High amounts of animal blood, skinning residue, wash water from cleaning animal carcasses, as well as ambient air, are all present in slaughterhouses' high-strength waste, which is mostly made up of biodegradable organic carbon, lipids, and proteins (Grosser *et al*.,2019).

Four types of products from animals slaughtered for meat are meat (high-value product), inedible parts that can be used in industry (such as animal skin, skeletons, appendages, and blood), offal and meat meal (low-value components), and items that serve no purpose such as intestinal content, wool slip, and pollutants (that are thrown away as trash). Unwanted byproducts of the meat industry include blood, which can make up up to 4% of the live animal's weight or 6% to 7% of the carcass's lean meat content (Fallows *et al*., 1982). Blood contains a number of items with potential for economic gain and is a strong source of protein (Bah *et al*., 2013).

According to the Global Slaughter Index, this figure of slaughtering animals is steadily expanding. In 2003, 2014, and 2016, the Food and Agriculture Organization of the United Nations reported that more than 49.2, 70.3, and 74.2 million animals were butchered, respectively (FAO). Based on the kind, size, manner, length of blood collection, and size of the animal being slaughtered, all animals bleed to varied degrees during the procedure (Nazifa *et al*., 2022).Animal bleeding generated in slaughterhouses is a dangerous waste of the meat processing units because of the huge volumes produced and the cause of excessive pollution when thrown directly into the environment (Joseph *et al*., 2009). The importance of slaughterhouses is greater than that of the other units in terms of pollution.

Particularly in the developing countries, slaughterhouses are a significant source of water pollution and greenhouse gases emissions. Abattoir-specific standards are either absent or ineffectively checked and implemented, resulting in effluent that frequently goes into nearby rivers and water sources untreated. This is an urgent environmental issue that will have an impact on the growth of aquatic life (Siddiki *et al*., 2021).

Water contamination, turbidity, and even algae blooms are brought on by high blood, protein, and fat levels (Businge *et al*., 2021). Additionally, trash from slaughterhouses frequently contains zoonotic infections, which are illnesses that may spread from animals to people (Bandaw *et al*, 2017). According to reports, the

organic contamination level in wastewater increases by 35–50% when raw blood is released directly into the sewage system (Wang, 2015).

The development of a renewable energy sector and society's struggle to go in that direction are now two of the most demanding topics. Due to current energy short fall and global dependency on fossil fuels, environmental concerns have grown significantly. An appealing method for treating the waste blood from slaughterhouses is anaerobic digestion (AD), which may be utilised to extract energy and balance off the energy spent in the slaughtering. Anaerobic digestion (AD) has a number of benefits, including reduced pollution, the creation of useful byproducts like fertilizers, and the generation of renewable energy. Because of its nutritional and biogas potential, slaughterhouse blood can be regarded as a viable alternative.

It is well recognized that blood is a highly potent waste product of the abattoir that, rich protein composition, is hard to handle in AD. Only a small number of investigations have so far been conducted (Lopez *et al*., 2006; Wang *et al*., 2018). Analyzing the efficiency of energy regeneration from organic by-products from a slaughterhouse is the target of the current study.

METHODOLOGY

Sample Collection: The slaughtering waste sample considered for this study was pure blood. Pure blood was collected from a local slaughter house facility in sterilized screw capped plastic bottles.

Experimental Set up

BMP bottle sets

5 bottles with rubber septa were used for BMP testing. Volume of the bottles were enough to accommodate the possible gas generation from the selected substrate. To avoid loss of biogas, the connections were securely tightened with gastight butyl rubber septa. The entire set up was connected to a storage reactor which was later on subjected to volumetric measurement.

Conditions for BMP bottles

For proper working of BMP bottles following conditions were manually ensured;

- Temperature-controlled environment: The bottles were maintained in an oven set to a mesophilic temperature of 35°C.
- Proper mixing: Bottles were shaken manually once a day.
- Incubation time: For 77 days bottles were incubated.

BMP bottle content

Inoculum and Substrates

Animal excrement and 150 ml of distilled water with micronutrients prepared by Angelidaki *et al.,* method (Angelidaki *et al*., 2009) were used to prepare the inoculum. Per bottle,10 ml of substrate was added, and each container held 200 ml of the finished product. To eliminate any big particles, the inoculum was passed through a 4.75 mm sieve. The inoculum and substrate was then examined using the Standard Method to determine the total solids (TS), volatile solids (VS), fixed solids (FS), and pH.(Method 2540-1997, and EPA Method 160.4).

Total solids: In an oven set to 103 to 105°C, a well-mixed sample was evaporated and dried to a consistent weight. The entire solids were represented by the weight that rises over the weight of the empty dish (Symons *et al*., 1941).

Fixed and Volatile solids: At 550°C, the remaining total solids were ignited to a constant weight for 1 hour. The dish was partially cooled in the air until the majority of heat had been removed. As soon as it was cooled in a desiccator, a dish was weighed. Fixed and volatile solids were then calculated (Fischer *et al*., 1944).

pH: pH was measured by pH meter.

SCOD (Soluble Chemical Oxygen Demand): SCOD of substrates was performed by using the principle of open reflux method of Pitwell (Pitwell *et al*., 1983). 0.5 ml of blank substrate was diluted to make volume up to 100ml. In a flask, volume up to 20 ml was prepared in which 10 ml of substrate and 10 ml of distilled water was added. Further a pinch of mercury sulphate, 5 ml of sulfuric reagent was added into flask. After mixing 10 ml of potassium dichromate and 25 ml of sulfuric acid reagent were added and then the flask was subjected to heating in reflux for about 2 hours. After cooling the reflux, titration was taken. Ferroin indicator was used to change the color of the sample and the color varied from green to blue and then blue to reddish brown. Ferrous ammonium sulphate was used to titrate the sample.

Substrate Pretreatment Strategy

- NaOH with Sonication: 0.05% NaOH in 10ml of substrate sample was added in a test tube and was sonicated for about 10 mins in ultrasonic water bath tub.
- NaOH with Heat: 0.05% NaOH in 10ml of substrate was added in a test tube and heated at about 70˚C for 10 mints.
- Only Sonication: 10ml of substrate was added in a test tube and sonicated for 10 mints in ultrasonic water bath tub.
- H_2O_2 with Sonication: 0.05ml of H_2O_2 in 10ml of substrate in a test tube was added and sonicated for about 10 mints in ultrasonic water bath tub.
- Blank Substrate: 10ml of substrate in a test tube was added and considered as a blank.

SCOD (Soluble Chemical Oxygen Demand) of pretreated substrates: SCOD of pretreated substrates after pretreatment is performed by using the principle of open reflux method of PITWELL (Pitwell *et al*., 1983).

BMP (Biochemical methane potential): Following Hamilton *et al* (2012) protocol methane potential was determined by using Biochemical Methane Potential (BMP) assays (Hamilton *et al*., 2012). To prevent overloading of inoculum, inoculum and substrate were supplied on the basis of volatile solids. The final reactor capacity was around 210 mL of liquid, with the remainder serving as head space in a 500 mL BMP container. Five sets were tested, including a sludge blank and four pretreatment groups. Bottles were incubated in the oven for around 77 days at a constant temperature of 35 °C and readings were taken every week with a one-week interval. Carbon dioxide was eliminated from the biogas after one month of reading using a 500ml solution of sodium hydroxide in water, which concentrated the CO_2 and produced sodium bicarbonate and methane (CH_4). After adding the NaOH solution, 2nd dose of substrate was given and readings were collected roughly for a month with a one-week interval. Methane volume was determined using the Volumetric Methane Yield Method after collecting readings for both CH_4+CO_2 and Pure CH_4 (Hamilton *et al*., 2012).

Kinetic Modeling

i. Zero Order Model (Goss *et al*., 2007).
ii. First Order Model (Parameswaran *et al*., 2012).
iii. Second Order Model (Helmenstine *et al*., 2020)
iv. Chen & Hashimoto Model (Kafle *et al*., 2016; Zhao *et al*., 2004). The coefficient of determination or regression analysis (R2) was used as a factor to determine the most feasible model. Following Chen & Hashimoto model, parameters such as intercept (1/μm) , slope(Kch/μm) , μm(1/ intercept) and Kch (rate constant) were determined.

RESULTS

Substrate and Inoculum Characterization: The values indicated that inoculum and substrate housed large amount of total solids (TS), fixed solid (FS), volatile solids (VS) . Total solids represented total fixed solid andvolatile solids present in inoculum and substrate. Biogas production increased simultaneously with the consumption of total solids. Total solids, fixed solids, volatile solids were estimated to be 57g/L, 28g/L, 29g/L respectively where as in substrate TS, FS and VS were 191.3g/L, 25.8g/L, 161.5g/L respectively.

pH: pH from 6.0 to 8.0 proved to be the best for efficient digestion and subsequent gaseous methane production. Results presented optimum pH levels for the efficient production of biogas (Table 1).

Soluble Chemical Oxygen Demand (SCOD): SCOD indicated the amount of oxygen needed for the oxidation of organic matter in sample. Higher COD level meant greater amount of oxidizable organic material in the sample. Results showed sufficient amount of oxygen in pretreated sample required to convert organic matter into biogas (Table 1)

Table 1: pH and SCOD of BMP bottles/ Digesters.

Pretreatments	pH	SCOD
Blank Substrate	7.3	132,480mgO2/L
H_2O_2 / Sonication	7.2	136,160mgO2/L
Only Sonication	7.7	138,000mgO2/L
NaOH/ Sonication	7.5	141,680mgO2/L
NaOH/Heat	7.4	147,680mgO2/L

Biomethane Production Potential (BMP): *Biogas production potential of 1st batch before addition of NaOH*

During the initial phase of the experimental set up 10ml of substrate was added for each 200 ml of inoculum, for the estimation of bio methane production, extending the duration till 28 days in total. Results clearly elucidated the gradual increase in biogas production with the passage of time in the pretreated batches subjected to NaOH/Sonication followed by NaOH/Heat, Only Sonication, H_2O_2/Sonication and blank simultaneously (Figure 1).

Biogas production of 2nd batch after addition of NaOH

At 28th day due to the addition of 20ml of NaOH solution (98%) there was sudden decrease in the level of biogas production occurred that could be attributed to the NaOH induced CO_2 concentration leading to the formation of sodium bicarbonate($NaHCO_3$) and methane (CH_4) as reaction products. At 29th day a second dose of substrate was added in BMP bottles and at 30th day there was a gradual increase in methane production (Pure methane). Pure methane production was evaluated for total 49 days (Figure 1).

Combined effect of biogas production evaluation of 1st& 2nd batch

Results with different pretreatments showed higher efficacy for biogas production. NaOH/Sonication showed higher efficacy than other pretreatment options. Biogas production evaluation from 1st and 2nd dose and the combined effect of dose 1 and 2 for the production of biogas are presented (Figure 1)

Biogas/g VS and Methane/ g VS production of 1st batch and 2nd batch

Values of Methane/gVS and Biogas/VS for comparing the 1st and 2nd dose production potential were calculated. Result presented higher Methane/gVS and Biogas/gVS production potential in the following descending order: NaOH/Sonication with maximum biogas generation was followed by NaOH/Heat, Only

Sonication, H_2O_2/Sonication and Blank as the least potential option. (Table 2 & 3).

Biogas/g VS and Methane/ g VS production of 1st batch

After adding 2nddose ,there was a gradual increase in biogas production as compared to 1st dose by at the time of 2nd dose , inoculum got acclimated for pretreated substrates (Figure 2).

Percentage Methane Production

Percentage methane production results clearly indicated maximum biomethane production (% Methane) from NaOH/Sonication treatment followed by NaOH/Heat, Only Sonication, H_2O_2/Sonication treatments and blank with 86%, 85%, 83%, 82%, 76% yield

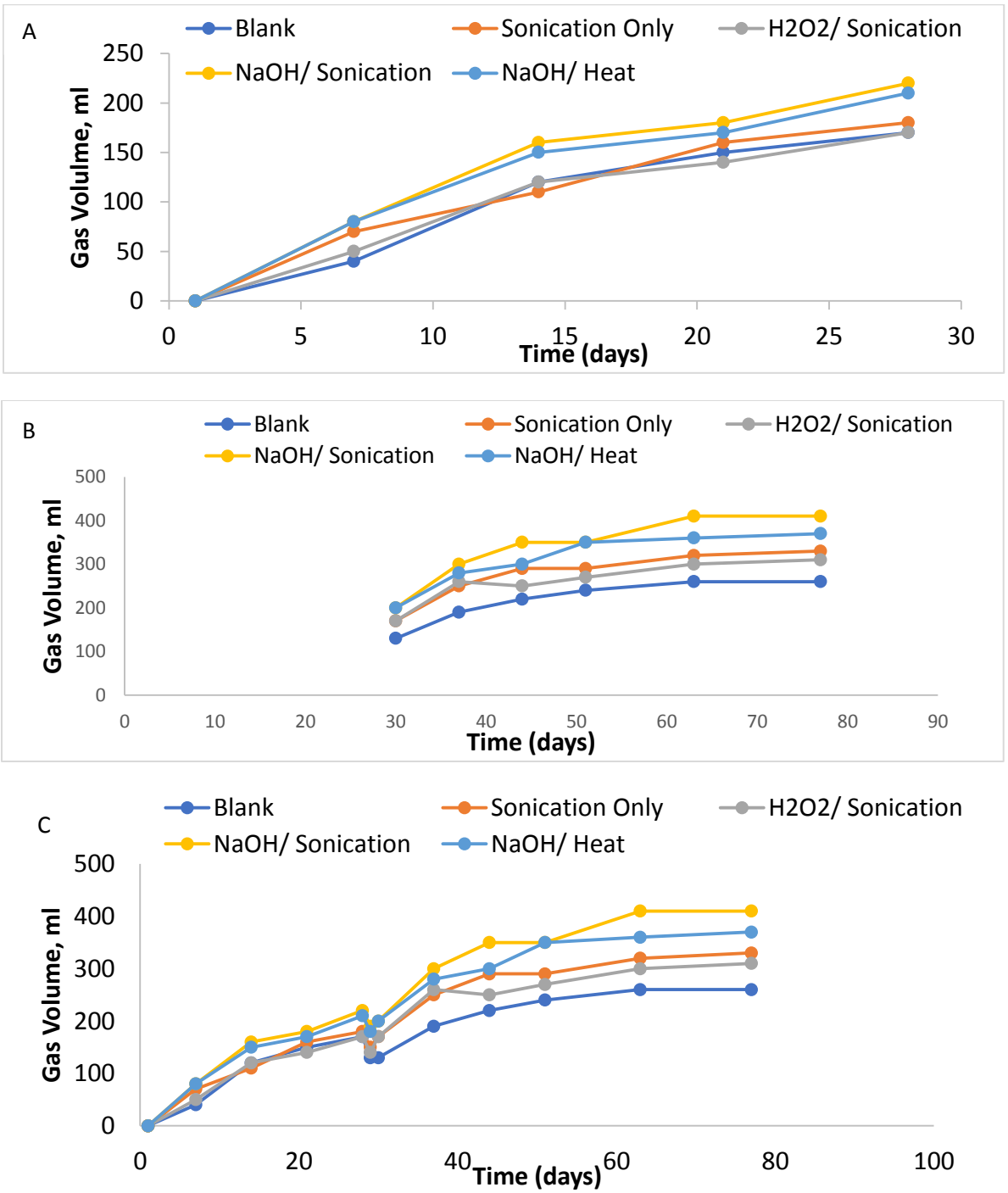


Figure 1: A) Biogas production of 1st batch before addition of NaOH, B) Biogas production of 2nd batch after addition of NaOH, C) Combined effect of biogas production evaluation of 1st& 2nd batch

Table 2: Biogas/g VS and Methane/ g VS production of 1st batch.

	Days	Blank	Sonication Only	H_2O_2/ Sonication	NaOH/ Sonication	NaOH/ Heat
Methane Production (Duration vs Treatments)	1	0	0	0	0	0
	7	40	70	50	80	80
	14	120	110	120	160	150
	21	150	160	140	180	170
	28	170	180	170	220	210
Cumulative Methane (ml)	29	130	150	140	190	180
Biogas/g VS		103	109	103	133	127
Methane / g VS		79	91	85	115	109

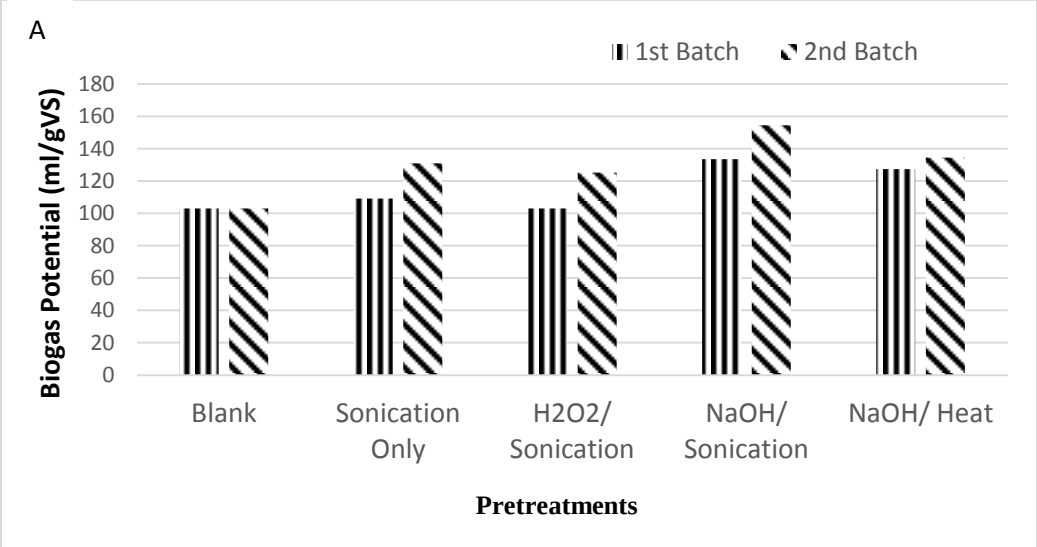
Table 3: Biogas /g VS and Methane/ g VS production of 2nd batch.

	Days	Blank	Sonication Only	H_2O_2/ Sonication	NaOH/ Sonication	NaOH/ Heat
Methane Production (Duration vs Treatments)	1	0	0	0	0	0
	2	0	20	30	10	20
	9	60	100	120	110	100
	16	90	140	110	160	120
	23	110	140	130	160	170
	35	130	180	160	220	180
Cumulative Methane (ml)	49	130	190	170	220	190
Biogas/g VS		103	138	125	154	134
Methane / g VS		79	115	103	133	115

Kinetic Modeling: Zero order, first order, second order and Chen and Hashimoto models were used to simulate the methane yield of BMP set up. Compared to the other three kinetic models , Chen and Hashimoto Model was found to be excellently fitted to define the results of BMP experiments (Table 4).

Regression values calculated for all five options i-e blank, H_2O_2/Sonication, only sonication, NaOH/Heat and NaOH/Sonication; using Chen and Hashimoto model, gave R^2 values *i.e*0.983, 0.986, 0.992, 0.982 and 0.985 respectively. Chen and Hashimoto kinetic models agreed well with the experimental data with R2 values ranging from 0.982 to 0.992.

Following this model, values calculatedfor parameterssuch as intercept (1/μm) , slope(Kch/μm) , μm(1/ intercept) and Kch (rate constant) are presented in detail (Table 5).



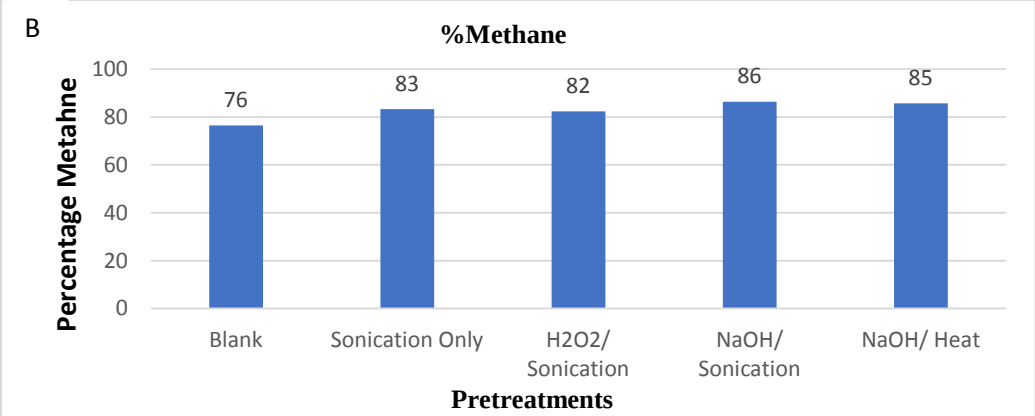


Figure 2: A) Comparison of Biogas/g VS production from 1st and 2nd batch, B) Percentage Methane Production

Table 4: Comparative regression analysis ; fitting of models.

Models	BlankSubstrate	SonicationOnly	H2O2/ Sonication	NaOH/Sonication	NaOH/Heat
ZeroOrdermodel	0.945	0.958	0.950	0.933	0.935
1stOrdermodel	0.796	0.9457	0.835	0.858	0.882
2ndOrdermodel	0.705	0.893	0.744	0.778	0.804
Chen andHashimotomodel	0.983	0.992	0.986	0.982	0.985

Table 5: Chen and Hashimoto Model parameters for pretreated substrates.

Substrates	Intercept = 1/µm	Slop = Kch/µm	µm = 1/ intercept	Kch
Blank substrate	1.7924	25.134	0.55791	14.0225
H_2O_2/Sonication	1.1073	32.081	0.903098	28.97227
Only Sonication	0.8229	31.69	1.21521	38.5101
NaOH/Heat	0.5461	29.002	1.831166	53.10749
NaOH/Sonication	0.5059	31.36	1.97668	61.9885

DISCUSSION

Slaughterhouse wastes such as blood and other animal by-product are inappropriate and exposed discarding of these wastes is an environmental challenge. Biodegradation of organic matter under anaerobic and aerobic conditions is an important process in the natural metabolism in the ecosystems (Lissens *et al*., 2014).

In the current experimental study, the pH of digesters was maintained between 6-8. During the initial time interval, the work efficiency of digesters was enhanced by increasing alkalinity which may have a direct impact on methane gas production. Under acidic conditions production efficiency of methane would be decreased (Reference). In our study only sonication at the pH level of 7.7, was found to generate more methane. NaOH/ Sonication, NaOH/ Heat, and H_2O_2 /Sonication presented the likewise trend by giving efficient yield at pH 7.5, 7.4, and 7.2 respectively. In a study by Lay *et al*., (1997) and Ouahabi *et al*., (2021) it was reported that high-solids digestion of methane production adjusted from 6.6 to 7.8 pH, an optimal pH was found to be 6.8 if moisture level was maintained up to 90 to 96%. But the process could prove ineffective if the pH was around 6.1 or more towards acidic side or 8.3 or more towards basic side. In addition, a lowest lag-phase time for methanogenesis was found near pH 6.8.

In this study, SCOD of the substrate was performed after pretreatment indicative of the solubility of organic matter in the sample. The results of SCOD varied for differently pretreated substrates. SCOD of Blank Substrate, H2O2/Sonication, Heat/NaOH, Only Sonication, and NaOH/Sonication were found to be 132480 mg²/ L. 138000 mg²/ L, 141680mg²/ L, 136160 mg²/ L, 147680 mg²/ L by the end of the experimental duration, projecting per day SCOD values as 1720mg²/L/ d, 1790mg²/ L/ d, 1830mg²/ L/ d, 1760mg²/ L/ d, 1910mg²/ L/ d, respectively. The higher level of SCOD meant a greater amount of oxidizable organic material in the sample ,showing a sufficient amount of oxygen in the pretreated sample that was required to convert organic matter into methane. In our current investigation, it was observed that NaOH/Sonication pretreated substrate attained a higher SCOD level i.e. 147680 mg²/ L indicating the highest feasibility of the substrate with the higher solubility and ability to convert volatile solids into

methane evincing greater biogas production. Nikolaeva*et al*., 2002 reported the treatment of pig waste by using an anaerobic fixed bed reactor (AFBR) at 18-32°C. The pilot AFBR functioned at two different volumetric organic loading rates, 5700 and 24000 mg²/ L/ d COD. As a result, 73% COD was achieved. Luste*et al*., 2009 investigated the results of five pre-treatments (acids, bases, heat, ultra-sonication, and bacterial products) on the potential of hydrolysis and methanogenesis of four by-products which were from the meat processing industry. In their study, it is observed that the Liquid Certizyme 5, a bacterial product improved soluble chemical oxygen demand (CODsol) of gastrointestinal contents and drum sieve waste mostly in contrast to raw material (62-96%, respectively), whereas sonication with grease trap sludge were highly efficient in increasing CODs ol (188%) and dissolved air flotation sludge (88%). In batch experiments, high temperature treatment increased the potential of methane production from drum sieve waste, acid of grease trap sludge, and all DAF sludge pre-treatments. Yet, by the total pre-treatments, potential of production of methane reduced in comparison with un-treated materials.

In the current study, different pretreated substrates showed different biogas production potentials. NaOH with Sonication showed the highest potential for biogas production which was estimated to be 200ml by the end of total 28 days duration and other digesters subsequently followed this trend by showing successively less potential as compared to NaOH/ Sonication. In the 2^{nd} batch, on 28^{th} day, after the addition of 10ml of 98% NaOH solution, there was a sudden decrease in the level of biogas production that could be attributed to the NaOH-induced CO_2 concentration leading to the formation of sodium bicarbonate (NaHCO3) and methane (CH_4) as reaction products. On the 29^{th} day, a second dose of the substrate was added to BMP bottles, followed by a gradual increase in methane production (Pure methane) at 30^{th} day, showing the inoculum's acclimation with the pretreated sample. Pure methane yield was evaluated for a total of 77 days. NaOH/ Sonication, only sonication, NaOH/ heat demonstrated higher methane yields of 350ml, 320ml, and 300ml respectively. The combined effect of biogas production of 1^{st}& 2^{nd} batch after 1^{st} and 2^{nd} dose of substrate revealed that NaOH with Sonication showed greater efficacy which was 410 ml after 2^{nd} dose of substrate possibly indicating that inoculums of the substrate got favorably acclimated than other pretreated digester set ups. Biochemical methane potential test indicated that the NaOH with ultrasonic pretreatment greatly favored the anaerobic degradation of the substrate (animal blood). Our findings are in concordance to the study showing the effectiveness of pretreatments *i.e.*, alkali pre-treatment (AP), ultrasonic pre-treatment (UP), and alkali-ultrasonic pre-treatment (AUP) applied on wheat straw (WS), solid fraction of cattle manure (SCM) and solid fraction of slaughterhouse waste (SSHW) that were examined by monitoring solubilization, anaerobic biodegradability and methane yield. The results indicated that the solubilization ratio of the substrates improved regardless of the types of pre-treatment applied. Though, AP was more effective on WS and SSHW than other pre-treatments (UP and AUP), with approximately 47% and 17% extra methane, respectively (Wahid *et al*., 2020).

The results of present study explicitly demonstrate that the biodegradability of the majority of substrates may be improved by different pre-treatment options. The high protein content of blood can serve as an excellent substrate for Anaerobic Digestion (AD) processes, with the dual benefits of both eliminating waste material and energy production.

Conclusion: Abattoir waste contains organic matter and the anaerobic digestion technology has been employed for economic benefit, considerably advancing the production of bioenergy. Results showed that NaOH/Sonication produced more biomethane than other pretreated batches. The trend of biomethane generation (percent Methane) was NaOH/Sonication >NaOH/Heat >Only Sonication> H_2O_2 /Sonication> Blank with pertinent percentages of 86, 85, 83, 82, and 76 percent, respectively. The Chen and Hashimoto Model of Kinetics was the most feasible model for the current study. Since it was difficult to carry out BMP test for single type of substrate especially in the case of blood, so we can co-digest it with other type of wastes such as agriculture waste, sewage waste etc to encourage efficient digestion and methane production. Variations of the F/M (Food to Mass) ratio can be tested to optimize the efficiency through inoculum concentration by centrifugation. As the highest methane production is achieved from NaOH/Sonication pretreatment, so the concentration of NaOH, duration of sonication and amplitude of ultrasonication can be changed to get more production of methane. By using parameters of Chen and Hashimoto Kinetic Model we could safely predict the theoretical performance of the experimental design without bringing in to account the practical aspect of the study.

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