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The influence of combined low-strength ultrasonics and micro-aerobic pretreatment process on methane generation and sludge digestion: Lipase enzyme, microbial activation, and energy yield

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ABSTRACT

Low-frequency ultrasonics is a potential technology to reduce the hydrolysis phase period in anaerobic digestion process. In this study, the influence of combined low frequency ultrasonics and micro-aerobic (MA) pretreatment on sewage sludge solubilization, enzyme activity and anaerobic digestion were assessed. Initially, the effect of ultrasonic density (0.012, 0.014, 0.016, 0.018, 0.1, 0.12 and 0.14 W/mL) and irradiation time (1, 3, 5, 8, 9, 10 and 12 min) of 20 kHz frequency waves were investigated. Accordingly, the effect of micro-aerobic pretreatment (Air flow rate (AFR) = 0.1, 0.2, 0.3 and 0.5 VVM) within 20, 30, 40.48 and 60 h were examined. In addition, the effect of combined pretreatment on COD solubilization, lipase enzyme activation, ATP, percentage of live bacteria and methane gas production during the anaerobic process were examined. The results showed that the highest lipase activity (14.9 Umol/mL) was obtained under the effect of ultrasonic density of 0.1 W/ml within 9 min. The highest solubilization (65%) was observed under optimal micro-aerobic conditions: AFR = 0.2 (VVM) and micro-aerobic time: 40 h. Combined ultrasonic and micro-aerobic (US + MA) pretreatment increases the solubilization (70%), microbial activity (2080%) and lipase enzymatic activity (129%) compared to individual pretreatment. The Biogas production during anaerobic digestion pretreated with combined methods increased by 193% compared to the control, while the elevated values of biogas production in reactors pretreated by ultrasonic and micro-aerobic pretreatment alone were observed to be 101% and 165%, respectively. The net energy in reactor with the combined pre-treatment methods was calculated to be 1.26 kWh, while this value for control, pretreated ultrasonic and micro-aerobic reactors were obtained to be 0.56, 0.67 and 1.2 kWh, respectively.

1. Introduction

Increased population growth over the recent decades has amplified the energy demand. Overconsuming energy from different sources has aggravated the global warming and threatened the local and natural energy sources [1]. In pace with world's population growth, global energy consumption is estimated to increase from approximately 600 quadrillion BTU in 2017 to 739 quadrillion BTU in 2040 [2]. The limitation of energy sources coupled with population growth and global warming has turned the spot light of research into renewable energy sources. Anaerobic digestion (AD) technology can both meet the increased energy demand and reduce the problem of environmental pollution [3]. Increasing the production of methane gas as a valuable product from anaerobic digestion using pretreatment methods has grabbed much attention by the researchers over the recent years [4]. The anaerobic digestion take advantages of volume waste reduction, methane gas production and digestate with high quality. Overall, biomethanation in AD is executed by a consecutive four-stage process, namely, hydrolysis, acidogenesis, acetogenesis and methanogenesis; the three former stages are mediated by bacteria and the last stage is accomplished by archaea [5]. In spite of advantages mentioned earlier for anaerobic digestion, low hydrolysis rate due to low decomposition of biologically activated sludge and instability at high concentrations of organic matter is the leading cause of lower or failure of

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biomethanation. This is mainly due to presence of extracellular polymeric materials (EPS), protecting the sludge cells and make less release of extracellular or intracellular components in the media. Therefore, pretreatment methods are necessary to facilitate Waste activated sludge (WAS) degradation and improve methane production [6]. Under optimal conditions, pretreatment methods can accelerate the decomposition and conversion of organic matter into simpler and more biodegradable compounds including monosaccharides, amino acids, short-chain fatty acids (SCFAs) which provide the substrate needed by other active microbes [7]. In general, pretreatment methods are classified into several main categories: mechanical, chemical, thermal, thermochemical and biological pretreatments [8]. Compared to other pretreatment methods, biological pre-treatment has the valuable advantages including less energy consumption, less AD inhibitors, more environmentally-friendly process and less expensive in operation. The primary usage of biological pretreatments is to increase the rate of hydrolysis by various enzymes [3]. Among biological pretreatment methods, micro aerobic process with high efficiency has drawn much attention for sludge anaerobic digestion. Lu-Man Jiang and et al. (2018) studied the sludge volume reduction using micro-aerobic process and reported that this method improve the sludge production by 0.074 g SS/g COD [9]. In addition, the biogas improvement in two-stage anaerobic digestion of corn straw was investigated by Shan-Fei Fu and et al. (2020) and they reported that this pretreatment improve the methane production by 15.9% [10]. The micro-aerobic process involves a little dosage of air or oxygen (usually between 0.005 and 5 L O_2/liter of biological reactor per day) into an anaerobic system. The micro-aerobic process establish a unique environment with both aerobic and anaerobic conditions; this technology maintains favorable conditions for both anaerobic and microaerobic organism [11]. The micro-aerobic process accelerates the hydrolysis step, reduce hydrogen sulfide concentration, prevent the accumulation of VFAs and accordingly improve the biogas production in anaerobic digestion process. In addition, this system can aid the stability of anaerobic digestion process [12]. The literature indicated that micro-aerobic process influence the hydrolysis of proteins and carbohydrates [11,12]. However, no significant effects has been reported for fats hydrolysis [13], while the effect of low-power ultrasonic waves to stimulate lipase synthesis have been comprehensively reported [14]. Ultrasonic has been proposed as an alternative pretreatment for improvement the biodegradability of recalcitrant organic matter on a laboratory scale and real conditions. The effects of ultrasonic on sludge include particle size reduction, solubilization of organic matter, enzyme release and stimulation of biological activities [15]. Cavitation bubbles and high temperatures instantaneously generated by ultrasonics can degrade EPS and cell wall of existing sludge microorganisms, release organic matter into the liquid phase and accordingly accelerate the decomposition of biological sludge [16,17]. Therefore, this technology can improve the biogas production in anaerobic digestion [18]. Recent studies confirm the efficient application of this technology in full-scale municipal wastewater sludge decomposition, dairy industry hydrolysis and aerobic digestion of activated sludge [19,20]. However, the high capital cost and operation of ultrasonic technology is one of the issues that should be considered in priority [19–21]. Thus, the combination of pre-treatment methods can lead to improve sludge treatment efficiency in terms of energy consumption and economic efficiency [21]. For instance, thermal chemistry, ultrasonic enzymatic and mechanical alkaline techniques improve the decomposition and solubilization of sludge before the anaerobic digestion process [22,23]. Kim et al. (2010) reported that the combined ultrasonic and alkaline pretreatment improve the sludge decomposition by 16.6% [24]. As per the recent studies, individual ultrasonic and micro-aerobic process improves the hydrolysis of organic matter through the integration of physical and biological phenomena [18]. Therefore, the present study was developed to evaluate the effect of the combined ultrasonic and micro-aerobic technologies on solubilization, activity of microbial lipase enzyme, and improved methane production in the anaerobic digestion process. The additional aim of present study was to investigate the feasibility analysis in terms of the energy balance and energy recovery in full-scale facilities.

2. Materials and methods

2.1. Substrate and inoculum

Raw mixed sludge containing WAS and primary sludge (PS) with predefined proportion of 70:30 (WAS: PS) and inoculum samples were withdrawn from return sludge of anaerobic digestion of south of Tehran wastewater treatment plant (WWTP). The samples were stored at 4 °C in order to maintain physical, chemical and biological properties before further experiments. The characteristics of the sludge used in this study are summarized in Table 1.

The thickening process of primary and activated sludge in the mentioned WWTP is performed by gravity concentrator and strip, respectively. Sludge stabilization is performed by mesophilic anaerobic digester with incubation period of 21 days [25]. The biogas produced in the digestion is converted into electrical energy using combined heat to power (CHP) system after gas purification process. The produced electricity is considered as a partial source of municipal electricity distribution network.

2.2. Experimental design and operation

2.2.1. Ultrasonic experiments

A pretreatment reactor should provide desirable operational conditions for cavitation phenomena in ultrasonic process [26]. The ultrasonic (US) pretreatment experiments with low frequency (20 kHz power 70 W) were performed in device with homogenizer equipped with a probe with adiameter of 3 cm (Bandelin-SonopulsSonopuls GM model 2070, Germany). The effect of ultrasonics density (UD) (W/mL) and irradiation time (UT) (min) on sludge degradation were investigated in a constant duty cycle of 0.5 and sample volume of 500 mL. To this end, 500 mL raw sludge sample was placed inside in beaker with lid and the effect of different ultrasonic densities (UD) (0.012, 0.014, 0.016, 0.018, 01, 0.12 and 0.14 W/ mL sample) on sludge solubilization and lipase activity were investigated. Then, further experiments were performed based on optimal condition (UD: 0.1 W/ml) within different time to calculate the effect of specific energies (SE) (kJ/ kg TS). (See Table 2). Specific energy was calculated based on input power (P), SS content, ultrasound time (t) and sludge volume (V), according to the following equation [27].

$$SE = (P^{*}t) = (SS^{*}V) \quad (1)$$

where, SE is specific energy (kJ/ kg TS), P denotes the input power (W), T is ultrasound time (S), and V and SS are sludge volume withdrawn (mL) and solid content (g/L).

Table 1
The chemical characteristics of sludge used in the present study.

Characteristics	Waste activated sludge (g/L)	Primary sludge (g/L)	Inoculum (g/L)
Total solid	52.03 ± 0.05	32.13 ± 0.05	28.21 ± 0.05
Volatile Solid/ Total solids*	83.78 ± 0.15	76.34 ± 0.15	61.23 ± 0.15
Total Suspended Solids	51.28 ± 0/5	31.96 ± 0/5	23.29 ± 0/5
Volatile Suspended Solids	43.58 ± 0.4	24.54 ± 0.4	14.16 ± 0.4
Chemical Oxygen Demand	82.95 ± 0.12	56.00 ± 0.12	37.52 ± 0.12
Soluble Chemical Oxygen	5.6 ± 0.05	4.300 ± 0.05	4.88 ± 0.05

*VS/TS (%).

Table 2
Electricity energy consumption in different exposure time (UD: 0.1 W/ml).

	Sonicatin time(min)	0	1	3	5	8	9	10	12
Ultrasound density = 0.1 (W/ml)	Specific energy [kJ/kgTS]	0	130	391	652	1043	1174	1304	1565
	energy applied[J]	0	3000	9000	15,000	24,000	27,000	30,000	36,000
	Ultrasound dose [J/L]	0	6000	18,000	30,000	48,000	54,000	60,000	72,000

Besides, the experimental effect of different mixing ratio (0, 115, 215, 315, 415, and 515 rpm) in ultrasonic process on sludge solubilization and lipase enzyme activity were performed in optimized conditions (ultrasonic density = 0.1 W/ml and ultrasonic time = 9 min).

2.2.2. Micro-aerobic pretreatment

Micro-aerobic pretreatment (MA) was performed in 1-L reactor and the required air was supplied via air distributor equipped in the bottom of the reactor using an air compressor with a capacity of 1 L/min.

The effect of air flow rate (AFR) (0.1, 0.2, 0.3, 0.5 air volume per-liquid volume per minute (VVM)) and time 0, 20, 30, 40, 48 and 60 h, at a temperature of 38° C on sludge solubilization and lipase activity based on the method previously described in [23].

2.2.3. Combined ultrasonic and microaerobic pretreatment

Furthermore, the effect of combined ultrasonic and micro-aerobic pretreatment system on hydrolysis and enzymatic and microbial activity was surveyed through measuring methane production during anaerobic digestion and energy analysis. For this purpose, the reactors were operated under anaerobic conditions after pretreatment and the amount of methane produced was measured and recorded instantaneously by a digital gas meter connected to a PC.

2.3. Reactor set-up and operation

After the ultrasonic and micro-aerobic pre-treatment stage, the pretreated sludge samples were evaluated in terms of biomethane potential. To this end, the anaerobic reactions were performed (working volume of 1 L and 0.2 L of free space above the reactor) and one reactor was loaded as a control by sludge without any pre-treatment. The reactors were inoculated with proportional volume of 10% through addition of biologically active inoculum [28] in the mesophilic temperature (38 °C). To ensure anaerobic conditions during the digestion process, the reactors were completely sealed and the contents of the reactor were continuously mixed using a mechanical stirrer at 60 rpm [29]. These reactors were incubated in the mesophilic temperature (38 °C) with an incubation period of 24 days. The anaerobic reactors were equipped with two ports for biogas sampling. The biogas produced was measured after passing through a liquid containing 3 M NaOH by a digital gas meter connected to a PC. All parameters were tested in triplicates. A schematic of the pilot used in the present research is illustrated in Fig. 1.

2.4. Flow cytometry analysis

To this end, an apoptosis assessment kit containing specific anxin1 and propidium iodide 2 pigments was employed. At first, the microbial sample was transferred to special vials. Then, a buffer was added to solution as per instruction, then fluorochromic anxin and propidium iodide were added and mixed gently with a shaker. After approximately 5 min, the produced solution was injected in a flow cytometer (Mindray, China). Along with the samples, a control sample without any pretreatment process and without fluorochromes anxin and propidium iodide was also injected in flow cytometer (Mindray, China). Flow cytometry data were analyzed by software (Treestar Inc., Ashland, OR Annexin V-PI-). Furthermore, live cells and dead cells were reported as (Annexin V-PI-), (Annexin V-PI+), respectively [30]. The detailed information for procedure about sample preparation are described in E. Hoseinzadeh et al. [31].

2.5. ATP measurement

Biological ATP assay was performed to quantify metabolic activity in the biological environment. Metabolic activity was analyzed using ATP assay kit (Hygiena, AquaSnap ™ Total) [32,33]. After contacting the probe kit with the contents of the sample for desired time, ATP test was initiated with pressing the top of the probe to remove the membrane and

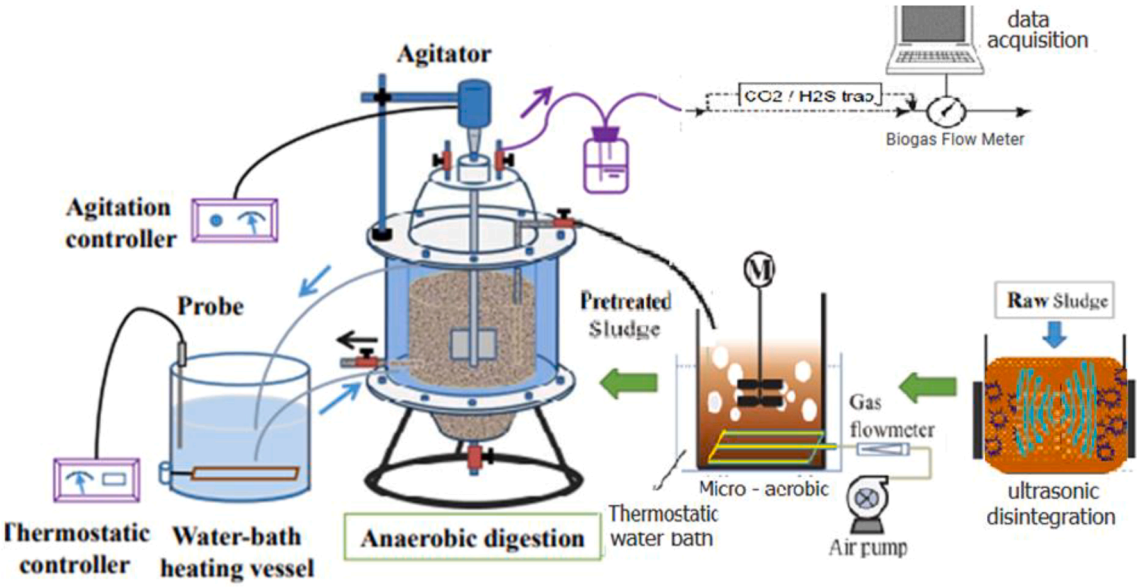

Fig. 1. Schematic of the low-strength ultrasonication with microaeration pretreatment and AD system schematic.

start the enzymatically reaction of microorganisms with all chemicals in the solutions. After shaking the probe for 10 s, the amount of light emitted by a light meter (NG III, 3 M), relative light units (RLU) was measured (log 10 RLU mL^{-1}). The procedure for sample preparation were described in detail in K Xiao et al. [34].

2.6. Microbial lipase enzyme

The oil-alcohol emulsion method was used to measure the lipase activity of bacteria. At first, 10% w/v olive oil was mixed with 5% w/v gum arabic and 0.1 M sodium hydrogen phosphate solution. Then, the resulting solution was mixed on a shaker for 3 min in order to ensure the homogenized products. Then, 1 mL sludge sample (raw or pre-treated) was added to the mixture and placed in an incubator shaker at 35° C for 15 min at 160 rpm. Furthermore, the alcohol-acetone mixture in ration of 1: 1 were added to resulting product and mixed. Finally, the resulting mixture was titrated with 0.05 M NaOH to reach pH 9.5. The amount of consumed NaOH will be equal to the amount of lipase activity (Umol/ml) [35].

2.7. Energy balance

Energy balance in terms of electrical and heating energy consumption as well as recovered energy related to biomethane production in anaerobic digestion process were calculated using the equations presented in Atelge et al. (2020) [36].

$$Energy\ Input(Ei) = ES + EM + TE \tag{2}$$

$$ES = P \times T/V \times TS \tag{3}$$

where ES is the energy spent (kWh/kg); P is the power consumed (kW); T is the pretreatment time (h); V is the volume of substrate sample (m3); and TS is the total solid concentration of sample (kg/m^3).

$$EM = Pn \times \rho m \times n_s \times D \tag{4}$$

where EM is the power needed for mixing (kW), Pn is the power number for impeller (no units), ρm is the density of biomass (kg/m3), ns is the revolutions per second (r/s), and D is the diameter of impeller (m).

$$TE = M \times SH \times \left(T_{fi} - T_{in}\right) \tag{5}$$

where TE is the thermal energy of biomass (kJ), SH is the specifc heat of biomass (for example, sludge −4.2 kJ/kg °C), M is the mass of biomass (kg), Tin is the initial temperature of biomass (°C), and Tf is the fnal temperature of biomass (°C). 1 J = $2.78*10^{-7}$ kW.

Energy Output (Eo) (kWh kg TS fed)

$$E_0 = \frac{P_{CH_4} * \varepsilon * \lambda_m}{VS\ \ removed} \tag{6}$$

where, E_0 is Energy output (kJ/g VS removed), P_{CH_4} refers to cumulative methane production in incubation period (m^3), ε expresses lower heating value of methane (35800 kJ/m^3CH_4), λ_m is Energy conversion factor of methane (0.9), and VS removed is g VS removed in incubation period [37,38].

Finally, the net energy were calculated based on Eq.7:

$$Net\ Energy = Energy\ Output(Eo) - Energy\ Input(Ei) \tag{7}$$

2.8. Analytical analysis and statistical analysis

The anaerobic digestate samples were taken in the predefined days to assess the performance of anaerobic co-digested reactors during the incubation period. The digestates taken from controls and pretreated anaerobic reactors were centrifuged (K240, Centurion Scientific, UK) at 3,000 r/min for 10 min at room temperature. Accordingly, the supernatants obtained were filtered through 0.45 m pore sized filters (PTFE-L) to determine sCOD. Soluble COD concentrations were measured by Hach COD high range vials and digestion and spectrophotometer method (MN Manocolor uv/vis).

3. Results and discussion

3.1. The effects of ultrasonic pretreatment

3.1.1. The effects of ultrasonic density on solubilization (SCOD) and lipase enzymatic activity.

The effect of ultrasonic density (0.012–0.14 W/mL) on SCOD variation was investigated within radiation time of 10 min (based on the optimum time presented in SM Joshi et al. ([39]. As shown in Fig. 2-a, the highest SCOD (5903 mg/L) and lipase enzymatic activity (14.9 Umol/ml) were observed when ultrasonic density 0.1 W/ml, indicating incremental 13.51% and 26.27% compared with control experiments, respectively. A downward phenomenon was observed when further increases in ultrasonic density more than 0.1 W/ml; SCOD decreased to 5860 and 5728 mg/L at 0.12 and 0.14 W/mL, respectively. According to literature, higher ultrasonic density significantly degrades the organic compounds and may reduce the strength and effects of cavitation [40]. Saurabh M et al.(2019) reported that ultrasonics with density 0.6 W/mL caused an increase in SCOD (62.8٪) in food industry wastewater with a solids content of 4.2% [39]. A similar trend to SCOD was observed for lipase enzymatic activity (Fig. 2a); density 0.1 W/mL increased significantly the lipase enzymatic activity from 11.8 to 14.9 (Umol/ml), and with a further increase in UD, a significant decrease was observed in enzyme activity (14.5 and 13.23 under UD of 0.12 and 0.14, respectively. In a similar study conducted by Si-Kyung Cho et al., ultrasonic with a density of 0.08 W/mL increased the metabolic enzymatic activity of anaerobic digesters [5]. In addition, increased lipase enzymatic activity in municipal wastewater sludge with TS 11.1 g/L with 75 W ultrasonic waves within 10 min has been reported by S. Anbazhagan et al. [41]. Therefore, it can be concluded that ultrasonics is an efficient pretreatment method in order to increase the degradability or increases the solubility of materials such as municipal sewage sludge, food waste, waste newspaper, sugarcane straw, etc. [42,43]. Furthermore, ultrasonic density of 0.1 W/mL was selected as the optimal condition for solubilization and activation of lipase enzyme for further experiments in the present study. In addition, the result related to effects of different mixing ratio on sludge solubilization and lipase enzyme activity are provided in Fig. S1. The results indicated an increased SCOD and enzyme activity with increasing the mixing ration until 215 rpm; the SCOD and lipase enzyme activity increased by 13.3% and 24.73%, respectively. However, the further increased mixing ratio had no significant increases in the SCOD and lipase enzyme activity; overall, increased 14.4% and 26.66% were observed in SCOD and lipase enzyme activity. Therefore, the mixing ratio higher than 215 rpm is not a cost-effective approach in ultrasonic process in terms of energy consumption.

3.1.2. The effects of ultrasonic time on solubilization (SCOD) and lipase enzymatic activity.

The effect of ultrasonic time (1–12 min) on SCOD variation and lipase enzyme activity considering a constant ultrasonic density 0.1 W/mL is shown in Fig. 2b. The results show that the highest SCOD (5918 mg/L) and lipase enzyme activity (14.9 Umol/mL) were observed at 9 min ultrasonic time, indicating an increase of 13.82% and 26.72% compared to the control sample, respectively. Further ultrasonic time caused a decrease in SCOD and lipase enzyme activity. In a similar study, increased ultrasonic time improved the SCOD of the sludge as time proceeded and the highest value was observed at 5 min; while SCOD decreased with further increased ultrasonic and large amounts of organic matter was observed in sludge flasks [44]. In addition, an increase of 15% in the SCOD level of excess activated sludge under the influence of ultrasonic waves with a specific energy of 9350 kJ/kg TS has been reported by Bougrie et al. [43]. As for the lipase enzyme

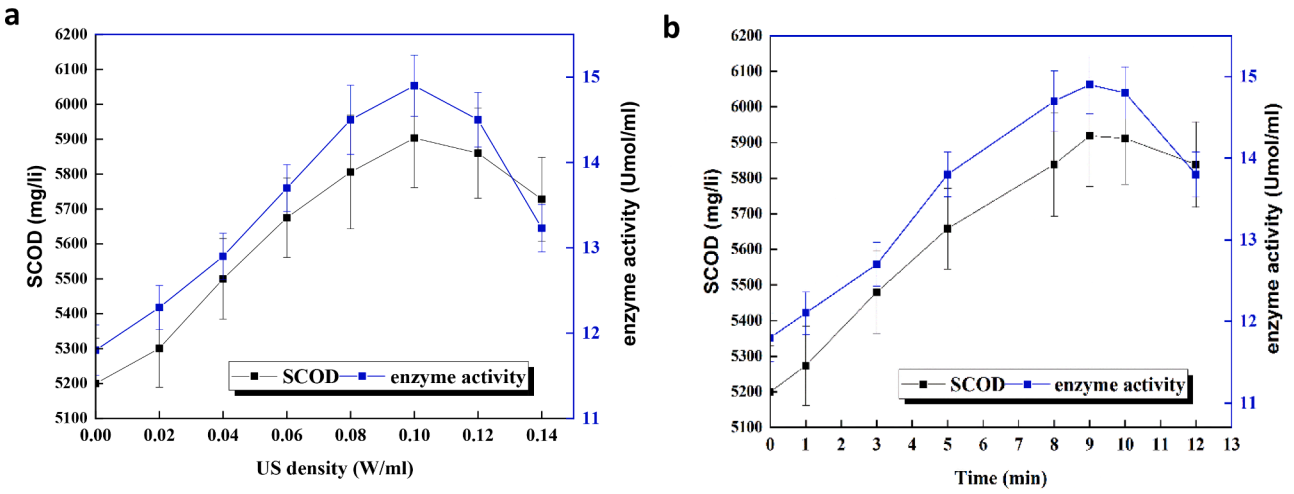

Fig. 2. The effects of ultrasonic density (a) and time (b) on solubilization (SCOD) and lipase enzymatic activity.

activity, a similar trend to SCOD was observed within ultrasonic time. In a study conducted by Anbazhagan, S. and S. Palani (2018) on extraction hydrolytic enzymes from excess sludge, they reported that increases in specific ultrasonic energy from 2703 to 27027 kJ/kg TS gradually increased the lipase enzyme activity. However, further increased specific energy declined the enzyme activity [41]. An increase in dehydrogenase activity has been observed in the Si-Kyung Cho et al with Ut = 10 min [5], which is consistent with the results of present study. S Kavitha et al reported that increasing the ultrasonic energy to 2.45 kJ/kg TS increased the activity of protease and amylase enzymes to 0.035 Umol/mL and 0.025 Umol/mL, respectively [45]. The optimal ultrasonic time depends on the complexity of the raw materials. Ultrasonic radiation for a longer period of time can also lead to excessive cavitation and degradation of the solution content. Therefore, ultrasonic irradiation for 9 min was considered as optimal condition in the present study. In a similar study conducted by Saurabh M et al., and Y Yan et al., Ultrasonic irradiation for 10 min was suggested to increase organic matter solubilization and lipase activity [39]. At the molecular level, ultrasonics can provide the impact or damage of enzymes, substrates, enzymes and substrates and their surroundings [46]. Ultrasonic in low frequency can cause cavitation, magnetic effect and mechanical oscillation effect [47]. Therefore, Ultrasonics improves the contact between enzyme and the substrate and accordingly increases the biological enzymes activity. In addition, ultrasonics can change the characteristics of the substrate and the type of reaction between enzymes [47]. Based on the above results, UD:0.1 W/mL and UT:10 min were selected as optimal condition for solubilization and lipase enzymatic activity.

3.1.3. The effects of ultrasonics pretreatment on metabolic activity

3.1.3.1. The effect of ultrasonics on ATP. Specific methanogenic activity (SMA) test is an easy way to assess the pre-treatment metabolic activity, however, it takes a long time to perform [5]. In this study, instead of SMA, ATP (microbial activity index) which requires much less time, and flow cytometry (determining the percentage of living and dead bacteria) were employed to evaluate the pretreatment efficiency as a complementary indicator.

Fig. 3 shows the effect of UT on microbial activity and microbial viability, expressed as relative light unit (RLU) and percentage (%), respectively by considering UD = 0.1 W/mL. The greatest increase in microbial activity was observed from (17 to 21 RLU) at 9 min, however, a significant decrease was observed in ATP with further increases in UT. In a similar study, when the anaerobic sludge flasks were exposed to UD = 0.1 W/mL for 10 min, the ATP content increased from 8 to 19 RLU [5]. In another study, anaerobic dehydrogenase activity abruptly decreased

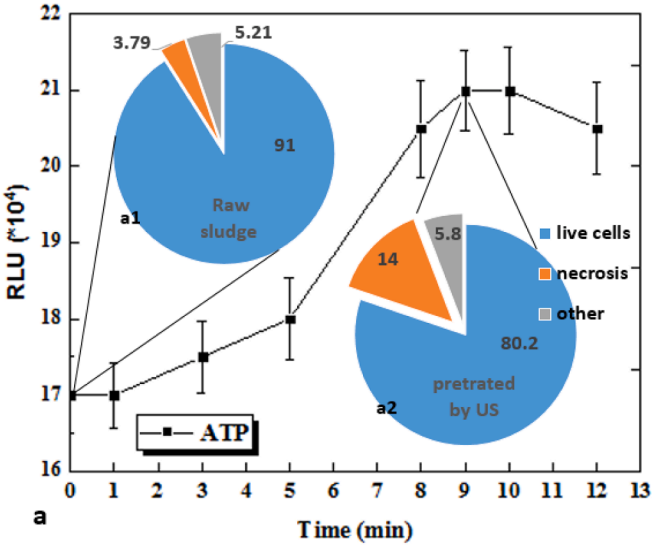

Fig. 3. The effects of ultrasonic pretreatment on ATP, microbial population before (a1) and after pretreatment (a2).

when UD exceeded a certain level [48]. When the sample is exposed to ultrasonic for a long time, the sludge flakes was loosen and broken in some extent [33]. Based on the above results, UD = 0.1 W/mL and UT = 9 min were selected as optimal conditions for microbial activity which are in consistent with DN Avhad et al. [49].

3.1.3.2. The effects of ultrasonics on microbial community. In this study, the sludge sample was exposed to an ultrasonic pretreatment with optimal condition UD = 0.1 W/m and UT = 9 min (Fig. 3). Apoptosis and flow cytometric analysis shows that the content of living cells in the control sample is 91% (Fig. 3 a1), while, ultrasonic pretreatment within 9 min decreased the living cell percentage to 80.02% (Fig. 3 a2). In addition, necrotic bacteria in the mentioned conditions increased from 3.79% to 14%.

The structure of biological membranes, cell morphology, cellular behavior, metabolism and vital processes in living organisms can be affected by the application of electrical currents which disrupt cell function and result in cell death [30].

In the present study, the percentage of live bacteria as an indicator of active biomass in the sludge sample decreased under the influence of ultrasonics. P. Foladori et al. (2010) reported that the percentage of live

bacteria under the influence of ultrasonic waves with a strength of more than 15000 kJ/kg TSS was sharply reduced and cell destruction were observed to initiate in SE more than 30,000 kJ/kg TSS [50]. The effects of ultrasonic on microbial growth and enzyme activity depend on some conditions such as frequency, density and temperature [47]. Similar studies using electron microscopy and scanning electron microscopy indicated that the mechanisms of cell membrane erosion and disruption, the formation of cavities on the surface and the slipping of cells cause the release of intracellular contents, cell membrane roughness and displacement of cell debris to the surface of other cells [47,51]. Low energy consumption obtained from the present study (ES: 1174 kJ/kg TS) compared to the effective values in the destruction of microorganisms (more than 10,000 kJ/kg TSS [50]), UD: 0.1 W/mL and UT: 9 min were selected as the optimal conditions. In addition, many studies have indicated that ultrasonic technology has many limitations. So far, the mechanism of ultrasonic treatment has not fully understood. In addition, energy consumption for sludge treatment with this technology is relatively high. Therefore, the combined ultrasonic sludge and other technologies has been proposed to improve the efficiency of treatment and lower energy consumption in field scale [44].

3.2. The effects of Micro-aerobic process

3.2.1. The effects of proportional volume of air on solubilization (SCOD) and lipase enzyme activity

The effect of AFR on SCOD (solubilization index) is shown in Fig. 4 (a). As seen in Fig. 4 (a), the amount of SCOD were increased in all air volume ratios (Fig. 4 (a)); the largest incremental SCOD (65.14%) was observed when AFR was set on 0.2 VVM for 40 h at 38 °C. In addition, the enzyme activity in the optimal solution conditions (0.2 VVM) had a slight increase of 2.6% compared to the control. The difference between pre-treatment and without pre-treatment processes shows that hydrolysis is faster in micro-aerobic conditions. More SCOD production in micro-aerobic conditions is due to the solubilization of complex organic compounds [52]. The results are in consistent with Lim and Wang [53], Jang et al. [54]. By contrast, J Diak et al. (2013) reported that the SCOD in micro-aerobic reactors decreases compared to the anaerobic digester sample [55]. This discrepancy is due to the time of micro-aeration and frequency of pre-aeration used.

Many studies have shown that micro-aerobic process has a positive effect on the hydrolysis of proteins and carbohydrates [11,12], however, this method has no significant effect on the hydrolysis of fats [13]. An increase in CMCase activity reflects the β-1.4-glucan activity for cellulose hydrolysis by 33.57% compared to the control sample reported in the study of Wanying Xu et al. [3]. D Ruan et al (2019) reported the increasing Protease and Glucosidase α activity in AFR of 4 VVM [11]. Increased production of amylase, protease and cellulase enzymes under the influence of micro-aerobic process has been reported by D Nguyen and SK Khanal et al. [56]. However, none of the mentioned studies has shown the effect of micro-aerobic process on lipase activity.

3.2.2. The effect of micro-aeration time on solubilization and lipase enzyme activity

The effect of micro-aerobic time on SCOD and lipase activity is shown in Fig. 4b. The results show that by increasing the micro-aerobic time up to 40 h, the solubilization rate increases. However, further micro-aeration more than after 40 h leads to a downward trend in the solubilization process. In addition, no significant change in lipase activity (maximum 3.5% in 60 h and 1.7% in 40 h) was observed as time proceeded. Increased peroxidase activity over 24 h has been reported in a similar study [3]. Increased peroxidase activity over 24 h has been reported in a similar study [57]. Based on these results, time 40 h (this study) is more desirable than the time of 48 h presented in the S Montalvo study [58]. (for solubilization only), which can be due to the higher percentage of sludge solids and the temperature difference. However, similar to the volume percentage of air, micro-aerobic process time does not have a significant effect on the activity of lipase enzyme.

Micro-aerobic process is an environmentally friendly and promising biological pretreatment method to increase the efficiency of resistant substrates. Increased production of extracellular hydrolytic enzymes (ie amylase, protease and cellulase) in diverse hydrolytic bacterial population, in particular, in micro-aerobic process increases the hydrolysis of carbohydrates, proteins and other organic substrates [59]. The primary mechanism of enhancing the AD process through oxygen exposure is to increase the activity of hydrolyzing and acidogenetic microorganisms under micro-aerobic conditions [12].

3.2.3. The effect of micro-aerobic process on metabolic activity

3.2.3.1. The effect of micro-aerobic on ATP. Fig. 5 (a) reveals the effect of micro-aerobic process on ATP. As shown in Fig. 5 (a), a significant increases in microbial activity (17 to 202 RLU) was observed at first 20 h, followed by a decrease in ATP. In addition, ATP values in time 40 h (optimal condition for micro-aerobic process) are 64 RLU. Lin et al. (2010) showed that the maximum amount of ATP produced (as an indicator of biomass growth) in the micro-aerobic process occurred at 30 h [60]. The trend of ATP variation observed in the present study is consistent with Lin et al. (2010) [60] and the difference in optimal time is due to the chemical nature of substrate used in the two studies.

Under micro-aerobic conditions, coenzyme A acetyl passes through the tricarboxylic acid (TCA) cycle to be completely oxidized to CO_2 through a highly energetic reaction. When O_2 acts as a final electron

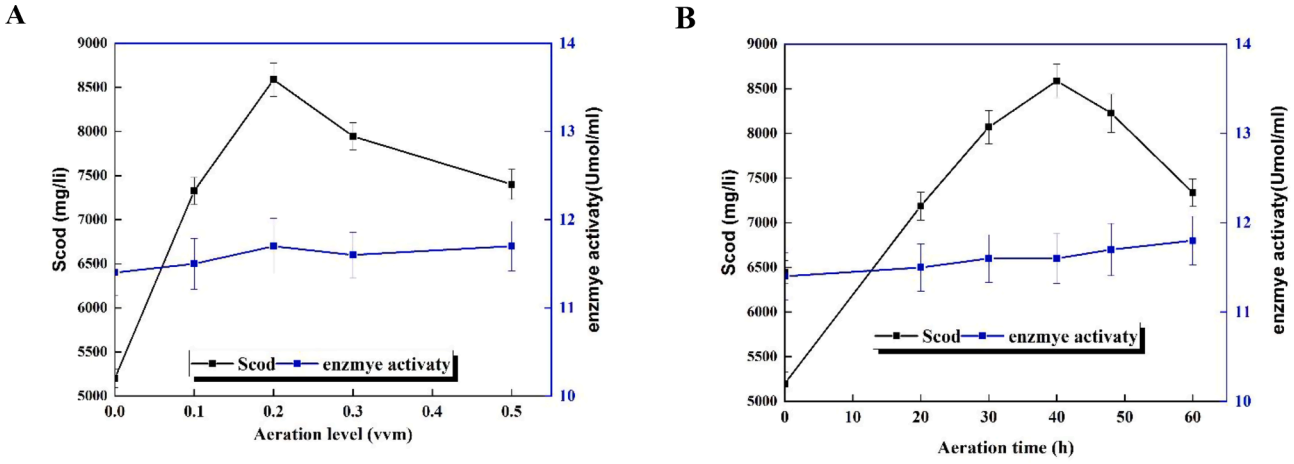

Fig. 4. Effect of volume percentage (a) and micro-aerobic time (b) on SCOD and lipase activity.

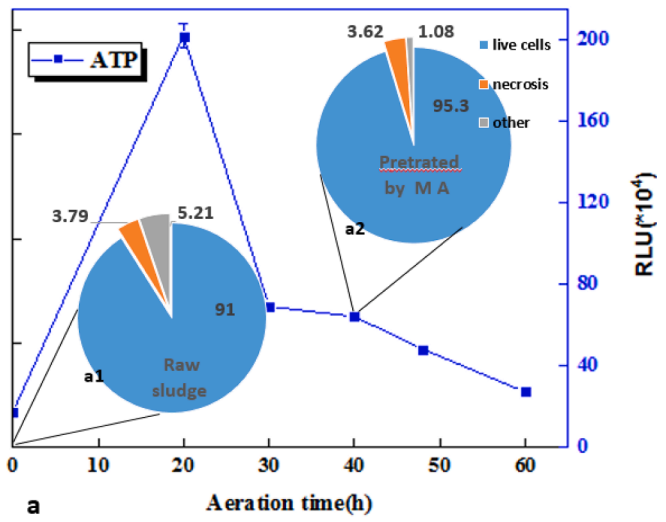

Fig. 5. The effects of micro-aerobic aeration on microbial activity (a) and percentage of live and necrotic bacteria in control (a1) and after micro-aerobic process AFR = 0.2 and time 40 h.

acceptor, aerobic oxidation of one mole of glucose through glycolysis, TCA cycle, and phosphorylation of reduced coenzymes produces 32 mol of ATP (NADH and FADH2) [56]. ATP is known as an indicator of electron transfer and microbial activity resulting from cellular respiration. On the other hand, ATP is produced as a microbial food and subsequently used for cell maintenance and synthesis of new cells in biochemical reactions [61,62]. A decrease in ATP over a period of 20 to 40 h and a subsequent increase in the number of living microorganisms over a period of 40 h confirms this theory. It seems that the increase in microbial activity up to 20 h and the subsequent decrease up to 40 h is due to the consumption of biodegradable organic compounds [23,63].

3.2.3.2. The effect of micro-aerobic process with FCM. In this study, the sludge sample was subjected to a micro-aerobic pretreatment process with AFR = 0.2 VVM at 38° C for 40 h.

The apoptosis method and flow cytometric analysis shows that the content of living cells in the control sample is 91% (Fig. 5 a1). However, the living cells content after micro-aerobic pretreatment increased to 95.3% (Fig. 5 a2), while no significant differences was observed between the Necrotic bacteria in the optimum conditions and the control sample (3.62% VS. 3.72%).

AS Dhoble et al (2016) investigated the microbiome composition in industrial-scale anaerobic digestion with varied hydraulic retention using flow cytometry-based technique [64]. It can be concluded micro-aerobic process, an efficient pretreatment method promotes the growth, activity and diversity of rapidly growing microbes, and enhance AD hydrolysis [12]. However, this method doesn't have significant effects on oil-rich wastewater. Therefore, in case of high fat content, the use of combined pretreatment techniques and methods along with the aerobic process seems necessary.

3.3. The combined ultrasonic and micro-aeration pre-treatment

3.3.1. The effects of combined ultrasonic and micro-aeration pre-treatment on solubilization and microbial activity

Organic matter is mainly found in the solid phase of sewage sludge. Prior to sludge digestion process, the organic fraction should be released into the liquid phase by decomposition of biological organic solids, which is the main step in lowering the rate of anaerobic digestion [22]. Therefore, the effect of combined pretreatment on sludge decomposition was also investigated using soluble COD.

Fig. 6a shows the SCOD in control (5210 mg/L), individual ultrasonic (5918 mg/L), individual micro-aeration (8580 mg/L) and combined ultrasonic and micro-aeration (8660 mg/L) experiments. Pretreatment efficiency in combined US and MA experienced approximately 41.19, 33.20 and 3.16% higher than that in control, US and MA experiments, respectively. These results showed that combined ultrasonic and micro-aerobic pretreatment leads to the disintegration of biological sludge solids, and release more soluble material in the environment [22]. In a similar study, combined ultrasonic and calcium peroxide (CaO_2) pretreatment increased SCOD by 4.25, 1.75, and 1.69-fold compared to the control sample, us alone, and CaO_2 alone, respectively. In addition, G Mancuso (2019) reported that the combined thermal pretreatment with low temperature and alkalinity increase more solubilization compared with the combined thermal pretreatment with high temperature and alkalinity [51]. According to studies, the instantaneous temperature and high pressure produced by ultrasonic can have strong biochemical effects on organisms and lead to cell disruption [65]. It should be noted that the effect of combining US with MA was merely synergistic, not antagonistic. When US is combined with MA, ultrasound can greatly reduce the particle size of sludge by affecting the structure of the sludge [44]. This increased solubility (permeability) can cause leakage and excretion of cellular content and extracellular glycoproteins from bacterial metabolism, which in turn affects the synthesis and production of ATP [30].

The results of enzymatic and microbial activity are shown in Fig. 6b. As seen in Fig. 6b, Lipase enzyme activity after pretreatment with US, MA and combined US + MA were found to be 15.2, 11.8, 14.8 Umol/mL, respectively, while the amount of lipase enzyme activity in the raw

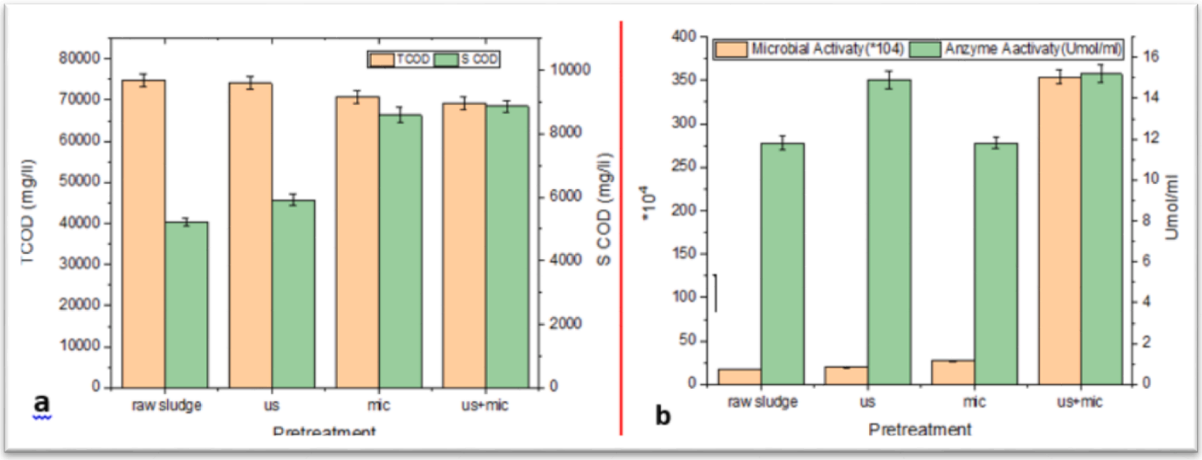

Fig. 6. The combined pretreatment on SCOD (a) and microbial and enzymatic activity (b) compared with Raw, US and MA experiments.

(control) sample was 11.4 Umol/mL. In addition, microbial activity (ATP) in the control sample, US, MA and the combined US and MA were between 17, 20, 64 and 354 RLU, respectively. Patricio Neumann et al. (2011) reported that combined ultrasonic pretreatment (SE = 15,500 kJ/kgTS) and low temperature thermal hydrolysis increased amylase and protease enzyme activity (1.8–4.3 times, respectively) compared to the sample without pretreatment. P Jenicek et al (2011) reported that the activity of methanogenic bacteria in micro-aerobic pretreatment digestion with sulfide injection was higher than the sample without pretreatment [66]. The effect of pretreatment on hydrolysis and acidification processes is surveyed by measuring the activity of specific key enzymes [67]. In case of hydrolysis process, protein and carboxylate are the two main constituents of WAS; the activity of two common enzymes, namely protease and α-glucosidase, is more important in pretreatment processes [67]. The combined pretreatment microwave and APG (biosurfactantalkylpolyglucose) increase the activity of key enzymes in SCFA production shows that the relative activities of hydrolysis and acidase enzymes are significantly higher than the activities of enzymes affected by pretreatment Microwave or APG was alone. It is well understood that MW with APG can enhance WAS hydrolysis and acidogenesis [8]. Hydrolytic and methanogenic activity depends on the type of biomass and the type of substrate or operating conditions cause the formation of specific microbial populations (biomass). Leticia Regueiro et al. (2018), reported that the hydrolytic activity was significantly changed by changing the substrate, while no significant difference was observed in the activity of acidogen and methanogenics [67]. According to the results obtained from the present study, combined pretreatment can increase the solubilization and activation of hydrolytic enzymes, providing growth conditions and microbial activity and thereby accelerate the hydrolysis process. In addition, combined pretreatment by creating active biomass reduces the incubation period of anaerobic digestion (lag phase is the most critical stage of the anaerobic digestion process) and, consequently, will make the anaerobic processes economically competitive [68].

3.3.2. *The effects of combined US and MA pretreatment on live and necrotic bacteria*

A healthy and efficient microbial population is critical to the stability and performance of anaerobic bioreactors. The microbial functional stability is governed by dynamic interaction in these microbial communities. The main hypothesis is that flow cytometry can classify microbial components based on characteristics such as viability, metabolic activity, and morphology. The main reason for the proposed method is that the combination of these properties constitutes a unique "cytometric fingerprint" which can complement existing technologies and may facilitate the rapid description of the dynamics of microbial communities [64]. The apoptosis method using flow cytometric analysis shows that the content of living cells in the control sample is 91% (Fig. 7a), and reduced to 80.02% (Fig. 7b) in US pretreatment. While, the percentage of living cells were reduced to 95.3%, 80.02% after MA and combined US + MA, respectively (Fig. 7c and 7d). While necrotic bacteria in the control sample, US, MA and US + MA were found to be 3.79, 14, 3.62 and 12.5%, respectively.

Physical methods such as ultrasonic have the highest efficiency on solubilization (SCOD) and destruction of membrane integrity of microorganisms. However, ultrasonic leads to excessive energy consumption. While the use of ultrasonic for the purpose of disrupting the structure of sludge flasks does not consume much energy [50]. Proper ultrasound has been shown to enhance the growth of microbial cells. Due to the fact that low frequency ultrasonics cause stable cavitation and repairable damage to cells, this condition accelerates their proliferation and increases the products of metabolism. However, high intensity ultrasound due to its irreparable damage are not able to accelerate the proliferation of microbial cells [63]. Low dosage of O_2 increase the diversity and activity of hydrolytic and fermenting microorganisms in the AD process. Improving the growth and metabolism of these bacteria is the basis for regulating and controlling the concentration of VFA, which leads to the promotion and overall stability of the anaerobic digestion process. The results of study conducted by SF Fu et al. (2016) indicated that under the influence of micro-aerobic process, the relative abundance of hydrolysis microorganisms is increased compared to anaerobic conditions [69].

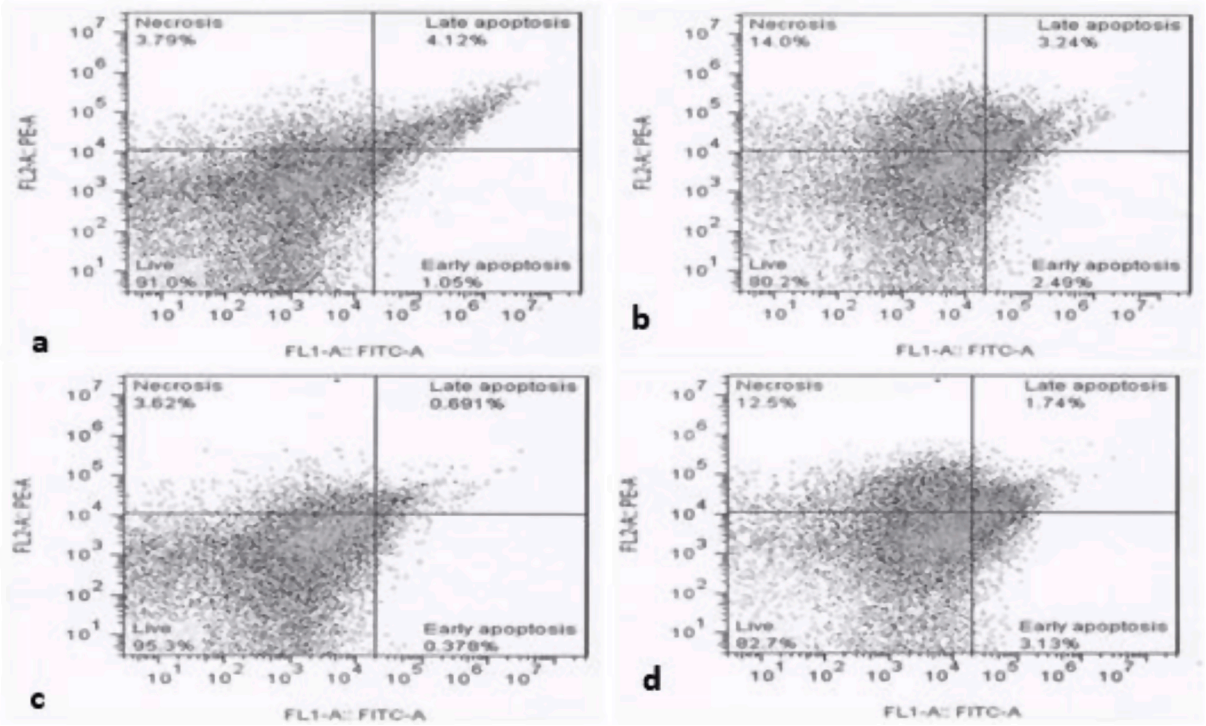

Fig. 7. Live and necrotic bacteria in control (a), after US (b), MA (c) and combined US and MA (d) pretreatment experiments.

However, all pretreatment methods have their own disadvantages. For instance, if used alone, they will have disadvantages such as high energy demand in ultrasonic pretreatment and lack of effect on micro-aerobic lipid hydrolysis. To solve this problem, one of the best options is to use combined pretreatment methods simultaneously or sequentially. In some cases, combined pretreatment will not only increase the efficiency of the pretreatment but also reduce the input energy/operating cost of the pretreatment. The results obtained from the present study show that combining two ultrasonic and micro-aerobic pretreatments, in addition to reducing the US impact on living bacteria, leads to optimized energy consumption.

3.3.3. *The effects of combined US and MA pretreatment on biogas production*

The cumulative methane gas production within incubation period of anaerobic digestion pretreated with combined US and MA compared to individual pretreatment method and control are shown in Fig. 8. As shown in Fig. 8, the cumulative amounts of methane in all reactors gradually increase as the incubation period time proceed. Compared to the control reactor without any pretreatment (120 mL CH4/initial g VS), combined pretreatment (US + MA) yielded higher performance after 24 days of anaerobic digestion. The amounts of methane production in individual US and micro-aerobic pretreated reactors were 182 and 297 mL CH_4/initial g VS, respectively. While the amounts of methane production in combined US and micro-aerobic pretreated reactors was calculated to be 340 mL CH4/initial g VS. These results confirm that ultrasonic pretreatment has led to an increase in the efficiency of micro-aerobic pretreatment. In a similar study, the use of ultrasonic energy with a specific energy (30,500 kJ/kg TS) before thermal pretreatment with a time of 13 h increased methane production in the anaerobic digestion process by 50% [18].

Furthermore, Akgul Deniz et al (2018) revealed that combined pretreatment of lignocellulosic wastewater by steam-explosion (210 °C and 10 min) and biological pretreatment (cellulolytic bacteria) leads to an increase in methane production by 140% compared to the control. While, steam-explosion alone increased methane production by 118% [70].

According to the results obtained from the present study (Fig. 8), combined pretreatment US and MA can increase the efficiency of anaerobic digestion by 2.81 times higher compared to the control. In addition, using combined pretreatment, the amount of methane production compared to the sample of US and MA alone increases 1.146 and 1.87 times, respectively. Another important point is that the amount of methane production with combined US and MA pretreatment in 19 days is more than the amount of methane produced in 24 days using MA pretreatment alone. Therefore, combined pretreatment has the potential to reduce the volume of anaerobic digestion by more than 20%. Proper combined pretreatment can increase the availability of substrate components (SCOD), providing affordable substrate digestibility [36]. Studies show that combined biological and physical or chemical pretreatment is more effective compared with than when a single pretreatment (with a specific substrate) is used. For instance, while biological pretreatment of fungi is economically viable and environmentally friendly, it is not suitable for commercialization due to its low hydrolysis rate and long shelf life. On the other hand, when pretreatment is combined with other pre-treatment methods such as physical or chemical, the performance of anaerobic digestion are improved [71].

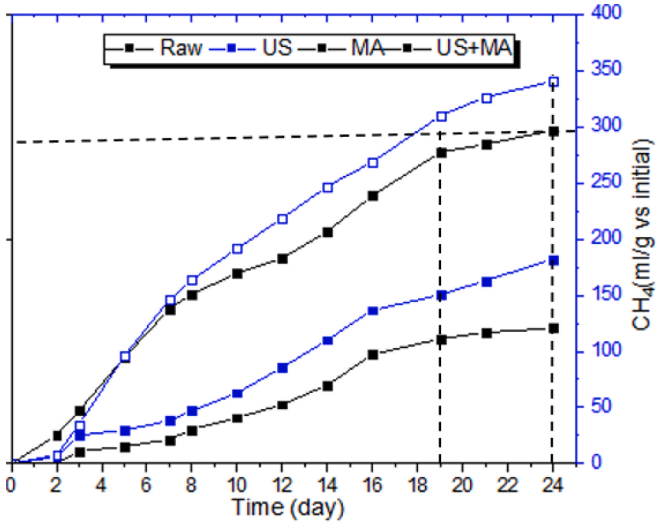

Fig. 8. The trends of methane production in anaerobic digestion incubation period in control, after US, MA and combined US + MA.

3.4. *Energy analysis*

A summary of the performance and energy recovery results for different scenarios are given in Table 3. Energy balance analysis shows that the total energy consumed (electrical and thermal) in control, US, MA and US + MA were calculated to be 0.0205, 0.076, 0.022 and 0.083 kWh, respectively. The energy obtained from methane production during the anaerobic digestion process for (control), US, MA and US + MA were calculated to be 0.581, 0.748, 1.218 and 1.337 kWh, respectively.

Net energy is considered to be the most important indicator for evaluating energy efficiency in a pretreatment process [4]. Achieving positive net energy proves the optimal energy production. In the present study, a net positive energy of 1.26 kWh was obtained when combined US and MA were used as pretreatment, while in the control sample, US and MA the net energy were calculated to be 0.56, 0.67 and 1.2 kWh, respectively. Although some energy is wasted by heat and energy by combining the two pretreatments, it can be offset by the energy from the methane (output). This energy performance shows the combination of MA + US relative to US and MA. In a similar study, combined pretreatment fine anaerobic granules (FAG) and bacterial pretreatment (BP) significantly improves the methane production [4]. Furthermore, Houtmeyers et al. (2014) reported that the net energy obtained from combined ultrasonic and microwave pretreatment is higher than that when individual system was used as pretreatment [72].

4. Conclusion

This study investigated the improvement of biomethane production using low frequency ultrasonics with micro-aerobic pretreatment prior to anaerobic digestion of municipal wastewater. The results showed that the combined pretreatment of sludge improves the hydrolysis of fat compounds and finally increases the production of biomethane compared with individual pretreatment. The results indicated that the optimal ultrasonic conditions are density 0.1 w/ml within 9 min and micro-aerobic process with AFR = 0.2 VVM and time 40 h. Combined pretreatment methods showed significant effects on solubilization, microbial activity and lipase enzyme; the production of methane in anaerobic digestion was 2.9 times higher than the control. In general, the results of the process in terms of energy show that combined pretreatment leads to energy recovery of 2.26 times higher compared with controls.

CRediT authorship contribution statement

Reza Barati Rashvanlou: Software. **Mahdi Farzadkia:** Supervision, Methodology. **Abbas Rezaee:** . **Mitra Gholami:** Writing - review & editing. **Majid Kermani:** Software. **Hasan Pasalari:** Writing - review & editing, Software.

Table 3
Energy analysis.

pretreatment method	Optimal condition	Feed concentration	VS destruction	Energy Input (kWh/kg VS fed)			Energy Output (kWh kg VS fed)	Net Energy (NE)(kWh)
				Energy spent (ES)	power needed for mixing (kW)	Thermal energy (TE)		
None-mesophilic AD	–	4.65	0.23	0.0015	0.0001	0.019	0.581	0.56
Sonication(US)-mesophilic AD	50 W, 540 S, Specific energy 1174 kJ/kgTS	3.97	0.34	0.06	0.0001	0.016	0.748	0.67
Micro aerobic(Mic)-mesophilic AD	AFR 0.2 vvm, 38 0C,39 0C, 40 h	4.08	0.56	0.001	0.002	0.018	1.218	1.2
Sonication + Micro aerobic(US + Mic)-mesophilic AD	50 W, 540 S, Specific energy 1174 kJ/kgTS + AFR 0.2 vvm, 38 0C, 40 h	3.94	0.64	0.062	0.002	0.017	1.337	1.26

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2021.105531.

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