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Application of rumen culture forenhancing hydrolysis during dry anaerobic mono-digestion of grass silage in leach-bed reactors

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Abstract: *Four, one-stage leach bed reactors (LBRs) were studied for application of rumen culture as an inoculum source for enhancing hydrolysis during dry anaerobic mono-digestion (AD) of grass silage at 35^{o}C.Two LBRs were employed to test the effect of rumen culturewith (25% rumen culture + 75% digested cow manure) and without combining the digested cow manure (i.e., 100% rumen culture). Another Two LBRs were employed to compare the hydrolytic degradation efficiencies of NaOH pre-treated and untreated grass silage using digested cow manure (100%) as inoculum. Results showed that, application of a combination of rumen inoculum @ 25% of rumen culture + 75% digested cow manure or 100 % rumen culture as an inoculum source, enhanced hydrolysis of grass silage by 10% than in control LBR. Further,methane potential assays were also carried out to test the effect of rumen culture with and without combining digested cow manure at different loading rates (v/v) and the results showed that AD of grass silage was enhanced by 36-42% from a 50:50 (v/v) combination of rumen culture and digested cow manure as inoculum with a methane yield of 0.50±0.01 m^3CH_4 $kg^{-1}VS$ when compared to the methane yields obtained with 100% of either of the inocula. In the II set of batch assays, AD of grass silage did not improve either with unfiltered whole rumen fluid (0.06±0.01 m^3CH_4 $kg^{-1}VS$) or with the solids fraction of rumen fluid as inoculum (0.005±0.00 m^3CH_4 $kg^{-1}VS$).*

Keywords*: Leach bed reactor, hydrolysis, grass silage, rumen fluid, methane, inocul*um

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I. Introduction

Anaerobic digestion (AD) of ligno-cellulosic substrates have slow conversion efficiencies, long solid retention times at low loading rates in comparison to wastewater treatment processes. This is due to the fact that hydrolysis is the rate limiting step in the anaerobic digestion of the ligno-cellulosics which is in turn due to the crystalline structures of cellulose with hemi-cellulose and lignin. Such complex structure offers higher recalcitrance for faster and efficient degradation (Strang et al., 2017). On the other hand, the process of AD occurs naturally in anaerobic microbial ecosystems wherein degradation of ligno-cellulosics takes place efficiently. The fore-stomachs (rumen) of ruminant animals such as cow, sheep, deer, goat and kangaroos and hindguts of termites are the examples for such anaerobic microbial ecosystems (Andrade et al., 2016).

The rumen is a complex anaerobic cellulolytic ecosystem with various bacteria, archaea, protozoa and fungi (Yıldırım et al., 2017).These microbial consortia collectively degrade plant polysaccharides to generate VFAs (acetic, propionic and butyric acids) as animals' energy source, biomass as the protein source for the animal and the methane is eructated via the mouth (Barnes and Keller, 2003). Therefore, the rumen uses the specific microbial community and the specific associated enzyme systems to anaerobically degrade the ligno-cellulosic materials that the animals consume (Haibo et al., 2016). The rumen can be used as a model to develop an efficient AD system which could enhance depolymerization and obtain higher degradation rates of the ligno-cellulosic substrates. The rumen-dominated AD reactors were reported to offer higher hydrolysis and acidification efficiencies for lingo-cellulosicsubstrates. Furthermore, VFAs which are released as the major intermediate product in rumen dominated reactors couldbe used to produce not only methane but also other energy products such as electricity, hydrogen, bioplastics and others (Yue et al., 2012).

Potential applications of rumen microorganismsboth as a pretreatment option and as an inoculum in operating artificial rumen reactors for the conversion of ligno-cellulosic materials were investigated (Haibo et al., 2016, Lazuka et al., 2015,Dalhoff et al. 2003, Barnes and Keller, 2003 and 2004, Hu and Yu, 2005). For example, Haibo et al (2016) investigated AD of rice straw by pretreating rice straw with rumen fluid and obtained about 82% of increase in methane yield with an improvement in VS degradation efficiency of 14-32% when compared to control.

Hu and Yu (2005) studied AD of corn stover in batch and semi-continuous cultures and obtained a high VS conversion efficiency of 65-70% after 10 days of incubation at 25-40^{o}C. In another such study by Yue et al. (2007), a degradation efficiency of 52.3% was reported during AD of aquatic plant such as *Canna indica L.* using rumen cultures in batch experiments. Furthermore, O'Sullivan et al. (2006) studied cellulose solubilization rates in batch reactors using rumen culture and MSW leachate as sources of inocula and micro crystalline cellulose as substrate. The authors obtained about 34-50% higher cellulose solubilization rates when rumen culture was used as an inoculum source than with the MSW leachate. Therefore, above studies have indicated that using rumen cultures as an inoculum source for enhancing AD of ligno-cellulosics was clearlyuseful in terms of obtaining higher degradation efficiencies and thus higher energy/methane benefits. However, none of the studies found in literature studied the effects of using rumen fluid as an inoculum source particularly during anaerobic, dry and mono-digestion of lignocellulosic biomass i.e., under high solid concentrations which can be created in reactors such as leach bed reactors (LBRs).

The objective of the present study was to test the effects of using rumen fluid as a source of inoculum for enhancing hydrolysis of grass silage during anaerobic mono-digestion in LBRs and compare the degradation efficiency/ rates of hydrolysiswiththe most commonly used inoculum such as digested cow manure from laboratory/farm scale biogas plants at mesophilic conditions. The suitability of the rumen fluid as inoculum was further evaluated in biochemical methane potential (BMP) assays as well as in leach bed reactor(LBR) studies.

II. Materials and Methods

2.1 Origin of materials

The substrate used was grass-silage and was collected from a dairy farm (Kalmari Farm, Laukaa) located in Central Finland. Grass-silage was a mixture of 75% timothy, *Phleumpratense* and 25% of meadow fescue, *Festucapratensis* harvested at the early flowering stage. After arriving at the laboratory, the material was further cut (approximately to 2-3cm) using kitchen scissors and was stored at -20°C until further use for about another 4 months.

Inoculum (digested cow manure) wasalso collected from the farm's mesophilic digester for LBR experiments and the batch experiments (BMP assays). The farm digester was co-digesting cow manure and energy crops and also by-products from confectionery industry. Rumen fluid was obtained from MTT, Jokioinenand SouthernFinland. Rumen fluid was filtered using double layers of nylon mesh (< 1mm) to filter the solid particles from the liquid, before experimental use in LBRs and in methane potential assays unless mentioned otherwise. Rumen fluid was referred as 'whole rumen fluid' when it was unfiltered and as 'rumen culture' when filtered. The characteristics of grass-silage and theinocula used are presented in Table 1.

2.2.LBR experimental set-up and application of rumen culture as inoculum

The LBR set up consisted of four, 1L acrylic column reactors with a working volume of about 780 mL andwere referred to as L0, L1, L2 and L3 operated at 35±1^{o}C. Grass silage and inocula were added in the LBRs at a $VS_{inoculum}$ to $VS_{substrate}$ ratio of 2. The LBR L0 was studied as a control reactor with grass silage and inoculum from biogas plant while, LBR L1 was tested with grass silage and rumen inoculum. The LBR L2 was tested with grass silage and a mixture ofinocula i.e., 25% rumen culture and 75% cow manure and LBR L3, was tested with NaOHpretreated grass silage and cow manure as inoculum. In all the LBRs 550 mL of water was added to make up the liquid (working) volume to about 780 mL.

Leachate collection systems used were as depicted and described in the study by Jagadabhi (2011). The leachate collection systems were placed at the bottom of the LBR columns, which consisted of a 2cm long acrylic cylindrical column on top of which a steel mesh (2 mm pore size) was placed to support the biomass weight. On top of this steel mesh a layer of foam (1cm thickness) and a layer of glass beads were placed to prevent microbial washout from the column. At the bottom of the cylindrical column two layers of nylon mesh (<1mm) were placed to further filter the solids from the leachate. Leachate was recirculated using peristaltic pumps connected to timers to pump every15 minutes (during the 24 hours).

2.3. Application of rumen culture in batch experiments

Methane potential assays (BMP) were carried outin two sets, the first set was to determine the effects of using a mixture of (filtered) rumen culture and digested cow manure as an inoculum at different ratios while the second set was to determine the effect of unfiltered, whole rumen fluid (as obtained from the animal) and the solid fraction of the rumen fluid(obtained after filtering the whole rumen fluid)on ADof grass silage. All the BMPs were carried out in triplicates in 120 ml serum bottles at 35^{o}C. To each assay,substrate grass silage and inoculum were added to achieve$VS_{inoculum}$ to $VS_{substrate}$ ratio of 1. Fermentation buffer (Raposo et al., 2006) was added to obtain a working volume of 50 mL. Of the total inoculum amount (gVS basis), rumen fluid was added at loading rates of 0, 25, 50, 75 and 100%. The corresponding ratio for digested cow manure was in reverse order i.e, 100, 75, 50, 25 and 0%. Prepared assays were sealed with butyl rubber stoppers and aluminium crimps.

The bottles were subsequently flushed with N_2 gas for 2 min in order to create anaerobic conditions. Incubation was carried out in a static incubator for about 80 days. By day 16, batch assays operating with rumen culture showed a drop in pH (5-6) with a decline in methane production and thus 2 of the replicates from these assays were opened and re-inoculated with cow manure and the 3rd replicates was continues as control against these re-inoculated assays.

2.4. Analyses and Calculations

pH was measured with a Mettler Toledo S20 Seveneasy pH meter. Chemical oxygen demand (COD) was measured according to SFS 5504 (Finnish standards association, 1988). Soluble COD (SCOD) and NH_4-N from crop samples were analyzed after extraction according to SFS-EN 12457-4 (Finnish standards association, 2002) and samples for soluble COD (SCOD) and NH_4-N were filtered using GF 50 glass fibre filter papers (Schleicher&Schhuell) before analyses. NH_4-N and Total Kjeldahl Nitrogen (TKN) were analyzed by Tecator Application Note (Perstorp Analytical/Tecator AB. 1995) with Kjeltec system 1002 distilling unit.

Methane, hydrogen content and volatile fatty acids (VFAs) were measured by gas chromatograph, Perkin Elmer Clarus 500 GC as described in Jagadabhi et al., (2008). Argon (for methane analyses) and helium (for VFA analyses) were used as carrier gases. A pressure lock syringe was used for sampling the gas and the volume of biogas produced was collected in aluminium gas bags and measured by water displacement method. Total solids (TS) and volatile solids (VS) were determined according to the standard methods (APHA, 1998). Specific methane yields were calculated as cumulative methane (mL) per g VS added and were expressed in m^3 CH_4 $kg^{-1}VS_{added}$. The methane production of the inoculum was subtracted from those of the substrates. Specific SCOD production (g SCOD g^{-1} VS), specific NH_4-N (mg NH_4-N g^{-1} VS) were calculated by considering the total leachate volume and the sample volume removed during the operation of LBRs.

III. Results and Discussion

3.1. Substrate characteristics

The chemical characteristics of the substrate grass silage, inoculum from the biogas plant and rumen fluid are shown in Table 1. It can be seen that grass silage showed an acidic pH (4.09) and low NH_4-N content (Table 1) indicating thatthe AD process in the LBRs wouldsuffer low buffering capacity. High solids content in the grass silage indicated the complexity involved in the degradation of this substrate.

3.2. Effect of different combinations of inocula in batch experiments

Batch experimentsperformed at different loading rates of both inocula(0, 25, 50, 75, and 100%) showed that AD of grass silage with a combination of 50% inoculum from biogas plant and 50% of rumen culturegave36-42% higher methanemethane yield (0.50±0.013 m^3CH_4 $kg^{-1}VS$) when compared to the methane yields obtained with 100% of either of the inocula. In a previous study conducted by Ke Li et al., (2017) the effect of rumen fluid concentration on the methane production from wheat straw was tested in batch experiments. Six levels of rumen fluid concentration were included from 1%, 5%, 10%, 15%, 20% and 25% (v/v) in reactors R1, R5, R10, R15, R20 and R25 respectively. The authors reported that rumen fluid concentrations lower than or equal to 5% resulted in reactor acidification and low methane production. The highest methane yield of 0.1 $m^3CH_4kg^{-1}VS$ was achieved in the reactor with 10% rumen fluid (in R10), whereas higher rumen fluid concentrations than 10% could not improve the methane production significantly. The methane yield obtained in the present study when 25% rumen fluid concentration was tested was higher (Table 2)thanthat obtained in the above study, which could be attributed mainly to the dryness/moisture content of the substrate that directly influences its chemical composition such asthe high solids content in wheat straw than in grass silage. High solids content in the substrate offers more structural complexity and recalcitrance for undergoing efficient microbial degradation (Kabir et al., 2015).

Higher methane yields from the assays with 50% inoculum from biogas plant and 50% of rumen culturecould be due to higher hydrolytic/cellulolytic and methanogenic rates of the process and also possibly a balance achieved in these two phases. In the assays with >50% of rumen culture as inoculum lower methane yields were obtained.This could be due to an imbalance in the processdue to higher hydrolytic/cellulolytic activity (mainly offered by rumen inoculum) and lesser methanogenic activity available (mainly offered by inoculum from biogas plant)for conversion of the formed VFAs to methane. Rumen culturealone showed about 89% higher methane yield than the inoculum from the biogas plant indicating high microbial activity of the rumen bacterial population.

In the second set of batch assays, unfiltered, whole rumen fluid alone showed high methane yield of about 0.38±0.01m^3CH_4 $kg^{-1}VS$. AD of grass silage did not improve when unfiltered whole rumen fluid was used as inoculum and resulted in a methane yield of only 0.06±0.01m^3CH_4 $kg^{-1}VS$ (Table 2). On the other hand, solids fraction of the rumen fluid resulted in 0.03±0.01m^3CH_4 $kg^{-1}VS$ while, AD of grass silage with the solids fraction as inoculum resulted in only 0.005±0.00m^3CH_4 $kg^{-1}VS$ (Table 2).This could be simply due to the microbial washout during filtration of whole rumen fluid while the microbial population adhered to the solids may not have been enough to carry out the degradation process and thus resulted in lower methane yields.

3.3. Effect of rumen culture application in LBR experiments

LBRs were operated for a period of 64 days. During the first 20 days, pH varied from 5-6 and after day 20, pH increased and remained 6.7-7.6. Consequently, maximum SCOD production was obtained by day 20 in all LBRs (7-16gL^{-1}) except in LBR with NaOH pretreated grass silage (L3) indicating that maximum hydrolysis was completed in the first 20 days (Chanakya et al., 1992)and before pH rose above 6.5. The increase in pH above 6 after day 20 also confirmed that maximum hydrolysis was obtained after which methanogenesis step was subsequently initiated. In LBR with NaOH pretreated grass silage, pH was above 7 throughout the experimental period and maximum SCOD production was observed on day 7 (8.3gL^{-1})and after which it was found to be more or less stabilized (Fig. 1).

Specific SCOD production obtained from the LBRs with rumen cultureas inoculum (L1- 100% and L2- 25 % rumen culture) was 10% higher (0.33g SCOD g^{-1}VS, Fig. 3) than the LBR with 100% inoculum from biogas plant (L0, 0.30g SCOD g^{-1}VS) following LBR with NaOHpretreated grass silage (0.17g SCOD g^{-1} VS). Maximum SCOD production was obtained in the first 20 days in all LBRs (7-16gL^{-1}) except in LBR with NaOH pretreated grass silage (L3). This result is in agreement with a previous study conducted by Chanakya et al. (1992) who studied AD of fresh and dry biomass feedstocks in solid phase biogas fermentors and observed that dry biomass feedstocks need 20-30 days of solids retention time to cross hydrolysis/acidogenic phase. The increase in SCOD solubilization was previously reported by O'Sullivan et al. (2006) during AD of microcrystalline cellulose tested with rumen inoculum against MSW leachates as inoculum sources. The authors reported that SCOD solubilization was enhanced by 34 - 60% in the reactor in which rumen culture was used as an inoculum source. The lower SCOD solubilization obtained in the present study could be due to the fact that the substrate grass silage was ligno-cellulosic in nature which offers more complex cell-wall structure than purified cellulose. Specific NH_4-N production was found to be highest in the LBR with 100% rumen inoculum (L1, 26.4 mgNH_4-N g^{-1} VS) (Table 3, Fig. 3). Total nitrogen in the LBRs varied 1-1.5gL^{-1} (Fig. 2) throughout the experimental period. Therefore, specific NH_4-N production was about 27-43% higher in the LBR with 100% rumen culture (L1) than the LBRs with inoculum from biogas plant indicating higher nitrogen solubilization into the leachate. This also confirms the higher SCOD solubilization obtained in the LBR with 100% rumen culture (L1).

The results for solids destruction showed that highest rate of degradation took place in the LBR with inoculum from biogas plant (72 and 74% of TS and VS removals, Table 3) following LBR L1, with rumen culture (100%) as inoculum and LBR L3, with NaOH pretreated grass silage. It has to be also noted that, although the I/S ratio was same in all the LBRs the VS contents in the LBRs were different as seen in Table 3 (foot notes). Higher SCOD solubilization was obtained in the LBRs with rumen culture (L1, 100% rumen culture and L2, 25% rumen culture + 75% inoculum from biogas plant) than in LBRs with 100% inoculum from biogas plant (L0 and L3) which could be a result of lower substrate concentration in these LBRs (L1-9, L2-16.5 gVS while L0-19.2 and L3-19 gVS). However, lower substrate concentration did not enable higher VS degradation efficiency from the LBRs with rumen culture. Previosly, Hu and Yu (2005), studied AD of corn stover at two different temperatures (35^{o}C and 40^{o}C) using rumen cultures at different substrate loads. The authors reported that as loading rate increased from 10-30gVS L^{-1} d^{-1} the VS degradation efficiency slightly decreased from 5-13%. This finding was in agreement to the present study as the LBR with100% rumen culture (L1) showed higher VS degradation as a result of lower VS content (9g VS) than the LBR (L2) with 25% rumen culture (16.5 gVS).

On the other hand, the differences in degradation efficiencies among the LBRs with rumen culture (L1 and L2) can also be attributed to the differences in pH conditions within these LBRs (Fig. 2). Rumen microorganisms are reported to be highly sensitive to pH fluctuations within the reactors (Hu et al. 2005). The pH conditions in the LBR with 100% rumen culture (L1) during the first 20 days, were higher (5.9-7) than the pH conditions in the LBR with 25% rumen culture (L2) (5.4-5.5) which could be the reason for higher degradation in the former LBR. Because, for rumen microorganisms pH conditions of 6-7.5 were reported to be appropriate for obtaining higher cellulose degradation (Hu and Yu, 2005, Yue et al. 2007). The lowest SCOD production in the LBR with NaOH pretreated grass silage could be clearly due to the unfavourable pH conditions in the reactor lowering the extent of hydrolysis of grass silage (Fig. 2 and 3). Because, unlike rumen microorganisms the ideal pH conditions for hydrolytic bacterial consortia are between 5.5-6.5 pH(Kim et al. 2003). On the other hand, pH conditions in the LBR with 100% inoculum from biogas plant had optimal pH conditions for better hydrolysis and thus resulted in higher specific SCOD production than LBR with NaOH pretreated grass silage.

The results of VFA production in the LBR with 100% rumen culture (L1) corroborate the results of pH and SCOD production and were found to rise upto 7.8gL^{-1} by day 12, after which VFA production started to decline due to gas production (Fig. 2). The lowest VFA production was found to be in the LBR with NaOH pretreated grass silage (L3), (3.6gL^{-1}, Fig. 2). During the first 20 days methane composition was <1% corresponding to the low pH conditions and increased only after day 20 when pH conditions rose to <6.5. High

methane yields were obtained in the LBR L1, with rumen culture (100%) as inoculum (0.22 m^3 CH_4 kg^{-1} VS) followed by LBRs L0> L2> L3 (Table 3). Higher VFA production in the LBR with rumen culture as inoculum (100%) confirms the higher SCOD solubilization and also the higher methane yield obtained from this LBR while the opposite was true for the LBR with NaOH pretreated grass silage.

IV. Conclusions

The present study showed that application of cellulolytic rumen cultures positively influenced the hydrolysis of the ligno-cellulosic substrate grass silage and improved its specific SCOD solubilization. The LBR with rumen bacterial culture as the sole inoculum source also resulted in 23% higher methane yield than its control indicating the potential for applying rumen cultures as sources of inocula for enhancing both hydrolysis and methanogenesis during dry anaerobic mono-digestion of lignocellulosic substrates in anaerobic bioreactors.

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Table 1. Initial chemical characteristics of grass silage, rumen fluid and inoculumfrom the local farm.

Parameter	Grass	Rumen inoculum (rumen fluid as obtained)	Inoculum from local biogas plant (digested manure)
pH	4.09	6.55	7.74
TS (%ww)	44.4	3.3	6
VS (%ww)	39.5	2.3	5
TCOD(gl^{-1})	62.3	32.7	50.3
SCOD (gl^{-1})	30.4	8.8	12.5
NH_4-N(gl^{-1})	0.1	0.01	0.8
TKN(gl^{-1})	2.69	1.57	3.04

Table 2. Specific methane yields obtained from grass silage with rumen culture and inoculum from biogas plant (set 1), solids fraction of rumen fluid and whole rumen fluid as inocula (set 2) (IV).

Set I	CH_4 yield (m^3 CH_4 kg^{-1} VS)
Inoculum from biogas plant	0.05±0.00
Rumen culture	0.44±0.00
Grass + 100% inoculum from biogas plant	0.32±0.03
Grass + 100% rumen culture	0.29±0.02
Grass + 75% inoculum from biogas plant + 25% rumen culture	0.20±0.00
Grass + 50% inoculum from biogas plant + 50% rumen culture	0.50±0.01
Grass + 25% inoculum from biogas plant + 75% rumen culture	0.33±0.01
Set II	**CH_4 yield (m^3 CH_4 kg^{-1} VS)**
Whole rumen fluid (unfiltered)	0.38±0.01
Grass silage + whole rumen fluid	0.06±0.01
Solids fraction of whole rumen fluid	0.03±0.01
Grass silage + solids fraction of whole rumen fluid	0.004±0.00

Table 3. Operational conditions and process performance of LBRs during theexperimental period.

	L0 (Gr + 100% inoculum from biogas plant)	L1 (Gr + 100% rumen inoculum)	L2 (Gr + 25% rumen inoculum + 75% inoculum from biogas plant)	L3 (NaOH treated Gr + 100% inoculum from biogas plant)
Specific SCOD production*[a] (g SCOD g^{-1} VS)	0.30	0.33	0.33	0.17
Specific methane yield[a] (m^3 CH_4kg^{-1} VS)	0.17±0.07	0.22±0.09	0.17±0.07	0.06±0.02
TS removal[a] (%)	72	66	52	61
VS removal[a] (%)	74	67	54	60
NH_4-N*[a] (mg g^{-1} VS)	19	26	18	15

* - average values

a - g VS added in the LBRs was different i.e., L0 -19.2, L1 – 9.0, L2 – 16.5 and L3 – 19 gVS

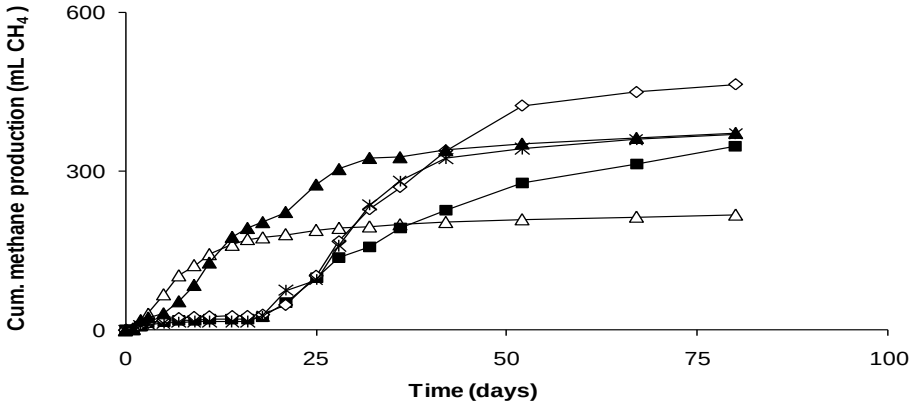

Figure 1. Cumulative methane production from the serum bottle assays. ▲- Gr + 100 % Inoculum from biogas plant, ∗ - Gr + 100 % rumen inoculum, △- Gr + 75 % inoculum from biogas plant + 25 % rumen inoculum, ◇ - Gr + 50 % inoculum from biogas plant + 50 % rumen inoculum, ■ - Gr + 25 % inoculum from biogas plant + 75 % rumen inoculum

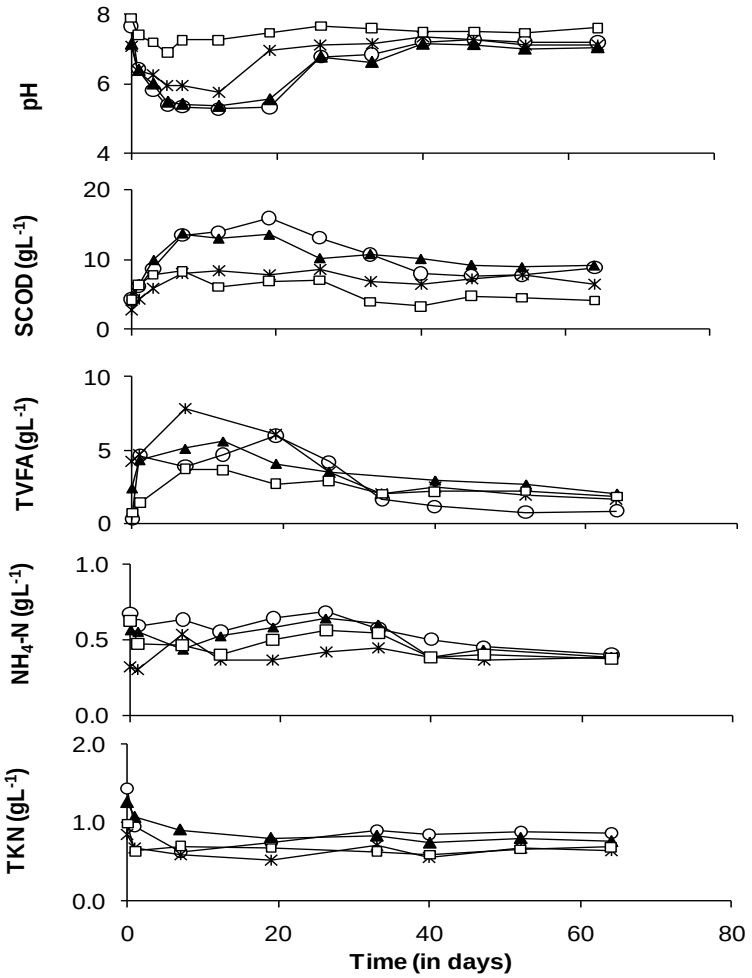

Figure 2.Performance of the LBRs during the experimental period; ○ - LBR with grass silage +100% inoculum from the biogas plant (L0), ∗ - grass silage + 100% rumen inoculum (L1), ▲ - grass silage + 25% rumen inoculum + 75% inoculum from biogas plant (L2), and□- NaOH pretreated grass silage + inoculum from biogas plant (L3).

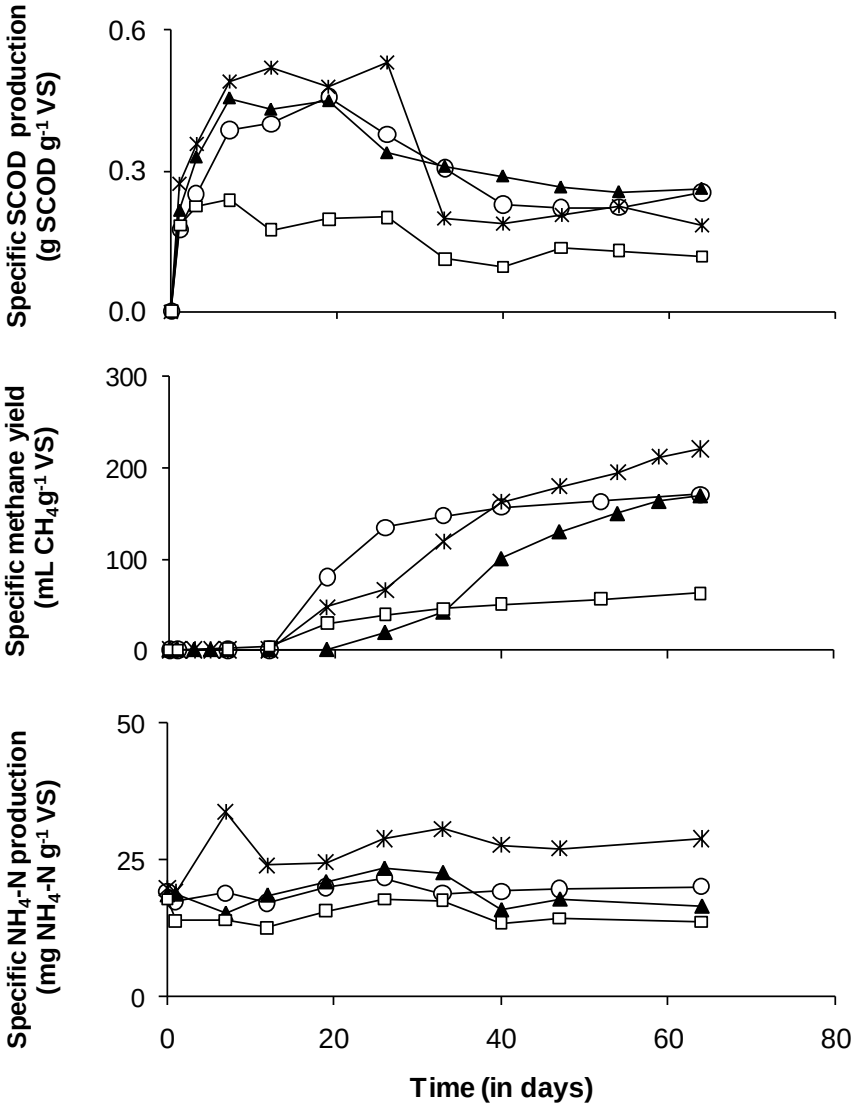

Figure 3. Specific SCOD production,methane yields and NH_4-N production from the LBRs from the LBRs. ○ - LBR with grass silage +100% inoculum from the biogas plant (L0), ✳ - grass silage + 100 % rumen inoculum (L1), ▲ - grass silage + 25% rumen inoculum + 75 % inoculum from biogas plant (L2), and □ - NaOH pretreated grass silage + inoculum from biogas plant (L3).

Padma Shanthi Jagadabhi. "Application of Rumen Cultures Forenhancing Hydrolysis during Dry Anaerobic Mono-Digestion of Grass Silage in Leach-Bed Reactors." IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS), vol. 10, no. 9, 2017, pp. 01–08.