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Influence of Oregano (*Origanum vulgare* L.), Fennel (*Foeniculum vulgare* L.) and Hop cones (*Humulus lupulus L.*) on biogas and methane production

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Dedicated

To my family and to the new generation

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Abbreviations

AD	Anaerobic Digestion
C/N	Carbon: Nitrogen ratio
T	temperature
V	Volume [m^3]
EC	European Commission
OECD	Organization for Economic Cooperation and Development
VFA	Volatile fatty acids
ADF	Acid detergent fibre
ADL	Acid Detergent Lignin
NDF	Neutral detergent fibre.
AOAC	Association of Official Analytical Chemists
XP	Crude protein
DM	Dry matter
EO	Essential oils
EU	European Union
n.a.	Not analyzed
GC	Gas Chromatography
SE	Standard Error
FID	Flame Ionization detector
ANOVA	Analysis of Variance
v/v	volume per volume
v/w	volume per weight
w/v	weight per volume
d	Day(s)
DIN	Deutsche Industrie Norm

EC	Entwicklungsstadium von Getreide
EEG	Erneuerbare-Energien-Gesetz
°K	Kelvin
Nl	Normliter
O.DM	organic dry matter
OD	optical density
ppmv	Part per million of volume
FNR	Fachagentur Nachwachsende Rohstoffe

1. Introduction

Over the last decade in Germany the biomass production for bioenergy purposes was clearly extended, particularly for biogas production via anaerobic digestion (Weiland, 2010). The number of biogas plants was increased from 1608 in 2002 to 7521 in 2012 (FNR 2013.). The growing number of biogas plants causes an increasing demand for crops as a feedstock for agricultural biogas plants in both mono- and co-digestion processes (Heiermann *et al*., 2009). The value of a crop as a substrate for anaerobic digestion depends on its biomass yield capacity compared to the effort for cultivation and on its ability to produce biogas with high methane content (Amon *et al.*, 2007; Heiermann *et al*., 2009; Weiland, 2010). Generally, the production of methane from organic substrates mainly depends on their content of substances that can be degraded to CH_4 and CO_2 (Hendriks and Zeeman, 2009). The key factors of methane production from energy crops are their composition and biodegradability. The content of carbohydrates such as cellulose, hemicellulose, starch and sugar as well as the primary compounds such as protein and fat markedly influence the methane formation (Amon *et al*., 2007).

Maize is the most predominant crop used for biogas production in Germany. It is characterized by the highest yield potential compared to other field crops grown in Central Europe (Vindis *et al*., 2008). Therefore, the reasons for expansion of maize production are: its high biomass yields with more than 40–60 t FM/ha per year (Weiland, 2010), good adaption of cultivars to the climate conditions in addition to the developed cultivation and silage techniques. Further advantage of maize is its auto-tolerance within the crop rotation which led to mono-cultivation in some regions (FNR, 2012). Since 2012 the share part of maize co-substrates in biogas plants is limited until 60% (EEG 2012). For that reason there is a need to find other alternative substrates or crops to feed biogas plants.

Anaerobic digestion of biomass to produce biogas has gained increasing value over the years mainly because of its positive energy balance (Nallathambi, 1997). Also it works as a waste treatment method and a recycling method for nutrients to farms and fields. Biogas is the end-product of a chain of biochemical reactions that occur in an oxygen-free environment (Zeng *et al*., 2007). The most common substrates for biogas production in farms are forage crops like maize, field grass (*Lolium perenne*,

Lolium multiflorum, *Dactylus glomerata*), cereals (triticale, wheat, rye), forage beets, sugar beets and animal manures (Amon *et al*., 2007). Biogas yield principally depends on the chemical composition of the used substrates. In other words, the characteristic of the used substrates for an anaerobic digestion to produce biogas should have wide ranges of bio-degradable materials with low lignin content and reduced part of compounds which may inhibit the methanogenes.

The design and the performance of anaerobic digestion processes are affected by many factors. Some of these factors are getting well along with the substrate characteristics, the digester design and the operation conditions. The physical and the chemical characteristics of the used organic materials are very important for designing and operating the anaerobic digesters (Parkin and Owen, 1986, Mata-Alvarez *et al*., 2000). Due to that those factors affect the biogas production and the process stability during the anaerobic digestion. The physical and the chemical factors include moisture content, volatile solids content, nutrient content, particle size, and biodegradability of the biomass. The biodegradability of a substrate is indicated by biogas or methane yield and percentage of solids (total solids or volatile solids) that are decomposed in the anaerobic digestion. Biogas or methane yield is measured by the volumetric amount of biogas or methane that can be produced per unit of volatile solids contained in the substrate after subjecting it to an anaerobic digestion for a sufficient amount of time under a given temperature (Zhang *et al*., 2007).

Due to the energy shortage problems and the environmental issues the world is facing some problems because of depletion of fossil fuels and accumulation of greenhouse gas emission from combustion of fossil fuels. These sustainability problems increase the demand for fuel produced from the renewable resources. Wide ranges of raw materials used in anaerobic digestion, as waste from households, animals and agriculture production. Generally, in some countries biogas is produced by anaerobic digestion of animal manure. Animal manure gives relatively limited rate of biogas production because all the components of the feed stuff were digested by the animal stomach. Therefore, in Germany and some European countries animal manure is used as co-substrate in combination with other substrates to enhance the biogas production. Utilization of the waste and residue of vegetables for the production of biogas offers some advantages. Beside waste management

strategy, the costs for raw material are cheap, available in high quantities and yield more biogas.

In addition to the current use of forage and energy plants also organic wastes from consumer households as well as from the processing of vegetables, spices and fruits can be used as co-substrates in biogas plants (Weiland, 2010). The main waste of vegetable plants are from agricultural cultivation practices, postharvest handling, storage, distribution, transportation and rest from consumption as household wastes. Wastes receiving from the production and processing of vegetable and spice plants are characterized by a wide and diverse range of compounds including secondary metabolites (Parthasarathy *et al*., 2008).

According to the EEG standards the medicinal and spice plants produce lower methane (59 Nm^3CH_4 /t FM), when compared with maize 120 Nm^3 CH_4 /t FM (FNR, 2012). By applying Baserga formula (1998) to oregano after analyzed to carbohydrates, protein and fat the theoretical biogas and methane were calculated. Our preliminary works on biogas production in mesophilic laboratory digester in Rauischholzhausen using oregano herbs gave low yield of biogas and methane. There is a big difference when comparing the theoretical calculated biogas and methane from oregano with its actual lower production. The low biogas production by oregano is attributed to the presence of the plant secondary metabolites found in oregano herbs and spices (Busquet *et al*., 2006; Macheboeuf *et al*., 2008). These secondary compounds figure the function and the characteristic of the medicinal plants and are correlated with the defense system in the plant itself, plant-insect interaction to attract pollinators and to reduce transpiration of the leaves by protecting the stomata (Hadacek, 2002). The medicinal plants and spices contain mainly essential oils, which are composing of terpenes and phenylpropane or aromatic compounds (Cowan, 1999). In addition some other groups of secondary compounds such as phenolic acids, flavonoids, coumarines, alkaloids and glucosinolates can be found (Wink, 2003). Until now there is a lack of information about the effect of terpenes found in spices and medicinal plants parts on anaerobic microorganisms.

Most of the essential oils components act as antimicrobial agents. For instance carvacrol, thymol and limonene, which occur in oregano, thyme, rosemary plants and citrus peel, may have antimicrobial function for the plant. For example limonene is reported to inhibit biogas production from citrus waste (Dorman and Deans, 2000,

Chang *et al*., 2001, Burt, 2004, Cos *et al*., 2006, Soylu *et al*., 2007, Martín *et al*., 2010).

Another study reported that carvacrol and thymol have antibacterial, antifungal and inhibitory effects against wide range of microbes and they improved the safety of food preservations (Bagamboula, *et al*., 2004; Kordali *et al*., 2008). Therefore, to increase the biogas yield of an agricultural biogas digester waste from spice plants must be in a right balance in relation to the other substrates (manure, maize and forage crops) which are used. Furthermore there is a need to analyze the composition of the used waste and their effect on methanogensis in a biogas plant.

Objectives

Medicinal and spice plants in addition to their processed products and wastes are characterized by a diversity of secondary metabolites mainly terpenes and phenolic compounds. Mono and sesquiterpenes including aromatic compounds are found in glandular trichomes especially in leaves, flowers, fruits and seeds but also in minor content in barks, woods, stems roots and rhizomes. However, in literature no studies are found focusing on the effect of plants containing secondary metabolites like terpenes on biogas and methane production. For that reason, the objective of this study was to investigate the inhibitory effect of oregano (*Origanum vulgare* L.*)* herbs, fennel (*Foeniculum vulgare* L.) fruits and hops cones (*Humulus lupulus* L.) on biogas and methane production and to determine their hazardous concentration range.

2. Literature review

2.1 Anaerobic digestion process

Anaerobic biodegradation of organic material takes place in the absence of the oxygen and in the presence of microorganisms in an anaerobic environment. It is a consequence of metabolic series and interactions among various groups of microorganisms. It can be characterized as a series of biochemical reactions during which organic materials are decomposed through the metabolic pathways of naturally occurring microorganisms in an oxygen free environment. Anaerobic digestion can be used to process any carbon-containing material, including crops, animal manures, food, paper, sewage, and solid waste, with various degrees of degradation. There are four steps of anaerobic digestion that include hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Deublein and Steinhauser, 2008; Weiland, 2010) as illustrated in Figure (1). Throughout the first step, large organic polymers that make up biomass are broken down into smaller molecules and monomers by chemicals and microorganisms. The second step is acidogenesis, in which acids are formed. The third step is acetogenesis, where anaerobic bacteria convert the products of the previous step into acetate, CO_2 and H_2. Methanogenesis, which is the last step, where methane is mainly produced from acetate, hydrogen with carbon dioxide or formate. In addition methanogenic *Archaea* also can utilize some metyl compounds such as methanol or methanethiol to produce methane. Each of these steps has its physiological unique microorganism's population. Some scientists mentioned three steps instead of four steps and their opinion for that acetogenesis is not complete step but it is included in acidogensis step (Arsova, 2010). In the following text the steps of anaerobic digestion will be explained.

Hydrolysis is the first step in anaerobic digestion, where the complex polymers such as carbohydrates, proteins and lipids are hydrolyzed into soluble organic molecules such as sugars, amino acids and fatty acids by extracellular hydrolytic enzymes, i.e. cellulases, amylases, proteases or lipases (Parawira, *et al*., 2005). In general, hydrolysis is the limiting step if the substrate is in particulate form (Vavilin *et al*., 1996). The rate of hydrolysis is a function of other factors such as pH, temperature, composition and particle size of substrate, and concentration of intermediate products (Veeken *et al*., 2000).

Acidogenesis is the second step in anaerobic digestion, where the soluble organic molecules like long-chain fatty acids and amino acids that are produced from hydrolysis step are used by either fermentive bacteria or anaerobic oxidizer to form acetate and other short-chain fatty acids, alcohols, hydrogen and carbon dioxide (Gujer and Zehnder, 1983, Garcia-Heras, 2003). The microorganisms in this step are obligate and facultative anaerobes. In a stable anaerobic digester, the main results are acetate, carbon dioxide and hydrogen. The intermediates, such as volatile fatty acids and alcohols, play a minor role. This degradation path way gives higher energy yield for the microorganisms and the products can be used directly by methanogenic microorganisms (Schink, 1997). However, the partial pressure of the hydrogen regulates what types of products are formed. Generally, a high partial pressure favors acetate production (Bryant, 1979, Klass, 1984; Ahring, 2003). In a well-balanced system, about 70-80% of the hydrolysis products will be transformed directly to methanogenic substrates i.e., hydrogen, carbon dioxide and acetate, with the remaining of 20-30% transformed into other intermediate products, such as volatile fatty acids (VFAs) longer than two carbon atoms and alcohols longer than one carbon atom (Gujer and Zehndr, 1983; Schink, 1997; Ahring 2003). If the conditions are not optimal, other intermediates are formed as well, such as alcohols and volatile fatty acids. These intermediates need to be further modified (acetogenic phase) before the methane-producing organisms are able to convert them to methane. Acidogensis step is usually considered the fastest step in anaerobic digestion of complex organic matter (Vavilin *et al*., 1996).

The acetogenesis is the third step in anaerobic digestion, where the intermediates products that formed in acidogenesis step, like fatty acids (longer than two carbon atoms), alcohols (longer than one carbon atom) and branched-chain and aromatic fatty acids. These products cannot be directly used in methanogenesis and have to be further oxidized to acetate and H_2 in acetogenesis step by obligated proton reducing bacteria in a syntrophic relationship with hydrogen utilisers. Low H_2 partial pressure (lower than 10^{-5} bar) is essential for acetogenic reactions to be thermodynamically favorable (Schink, 1997). The products from acetogenesis are then the substrates for the last step of anaerobic digestion, which is called methanogenesis.

Methanogenesis is the last step in biogas production, where the acetate and H_2/CO_2 coming from the previous step should convert to CH_4 and CO_2. This step is carried

out by two main groups of methanogens: the aceticlastic methanogens, which degrade acetate, and the other group, which is the hydrogen-utilizing methanogens. Methanogens are strict anaerobic group of microorganisms. It was estimated that, about 70% of methane is produced by the acetate-utilizing methanogens and 30% by the hydrogen consuming methanogens under stabilized conditions (Smith *et al*., 1980; Klass, 1984). Moreover, the inter-conversion between hydrogen and acetate, catalyzed by homoacetogenic bacteria, also it plays an important role in the methane formation pathway. Homoacetogens can either oxidize or synthesize acetate depending on the hydrogen concentration in the system (Kotsyurbenko, 2005). Hydrogenotrophic methanogens convert hydrogen and carbon dioxide to methane. While aceticlastic methanogens is independent on hydrogen partial pressure, it is sensitive to higher temperatures. At higher temperature the acetate oxidation pathway becomes more favorable and the hydrogenotrophic methanogens became more important (Schink, 1997). Moreover, the synotrophic relationship between the acetogenic and methanogenic microbes discussed above, is only feasible within the narrow hydrogen pressure range, from 10^{-4} to 10^{-6} atm (atm= 1.01 bar), (McCarty and Smith, 1986). Also Schink (1997) in his review mentioned that the H_2 partial pressure should be in the range between 10^{-6}–10^{-5} bar for the syntrophic relation between acetogens and methanogens to occur. In a biogas digester, the methane-producing microorganisms are most sensitive to changed process parameters, such as pH, temperature, and substrate concentration (Chen *et al*., 2008).

Methanogens are found in many habitats including anaerobic digesters, landfill sites, intestines and stomachs of ruminants and other animals, rice paddies, soils, freshwater and marine sediments (Lange and Ahring, 2001; Lange *et al*., 2005, Chaban *et al*., 2006, Angel and Conrad, 2013). In general, methanogens typically found in reduced anoxic environments (Aschenbach *et al*., 2013). The differences between *Archaea* and bacteria are: all *Archaea* lack a cell wall that contains muramic acid and the most common *Archaeal* cell wall consists of a single glycoprotein. Also in some *Archaea* the cell wall consists of polymers such as pseudomurein. Cultivation of methanogens need strict anaerobic techniques as described by hungate (Humane, 1969; Hungate and Macy,1973) or modifications of those techniques as the use of serum bottles with butyl-rubber stoppers as culture vessels (Miller and Wolin, 1974). Serum bottles are simple and flexible rather than the agar plates, which they need to be placed in an anaerobic chamber and need care in

handling. Examples of methanogens are: *Methanobacterium thermoautotrophicum, Methanosarcina barkeri* and *Methanosphaera stadtmania*.

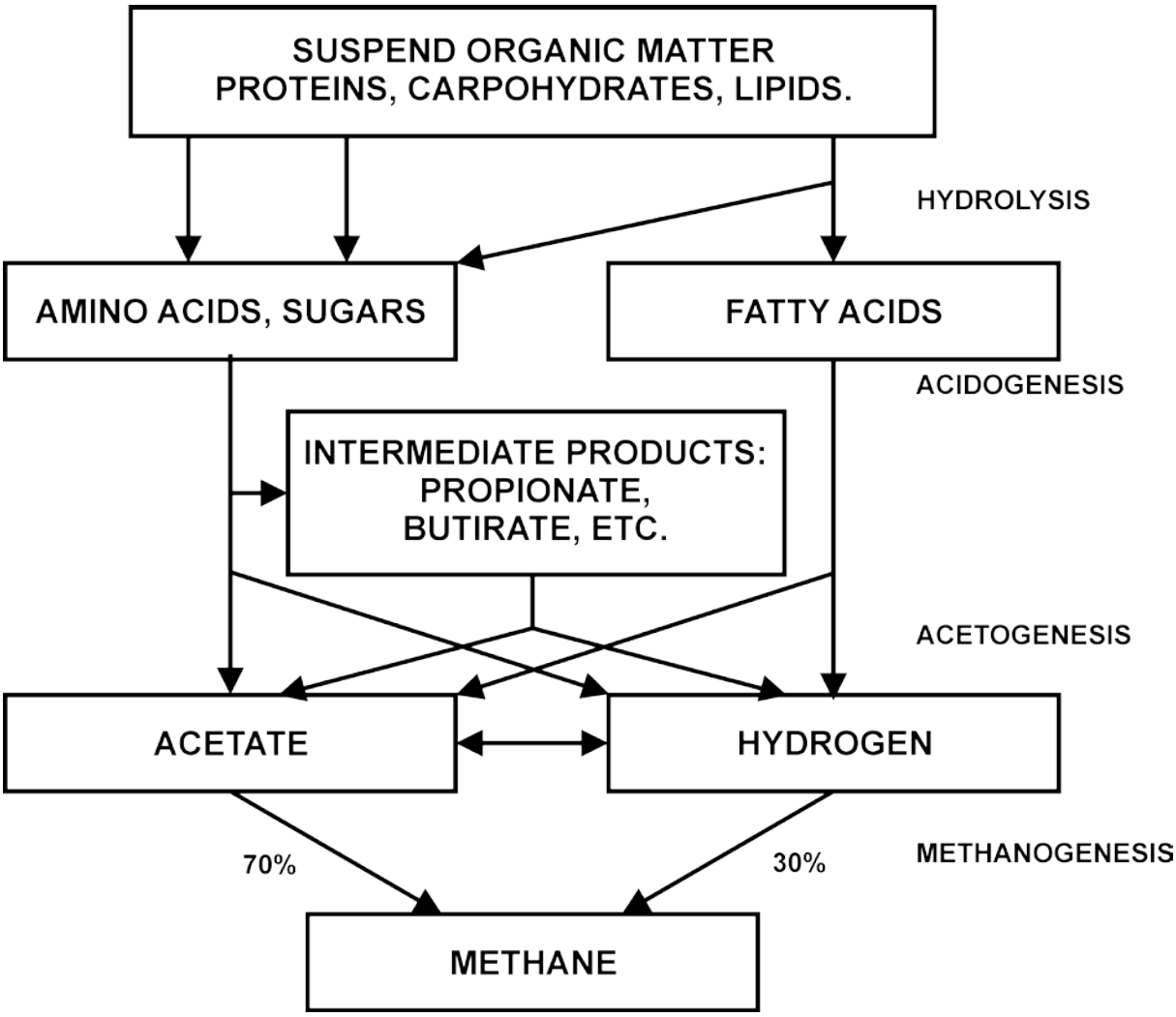

Fig. 1 Anaerobic digestion process (according to Gujer and Zehnder 1983).

2.2 Inhibition and toxicity factors in biogas production

The German standards (VDI 4630, 2006) defined the term inhibition in biogas production as the hindering of fermentation due to damage to the active microorganisms or to a reduction in the effectiveness (activity) of enzymes. Furthermore, Speece (1996) differentiate between inhibition and toxicity. Toxicity adversely affects microbial metabolism as a whole but inhibition is an injury of a particular microbial function. There are many substances found to inhibit biogas production via affecting the microorganisms that share in the biogas production. Examples are: long-chain fatty acids, ammonia, H_2S, Ca^{++}, Mg^{++}, K^{+}, Na^{+}, heavy

metals and a wide range of organic compounds (Gerardi 2003, Pereira *et al*., 2005, Schnürer and Nordberg, 2008, Fang *et al.*, 2011). However, several secondary metabolites in crops, such as phenols, terpenes, alkaloids, saponins, and *p*-coumaric acids are harmful to microorganisms (Turner *et al*.,1980). There are some studies investigated the concentration of plant secondary compounds that cause negative effects on biogas and methane producers. For instance Hernandez and Edyvean (2008) studied the inhibition of biogas process by seven phenolic compounds and they reported that 50% of inhibition is at the range of 120-594 mg of compounds per gram of O.DM and he concluded that inhibition will occur at concentrations of 800-1600 mg/l organic carbon. Borja *et al*. (1997) also investigated the impact of phenols in olive mill waste water on biogas production. Akassou *et al*. (2010) found that catchol and *p*-coumaric acid in olive mill waste water at concentrations of 1664 and 50 ppm respectively have strong inhibitory effect on methanogens.

There are some studies investigated the effect of plant secondary compounds on the enteric methanogens in the stomach of animals. For instance Prabhudessai *et al*. (2009) studied the effect of saponin on methanogenes and he concluded that saponins have negative effect at concentrations of 50,100,150 ppm. Lastly Zhou *et al.* (2011) studied the inhibition of the rumen methanogens by tea saponin. He reported that addition of tea saponin reduced CH_4 production mainly by inhibiting protozoa, which are in symbiotic relation with methanogens.

There are some studies that investigate the effect of terpenes on the methanogenes. For instance Sierra-Alvarez and Lettinga (1990) analyzed the methanogenic toxicity of wood resin constituents. He observed that terpenes in wood resin have high methanogenic toxicity ranged from 39-330 mg/l. Benjamin *et al*. (1984) found that *p*-cymene and *D*-limonene, which are terpenes found in essential oil of several medicinal plants cause 50% inhibition of methanogenes at concentrations of 500 mg/l and 250 mg/l respectively at in vitro tests. Crane *et al*. (1957) reported that in vitro tests D-limonene and α-pinene induce 50% inhibition of methanogens at concentrations of 122 mg/l for each.

Many inhibitory or toxic compounds enter the biogas digester with the contaminated waste. Other groups are naturally present in some substrates and include various types of plant secondary metabolites such as phenols, cresols, tannins, toluenes, terpenes and other aromatic structures. The type of inhibition is depending on the

concentration of inhibitory substances, retention time, temperature, pH; number and type of microorganisms present (Chen *et al*., 2008).

2.3 Methods for determining the biogas and methane potential

The biogas and methane yield (in Nl/kg O.DM) can be used to evaluate different organic substrates used in a biogas digester. Generally two methods are used to determine the biogas and methane potential:

1-Theoretical methods to calculate the biogas yield.

2-Practical methods carried out by anaerobic digestion**.**

Based on a theoretical equation called as Buswell equation the products from anaerobic breakdown of a common organic material can be estimated. The Buswell can be characterized as follows:

$$C_cH_hO_oN_nS_s + yH_2O \; xCH_4 + nNH_3 + sH_2S + (c-x)\; CO_2$$

Where: x= 1/8(4c+h-2o-3n-2s), c, h, o, n and s are the numbers of atoms

The Buswell equation can be used to estimate the biogas composition but not the biogas volume produced, as it assumes 100% of the material was breakdown into biogas. Moreover, Baserga formula is an alternative formula for Buswell, if the components of the substrates as carbohydrates, fat, protein, cellulose and hemicellulose are known. Therefore, Biogas and methane volume can be easily predicted using table 1. Theoretically carbohydrates yield 790 – 800 Nl biogas (with methane and CO_2 content of around 50% for each) per kg of O.DM. Similarly, fats yield 1200-1250 Nl biogas (with methane and CO_2 content of around 68% and 32% respectively) per kg of O.DM and that of proteins yield 700 Nl biogas (with methane and CO_2 content of around 71% and 29% respectively) per kg of O.DM (Table 1). VDI 4630 (2006) reported that 7% of O.DM will be used by the microorganisms to survive and produce their energy.

Table 1 Amount of theoretical biogas and methane found in the material components (Baserga formula).

Substrate	**Biogas (Nl/kg O.DM)**	**Methane %**	**CO_2%**
Proteins	700	71	29
Fats	1200-1250	68	32
Carbohydrates	790 -800	50	50

Practical methods on the basis of anaerobic digestion

The theoretical methods are not precise because:

a- Not all the organic contents of the substrates are biodegraded completely in the biogas digester.

b- The microorganisms use part of the substrate for their energy production; which is about 7% of the organic dry matter (VDI 4630, 2006).

c- Some substrates contain chemicals in addition to several inhibitors.

Therefore, the widely used method is the performing digestion tests for each substrate.

Added to that, it is a convenient tool for evaluating the actual biogas yield. Digestion tests can be done at different scales, and their results are commonly used for designing full-scale plants as example is a batch digestion assay, which is the simplest method of the digestion tests and can be used for determining the methane potential, and for kinetic measurements. Certain amounts of substrate and methanogenic inoculum are placed in the reactors, which then are sealed and placed in a controlled temperature until the substrate is degraded. The conditions are anaerobic and the temperature is kept optimal during the experimental period. These tests usually require 21-30 days, since anaerobic digestion is a slow process, but one advantage of the batch method is that many parallel tests can be done simultaneously. This makes it suitable for comparing the methane potential of different substrates or for evaluating different pretreatment methods and conditions. Typically, only the methane and carbon dioxide as the main compounds of biogas and in sometimes H_2S are measured.

2.4 Characterization of substrates used in biogas production

In Germany 46% of the substrate currently used in biogas plants is derived from energy crops, whereas 45% are from animal manure and 7% from biological waste (DBFZ, 2012). Over the last years it can be observed that energy crops are increasingly applied in most biogas plants (Weiland, 2003, Antoni *et* al., 2007). To be suitable for biogas production, a crop has to fulfill certain requirements (Weiland, 2006, BMELV, 2012): good suitability for storage, high methane yield per area land, low costs of production, and easy integration into existing farming systems. Maize fulfills these criteria to a high degree. For that reason the area under maize grown was about 1,470,000 and 2,028,800 ha in 2007 and 2011 respectively (BMELV, 2012). Maize yield is dependent on local and environmental conditions and may vary from 40 t fresh mass (Table 2) to over 65 t FM/ha (Weiland, 2010).

Great variety of substrates can be used in biogas production ranging from pure components to complex mixture of organic materials. Beside manure starch and sugar containing crops the most important materials for biogas production in addition to maize are sugar beet, molasses forage crops and the rest of food and house hold wastes (Table 2).

Table 2 Gross crop yield and biogas potential of different crops (modified from Weiland 2010, FNR 2012)

Crop	Yield(t FM/ha)	Biogas yield (Nm^3/t substrate)	Methane (Nm^3/t substrate)
Maize silage	40-60	170-230	89-120
WCC silage	22-43	170-220	90-120
Cereal grain	36	620	320
Grass silage	22-31	170-200	93-109
Sugar beet	40-70	120- 140	65-76
Fodder beet	80-120	75-100	40-54

WCC= whole cereal crop

Basically all agricultural crops can be applied for biogas production if the crop is not lignified and have enough carbohydrates, proteins, fats, cellulose and hemicellulose as main components. The most important crops are energy crops which have the highest potential in Germany (FNR, 2012). Forage beets have the highest gross

energy potential but also different cereal crops and perennial grasses have potential as energy crops (Table 2). Away from maize, beet cultivation provides large amounts of biomass (40-120 t FM/ha), which could be used in biogas production (Weiland, 2010). Normally, beets are used for sugar production or as animal feed. From table 2 similar to maize silage, the cultivation of beets has a high yield and the methane yield is comparably high. In biogas plants often the residues from sugar and fodder beet, leafs and tops are used (Börjesson and Berglund, 2007).

2.5 Characterization of terpenes containing plants

2.5.1. Secondary metabolites

Secondary metabolites are organic compounds, which are synthesized by plants in low concentration with high diversity of chemical structures. As opposed to primary metabolites they have no function in the life cycle of the plant cells. The production of specific secondary metabolites varies among species or genera. The boundary between primary and secondary metabolites is not well defined and the areas overlap. From a chemical point of view secondary metabolites are interesting for various reasons e.g. their structural diversity, their potential as drug candidates or as natural pesticides.

The function of secondary metabolites is that many of them are involved in the interactions between organisms, for example in plant defense against pathogens, in toxicity of the pathogens or attraction of organisms that beneficial for the producer (Bennett and Wallsgrove, 1994, Kimura *et al*., 2001, Hartmann, 2007). The uses of the secondary metabolites for humans are many and include uses as pharmaceuticals, agrochemicals, food additives and as ingredients in cosmetics. Figure 2 represents the main classes of plants secondary metabolites which are carotenoids, terpenes, alkaloids, glucosinolates and phenols, which are also, divided to phenolic acids, flavonoids, coumarins and tannins.

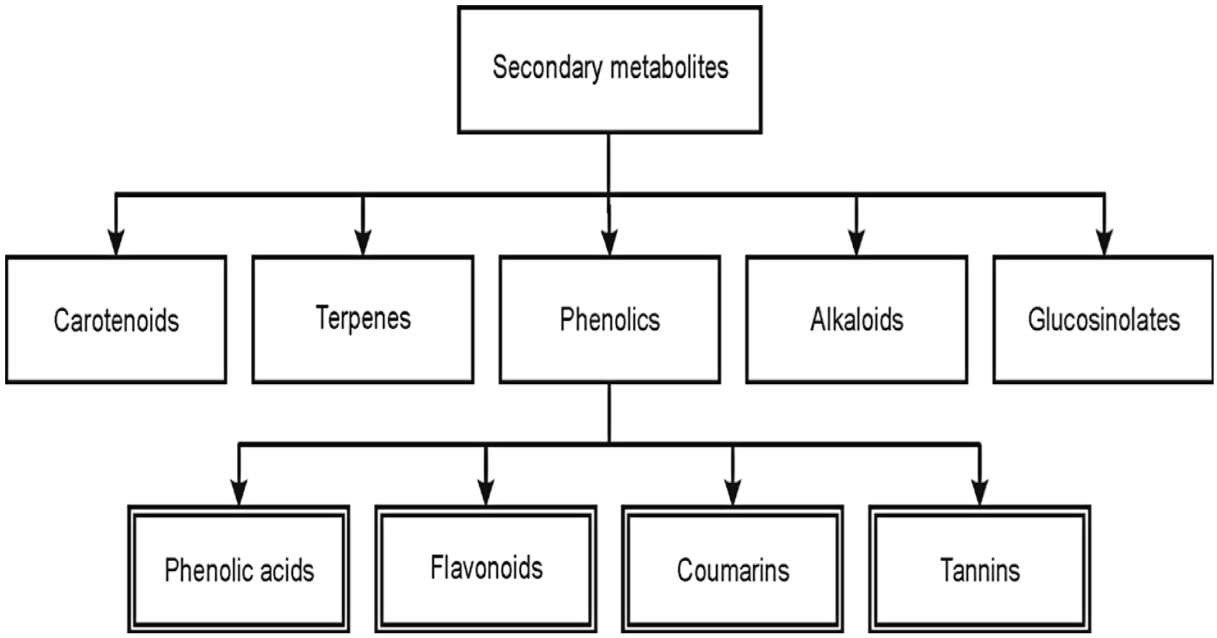

Fig. 2 Classification of plant secondary metabolites (modified from Liu 2004)

Terpenes

Terpenes are one of the largest group with more than 30,000 compounds of plant secondary metabolites synthesized in many plants (Sacchettini and Poulter, 1997; Dewick, 2002). Terpenes are classified according to the number of the five carbon atoms containing isoprene units in their structure: hemiterpenes C5 (1 isoprene unit), monoterpenes C10 (2 isoprene units), sesquiterpenes C15 (3 isoprene units), diterpenes C20 (4 isoprene units), triterpenes C30 (6 isoprene units), tetraterpenes C40 (8 isoprene units), polyterpenes (C5)n where ‘n’ may be 9–30,000 (McGarvey and Croteau, 1995). Terpenes with small molecules like mono and sesquiterpenes are mostly accumulated in essential oils (also called volatile or ethereal oils). Aromatic volatile compounds are only found in 10% of the plant kingdom and are stored in special brittle secretory structures in plants, such as glands, secretory hairs, secretory ducts, secretory cavities or resin ducts (Ahmadi *et al*., 2002; Bezić *et al*., 2009). Fig 3 below shows the chemical structures of some of the main terpenes found in oregano leaves, fennel seeds and in hop cones.

2.5.2. Oregano (*Origanum vulgare* L.)

Oregano is the common name for more than 60 plant species or subspecies used as spice all over the world. Four main groups commonly used for culinary purposes, Greek oregano (*Origanum vulgare* ssp*. hirtum* (Link) Ietswaart); Spanish oregano (*Coridohymus capitatus* L. Hoffmanns and Link); Turkish oregano (*Origanum onites* L.); and Mexican oregano (*Lippia graveolens* HBK) (Lawrence, 1984). All over the world and specially in Europe, the most commonly oregano species belong to the genus *Origanum* on the market the *O. vulgare* L. and *O. majorana* L*.* leaves are known as oregano and marjoram respectively (Olivier, 1996).

As a medicinal plant, European oregano traditionally has been used because of its carminative, diaphoretic, expectorant, stimulant, stomachic and tonic effects. In addition, it has been used against colic, coughs, headaches, nervousness, toothaches and irregular menstrual cycles (Kintzios, 2002).

Origanum vulgare L*.* botanically belongs to the family *Lamiaceae*, which can be divided in several subspecies as *hirtum, vulgare, viridulum, glandulosum*, *gracile vulgare, virens and* subspecies *viride (*Letswaart*,* 1980). The family Lamiaceae harbors many other aromatic plants of great scientific and economic interest such as rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis* and other *salvia species*) and mint (*Mentha species* and hybrids). These plants are associated with a characteristic aroma that arises from the essential oils found in the glandular trichomes located on the aerial parts. These glandular trichomes consist of specialized secretory cells in which the components of the essential oil are synthesized and accumulated in a sub-cuticular storage cavity (Gershenzon *et al*.,1989, Turner *et al*.,1999). The composition of the essential oil of oregano is dominated by mono- and sesquiterpenes (Skoula and Harborne, 2002; Stahl-Biskup, 2002). Chemically oregano contains carvacrol, thymol, *p*-cymene, α and β-pinene, myrcene, limonene, linalool and estragol (Sivropoulou *et al*.,1996, Milos*et al*., 2000, Aligiannis *et al*., 2001, Azizi *et al*., 2009). The essential oil of *O. vulgare* has great antimicrobial activity against bacteria, fungi and yeast species and therefore, it can be used as a natural preservative ingredient in food and pharmaceutical industry (Sahin *et al*., 2004). The phenolic components in the essential oil of oregano, such as carvacrol and thymol have a strong antifungal power (Curtis *et al*., 1996). Adam *et al.* (1998) reported that carvacrol and thymol showed higher antifungal activities against human pathogens than *p*-cymene and γ-terpinene. Furthermore, *O. vulgare*

has an antioxidant property and is applied in human health. Cervato et al. (2000) prove that the antioxidant activities of extracts of oregano's leaves (both aqueous and methanolic extracts) can inhibit all phases of lipid peroxidative process.

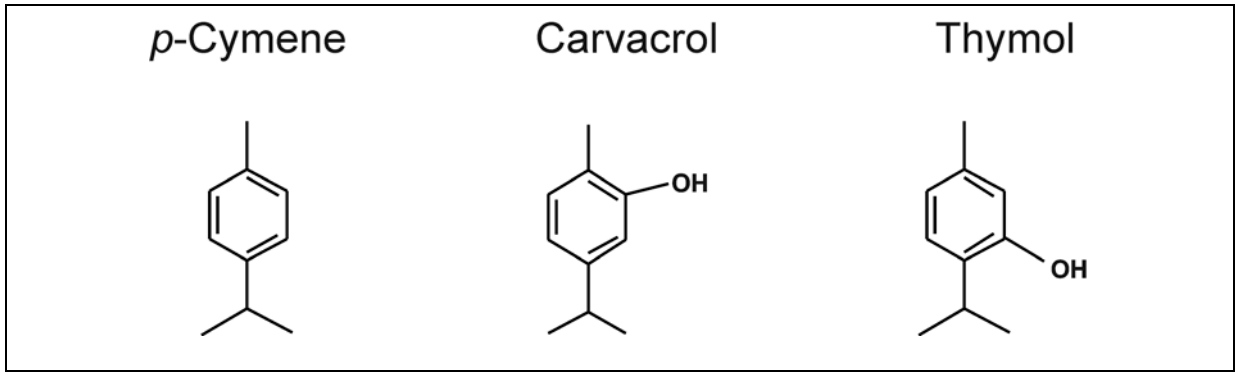

Fig. 3 Chemical structure of terpenes found as main compounds in oregano (Buddrus, 2003, Burt, 2004, Parthasarathy *et al*., 2008)

2.5.3. Fennel (*Foeniculum vulgare* L.)

Fennel (*Foeniculum vulgare* L.) is also called *Foeniculum officinale*, *Foeniculum capillaceum, Meum foeniculum Spreng or Anethum Foeniculum* (Chiej, 1984). It is a biennial aromatic edible medicinal plant belongs to the family *Umbelliferae* (*Apiaceae*). The plant is growing to a height of about 2 meters with a large spindle shaped root and it is cultivated in most countries, which are neighboring to the Mediterranean Sea and near Eastern regions. Besides it is used as a vegetable its fruits also used in the pharmaceutical concentrates. Its seeds are used for savoury formulations, sauces, liqueurs, confectionery, etc. (Guillén and Manzanos, 1996). The important ingredient is the essential oil located in the seeds. According to the composition of the essential oil two main chemo-types can be classified. Sweet fennel (*Foeniculidulcis fructus*) with about 2% oil content with about 80% trans-anethole and bitter fennel (*Foeniculiamari fructus*) with about 4% oil content, 60% trans-anethole and about 15% fenchone (Wagner, 1999). The fennel seeds are tiny and yellowish green, resemble the cumin seeds. Botanically the seeds are defined as fruits (Buntain and Chung, 1994).

Fennel is used in folk medicine as a stimulant, diuretic, carminative and sedative (Charles*et al*.,1993). Fennel fruits are used to treat diseases like cholera bile disturbances, nervous disorder, constipation, dysentery and diarrhea (Leung and

Foster, 1996). It is also used for control of diseases affecting chest, lungs spleen, kidneys and in colic pains (Brown, 2002). Furthermore fennel seeds are used in preparation of soups, sauces, pastries, confectioneries, pickles and meat dishes etc. (Bhati *et al.,* 1988). The essential oil of fennel is used to flavor different food preparations and in perfumery industries. The oil, which contains particularly trans-anethole and fenchone is of vital importance in pharmaceutical and other industries as well as in confectionery (Abdallah *et al*., 1978). Fennel oil is used as an expectorant component of cough remedies, and also as carminative component of stomach and bowel remedies in dosage forms including honey and syrup (Piccaglia and Marotti, 2001). Essential oil of fennel is used as flavoring agents in food products such as beverages, bread, pastries, and cheese. It is also used as a constituent of cosmetic and pharmaceutical products (Piccaglia and Marotti, 2001). The major components of the essential oil of fennel seeds are estragol, trans-anethole, limonene and the cyclic monoterpenes fenchone (Barazani *et al*., 2002).

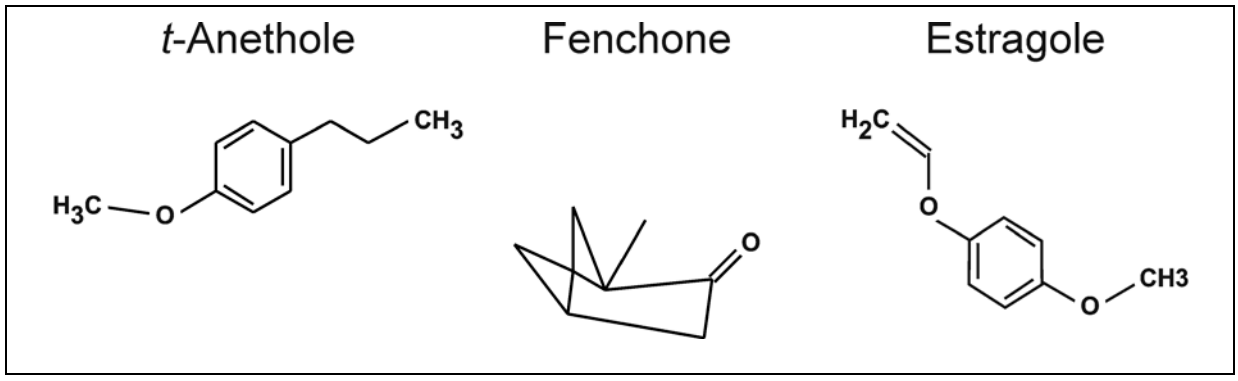

Fig. 4 Chemical structure of terpenes found as main compounds in fennel (Buddrus, 2003, Burt, 2004, Parthasarathy*et al*., 2008).

2.5.4. Hop cones (*Humulus lupulus* L.)

Hop (*Humulus lupulus* L.) belongs to the family *Cannabaceae*. It is a perennial, climbing, and herbaceous plant. New shoots are-growing during the spring from the rhizomes of the underground rootstock. In the fall season the plants are drying up and only the rootstock survives (Verzele and De Keukeleire,1991). *Humulus lupulus* L. is dioecious plant, which male and female flowers are found on separate plants. The economic value of hop depends on the secondary metabolites present in the lupulin glands of female cones. Hop is widely cultivated throughout the temperate

zones of the world. Therefore, it is grown for commercial purpose in Europe, America, South Africa, Australia, and New Zealand. The largest hop growing areas are in Germany which is 14086 ha (FNR 2013) and this equal to 37.1% of the total hop area around the world. Followed by USA (25.0%), Czech Republic (9.6%), and China (8.9%) (Hopsteiner, 2011). Hops have been employed for a long time as ingredient for beer production conferring aroma and flavor to beer, as well as for preserving it (Hopsteiner, 2011). Moreover, hops are also used as flavoring ingredient in other nonalcoholic beverages and foods. Female hop plants used for brewing purposes are derived from *H. lupulus* L., whereas male plants are essential for breeding to develop new varieties (Verzele and De Keukeleire, 1991). Hop plants are not only used in the brewing industry, they have also been used in the traditional folk medicine. Since middle ages, the sedative effect of hop has been recognized contributing to the treatment of the sleep disturbances and anxieties (Chadwick *et al*., 2006; Zanoli and Zavatti, 2008). Besides these properties, anti-proliferative, anti-oxidative, anti-mycotic, anti-bacterial, and estrogenic effects have been reported (Stevens and Page, 2004, Chadwick *et al*., 2006; Zanoli and Zavatti, 2008). Therefore, hop was named as “Medical plant of the year 2007” by the Study Group for the Historical Development of Medicinal Plant Science at the University of Würzburg in Germany (Biendl 2008).

The essential hop oil chemically contains monoterpenes and sesquiterpenes (Nickerson and Van Engel, 1992; Roberts *et al*., 2004) and in details it contains isobutyl isobutyrate, myrcene,β- pinene, geraniol, α–humulene and β-caryophyllene (Jirovetz *et al*., 2005). In addition to that limonene, *p*-cymene, *b*- pinenes, linalool, nerol, geraniol,nerolidol,citral, methylnonyl ketone and other oxygenated compounds could be found in essential oil of hop (Leung and Foster, 1996). Additionally hops contains resins, flavonoid, glycosides, phenolic acids and tannins (Leung and Foster, 1996).

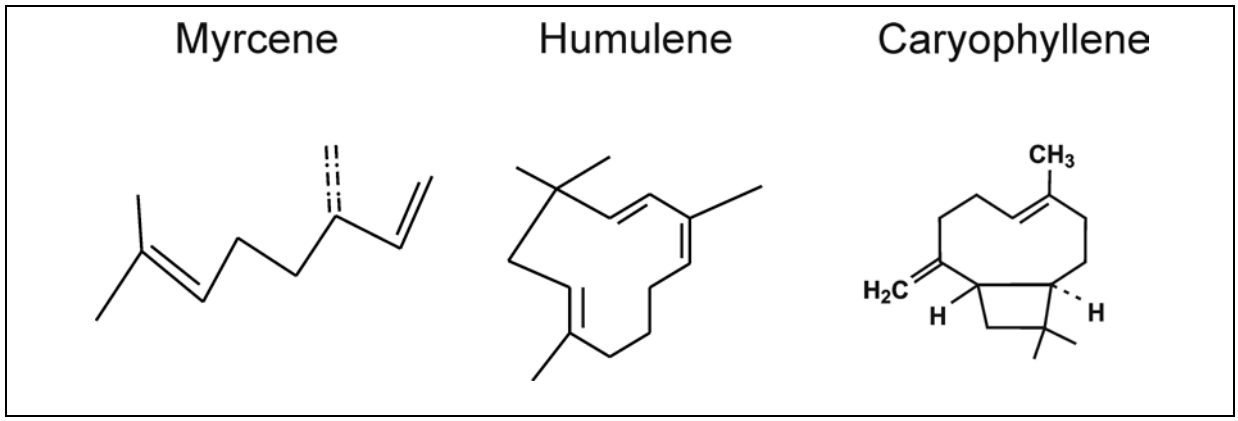

Fig. 5 Chemical structure of terpenes found as main compounds in hop cones (Buddrus, 2003, Burt, 2004, Parthasarathy *et al*., 2008).

2.5.5 Antimicrobial activity of spice plants

People around the world especially in developing countries depend on the traditional folk medicine to treat a variety of illnesses and diseases. Furthermore spices are used as a source of anti-microbial agents for maintaining a balanced microbial ecosystem especially of the gastrointestinal tract. Several hundred genera of medicinal and herb plants are used for these purposes (McGaw *et al*., 2000).

Recent bans and restrictions on the use of animal antibiotic growth promoters stimulated interest in bioactive secondary metabolites of plant source as alternative performance enhancers especially in animal nutrition (Greathead, 2003).

Some essential oils are known to have strong antimicrobial activity against a wide variety of food borne pathogens. Besides antibacterial properties, essential oils or their isolated components exhibit antioxidant properties (Baratta *et al*., 1998) as well as antifungal (Chao *et al*., 2000), antiviral (Ramadan *et al*., 2009), antiparasitic and insecticidal properties. Antimicrobial activities of several natural substances found in plants, such as in oregano had been reported in many studies (Burt, 2004).

Oregano is well-known as culinary herb, possessing antioxidant (due to the presence of phenolic acids and flavonoids) and antimicrobial (due to the presence of thymol and carvacrol). The oregano essential oils can inhibit some pathogenic bacterial strains, such as *E.coli*, *Salmonella enteritidis*, *Salmonella choleraesuis*, and Salmonella typhimurium (Penalver *et al*., 2005). Furthermore, it was found that oregano has strongest antibacterial properties against *Salmonella enterica* (Marques

et al., 2008). The activity of the oregano essential oil could be attributed to the existence of carvacrol and thymol as phenolic components. Moreover the essential oil from oregano has inhibitory activity against the growth of *Micrococcus luteus* and *Bacillus cereus* in addition, it possessed stronger antimicrobial activity compared with the antibiotics (Özkalp *et al*., 2010).

The essential oils of fennel seeds showed dramatically higher antioxidant activities. Fenchone, trans-anethole and estragol are the major components in the essential oil of fennel. It was observed that the antioxidant activities are related to the concentration of trans-anethole and estragol (Shahat *et al*., 2011). Furthermore, Kwon (2002) found that the extract of fennel fruits has antimicrobial activities against *Bacillus subtilis*, *Aspergillus niger* and *Cladosporium cladosporioides.* In another study it was found that the essential oils from the fruits of fennel showed significant antibacterial activity to *Escherichia coli* and *Bacillus megaterium* (EL-Adly *et al*. 2007). Chloroform soluble fraction from fennel fruits exhibited potent antimicrobial activity against bacteria and fungi (Kwon *et al*., 2002). Essential oils of fennel, trans-anethole, fenchone and dichloromethane fennel extracts showed antifungal activity against *Candida albicans* agent for candidiasis and other fungal diseases (Park and Seong, 2010).

The essential oil of hops showed high activity against both gram-positive, gram-negative bacteria and the yeast (Jirovetz *et al*., 2005). Also cones of hop are used in pharmaceuticals (Milligan *et al*., 2002, Zanoli and Zavatti, 2008) and also as replacement of antibiotics in livestock feed (Cornelison *et al*., 2006).

Essential oils of cinnamon (*Cinnamomum cassia*), were found to possess antimicrobial properties that inhibit the growth of *B.cereus* (Kalemba and Kunicka, 2003). Alcoholic extracts of cinnamon were found effective mostly against *Helicobacter pylori,* in reducing its growth (Tabak *et al*., 1996).

In another study it was found that essential oils of clove *(Syzgium aromaticum*) possess antimicrobial properties (Kalemba and Kunicka, 2003). Clove oil is affective against *E. coli*, *Listeria monocytogenes*, *S. enteric* (Friedman *et al*., 2002). The antibacterial activity of clove essential oil against two gram-negative bacteria, such as *Pseudomonas fluorescens* and *Serratia liquefaciens,* and four gram-positive

bacteria, such as *Brochothrix thermosphacta, Carnobacterium piscicola, Lactobacillus curvatus* and *Lactobacillus spec*.

The oil of thyme (*Thymus vulgaris)* and its different components have antimicrobial and an antioxidant agent (Dursun *et al*., 2003). Thyme showed broad antibacterial activity to both gram-positive and gram-negative bacteria. However, gram positive bacteria *Clostridium botulinum* and *Clostridium perfringens* appeared to be more sensitive than the gram-negative organisms (Nevas *et al*., 2004). The alcohol and ethanol extracts of thyme, essential oil (also contains thymol and carvacrol) were found to have strong inhibition activity against *Bacillus subtilis, Shigella sonnei* and *Eschrichia coli* (Fan and Chen, 2001). The essential oil of thyme or its constituent thymol decreased viable counts of *Salmonella typhimurium*on nutrient agar (Juven *et al*., 1994). Thymol showed antagonistic effect against *S. sonnei* in anaerobic conditions in vitro (Juven *et al*., 1994). Carvacrol, a compound present in the essential oil fraction of oregano and thyme showed a dose-related inhibition of growth of the pathogen *Bacillus cereus* (Ultee *et al*., 2000).

3. Material and methods

3.1. Microbiological investigation

These experiments were conducted in the laboratory of the research group of Prof: Dr. Sylvia Schnell (General and Soil Microbiology) of the Institute of Applied Microbiology at the Justus Liebig University of Giessen

3.1.1. Origin of microorganisms

The methanogenic culture of *Methanosarcina barkeri* were brought from German Collection of Microorganisms and Cell Culture Braunschweig (DeutscheSammlung von Mikroorganismen und Zellkulturen DSMZ)

3.1.2 Used chemicals

Microelements Sl 10 solution contained (in mg per liter): $FeCl_2$. $4H_2O$ (1000); $ZnCl_2$ (70); $CoCl_2$.$6H_2O$ (130), $NaMoO_4$ $2H_2O$ (36); H_3BO_3 (6); $MnCl_2$ $4H_2O$ (100) $CuCl_2$ $2H_2O$ (2), $NiCl_2$.$6H_2O$ (24) and HCl 25% solution (10 ml) in 1000 ml deionised water. The solution was sterilized in the autoclave (La-VA-ncs 2003, Wolf Adolf Sanoclav, Bad Überkingen, Germany) for 25 min at 121°C (Widdel, *et al*., 1983).
Selenite-Tungstate solution (Widdel *et al*., 1983) contains (in mg per liter): NaOH (400); Na_2SeO 5 H_2O (6) and Na_2WO_4 $2H_2O$ (8). The solution was sterilized in the autoclavefor 25 min at 121°C.The seven mix-vitamins solution contained (in mg per liter): pyridoxamine-di-hydrochloride (200), lipoic acid (50) nicotinic acid (200); Ca-D (+)-pantothenic acid (100), 4-aminobenzic acid (80); D (+)-biotin (20) and cyanocobalamine (10). The solution was filter-sterilized and stored at 4°C in the dark (Widdel and Pfennig, 1981). Thiamine solution contained (in mg per liter): Thiamine dihydrochloride (100), Na_2HPO_4/H_3PO_4, with pH value 3.4 (25 mM**).** The solution was filter-sterilized with whaman® filter (pore size, 0.2 µm, Whatman®, Dassel, Freiburg, Germany) in sterile 50 ml bottles and stored at 4°C in the dark (Widdel and Bak, 1992). Riboflavin solution contained (in mg per liter): riboflavin (50) and acetic acid (20mM). The solution was filter-sterilized through Whatman® (pore size 0.2 µm) filter (Whatman®, Dassel, Freiburg, Germany) in sterile 50 ml bottles and stored at 4°C in the dark. The vitamin B12 solution contained (mg per liter) cyanocobalamine (50) and deionized water. The solution was filter-sterilized through nitrocellulose membrane (pore size, 0.2 µm) in sterile 50 ml bottles and stored at 4°C in the dark. For the

preparation of the bicarbonate solutions 84.0 g of $NaHCO_3$ was dissolved in 1000 ml pure dionized water under CO_2 atmosphere and portioned in a volume of 30 and 60 ml in screw cap serum bottles, leaving approximately 1/3 of the bottle volume as gas head space. The head space was flushed and exchanged to saturated the solution with CO_2 by repeated flushing and vigorous shaking for 1-2 min. Lastly the solution portions were autoclaved and stored at room temperature (Pfennig 1978). Pure colorless crystals of $Na_2S.9H_2O$ was flushed with deionized water using a plastic sieve, weighted in and dissolved in pure deionized water at a final concentration of 240.12 g/1000 ml the solution was portioned in small narrow vial each contains 5 ml then it flushed with mixture of nitrogen and carbon dioxide gas and closed tight before autoclaving. Sodium sulfide acts as a reducing agent. For yeast extract 2.5 g of yeast was weighted, dissolved in 50 ml of deionized water, mix thoroughly in glassed beaker, lastly put in serum bottle, closed and crimped with black butyl rupper stopper, and aluminum crimp respectively, autoclaved for 20 min under 121 °C. For casitone extract 2.5 g of casitone was weighted, dissolved in 50 ml of deionzied water, mix thoroughly in glassed beaker, lastly it was put in a serum bottle, closed with black butyl rubber stopper, and crimped with aluminum crimp, autoclaved for 20 min under 121°C. Pure methanol (99, 9%) was sterilized by using Whatman® filter spore size 0.2 µm (Whatman®), Dassel, Freiburg, Germany) put in a 120 ml serum bottle, stoppered with black butyl rubber and crimped with aluminum crimp (All vitamins and other solutions were provided either by Dr. Ratering or Mr. Schneider from the work group of Prof. Dr. Schnell).

3.1.3 Preparation of the oregano extract

The oregano leaves were dried under the shade and ground to fine powder to pass a 1mm sieve by using a laboratory electric mill. A sample was extracted with 99% methanol and filtered after 24 h under shaking (Infors AG CH – 4103 BOTT MINEN); the speed of the shaker was 150 rpm. The plant residue was re-extracted with the addition of 99% methanol for 24 h, and filtered again. Filtrates were combined together and concentrated on a rotary evaporator (BÜCHI Rotovap®) at 42ºC to eliminate the methanol. Later the extracts were saved in sterile bottles under the cooled conditions until the use. The extracts' dry weight were achieved by evaporation the methanol and the concentration in mg/ml was determined according to Betoni *et al*. (2006).

3.1.4 Preparation of anaerobic liquid medium

Methanosarcina barkeri cultures were cultivated in anoxic, bicarbonate-buffered, sulfide-reduced, sterilized mineral medium (Widdel and Bak, 1992) containing: 1.0 g/l NaCl , 0.4 g/l $MgCl_2$ $6H_2O$,0.15 g/l $CaCl_2$ $2H_2O$ 0.5 g/l KCl, 0.2 g/l KH_2PO_4; and 0.25 g/l NH_4Cl. The medium was reduced with Na_2S9H_2O. The medium is prepared in a Widdel flask (1 or 2 liter volume) and autoclaved for 46 min at 121°C. Then after autoclaving the medium was cooled down to the room temperature under nitrogen atmosphere by flashing the headspace with oxygen-free gas mixture of N_2/CO_2 (80/20, v/v, from Air Liquide, Duesseldorf, Germany). Addition of oxygen free gas is to remove the oxygen from the headspace. After cooling under the room temperature and still connecting to the gas line a $NaHCO_3$ solutions (30ml/l), (1ml/l) of each of vitamin B_{12}, Seven-vitamin mix solution, riboflavin , thiamin, trace elements solution SL10, selenite and tungstate solution, and Na_2S 9 H_2O (5 ml/l) were added to the medium. The pH value (Microprocessor pH meter-pH 535 MultiCal®, WTW, Weilheim, Germany) of the medium was adjusted to 6.8 with the addition of 1 M HCl (Widdel *et al*., 1983).

For the cultivation of the methanogenic microorganisms, the medium was filled to 50 ml into each of 160 ml sized serum bottle (Ochs, Bovenden, Germany) and then sealed with the butyl rubber septa (Sigma-Aldrich, Steinheim, Germany), and crimped with the aluminum crimps. The 50 –ml filled serum bottles were left at the room temperature on the laboratory bench in the dark for overnight before inoculating the methanogenic organisms. The next day 0.1 ml of methanol, 4.0 ml of casitone and 4.0 ml of yeast extract were added aseptically before inoculation of the methanogens. The media were inoculated with 5.0 ml of a freshly grown stationary-phase culture. Kanamycin (0.5 mg/ml) per-culture (serum bottle) was added by N_2/CO_2 (80:20 [vol: vol]) gassed sterile syringes to make sure that the culture stay pure, and no other microbes invading the culture. The culture serum bottles were incubated statically and vertically but shaken manually from time to time briefly.

Cultures were routinely controlled by the optical density measurements and the microscopic examination (Axioscope; Zeiss, Jana, Germany). Optical densities of the cultures were measured after retrieving of one ml by N_2-flushed syringe in plastic cuvettes (Brand, Wertheim).

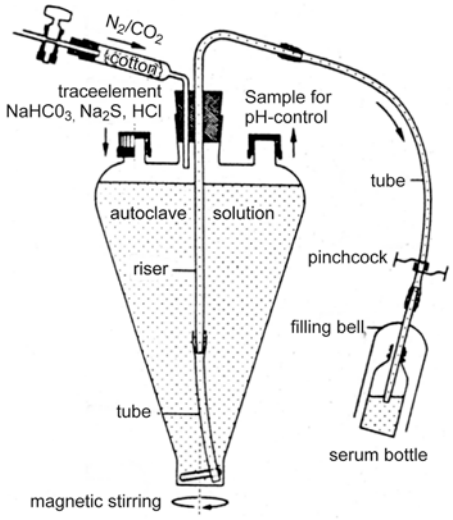

Fig 6 Flask for preparation of anaerobic media for cultivation of methanogens (Widdel 1980)

3.1.5 Addition of the tested materials

The investigated materials were representative samples (well homogenized) and they were added aseptically by sterile N_2/CO_2 mix gas flushed syringe through the black rubber stopper after the culture well grown (after 4 to 6 days) and the reason behind that is to make sure that all the cultures used in the experiment are active. Incubation occurred in 36°C in the dark and cultures were routinely checked and shacked manually from time to time.

3.1.6 Gas chromatography (GC) analysis

The concentration of methane produced by *Methanosarcina barkeri* was measured with the gas chromatograph (GC) Autosystem XL, Arnel (Perkin Elmer, Überlingen, Germany) combine with a flame-ionization detector (FID). The sample was taken directly from the head space of the serum bottle, after inserting the needle through the septum of the stopper. The sample was taken in a tight Pressure-Lok® series A-2 (Supelco, Oakville, Canada) gas sampling syringes (with side–opened needle to prevent coring of gas chromatography septa and it had zero dead volume). Samples were injected manually in the injector by injection of 20 µl. Nitrogen (30 ml min^{-1}) was used as a carrier gas on a packed (80/100 mesh) carboxen (5 Ă, Serva, Heidelberg, Germany) with hydrogen (30 ml min^{-1}) and air mix flow (300 ml min^{-1}) as burning gases. The column (3 m x 4 mm) heated to 50°C where as the temperature of injector and detector was 230°C.

Gas production in the head spaces of the serum bottles was measured every three days using the syringe technique. The gas sample was identified and quantified by

comparing the chromatograms (from the GC run) with the chromatogram of an external methane standard (100.4 ppmv, from Duste-Steininger, Muelhausen, Germany) by the retention time and the peak area respectively. The certified standard sample of methane is used to compare and to calculate the concentrations of methane that produced in the head spaces of the serum bottles by the methanogens. At each sampling time, triplicate samples from each culture bottle was analyzed, and the means were calculated.

3.2 Laboratory digestion

3.2.1 Used materials

Cattle liquid manure was used as source of microrganisms brought in big plastic containers from a second phase plant running for biogas production. Dried fine powders of *Origanum vulgare* L spp*. O. vulgare* leaves at treatments of 62.5, 125, 250 and 500 g. Seeds of *Foeniculum vulgare* at treatments of (25, 50,100,200 and 400 g). Dried powder of hops cone (*Humulus lupulus*) at treatments 25, 50,100, 200 and 400 g, dried powdered of whole maize plant at treatment of 400 g and standard fine cellulose powder at treatment of 100 g, the last two substrates used only as references materials. Each treatment was replicated three times. In inhibition experiments tests a mixture of cattle liquid manure and standard cellulose were used in all digesters as co-substrates with the tested materials to measure the inhibitory effects according to VDI rules (VDI4630-2006).

2.5.1. Prepration of the cattle liquid manure

Cattle liquid manure (3 to 5% DM) was brought in big plastic containers from a second phase plant running for biogas production. Directly after brought the manure was stirred mechanically for three days, sieved by 5 mm sieve and filled in other big tanks. The other big tanks of sieved manure were put inside an old store and stirred mechanically four to five times per day before distributed in the digesters. The organic dry matter of the manure was around 90%. The manure was mixed in all digesters with the co-substrates of dried fine powders of *Origanum vulgare* L spp, seeds of *Foeniculum vulgare*, dried powder of hops cone, maize and cellulose.

3.2.3 Preparation of the plant material

Origanum vulgare used in this study was grown in a field belong to the University of Giessen, research station in Rauischholzhausen (Germany) in 2009. The soil is

characterized by loess soil which is formed by the accumulation of wind-blown silt and variable amounts of sand and clay that are loosely cemented by calcium carbonate. The oregano plants were neither artificially irrigated nor chemically fertilized. The plants were harvested at the flowering stage. After harvesting, the plants were dried under the shadow for 15 days to protect the chemical components from chemically transforming. After drying the plants were separated into flowers, leaves and stems. The leaves were only taken because they contain more essential oils than other two parts. The dried leaves were ground to pass 1mm sieve by using electrical cereal grinder (Rotor beater mill SR2, Retsch GmbH, Haan, Germany).

Foeniculum vulgare used in this study also, was grown in fields belong to the University of Giessen, research station in Groß-Gerau (Germany) in 2010. The soils are mainly alluvial soil mixed with sand but also loamy texture. The soils are described as having a slightly loamy to loamy sand. The seeds had been harvested after ripening. After harvesting, the seeds were air dried under the shadow for 15 days as to protect the chemical components from been chemically transforming. The seeds were only taken because they contain more essential oils. The dried seeds were ground to pass 1mm sieve by using electrical cereal grinder.
Dried hop *(Humulus lupulus)* female inflorescences variety Herkules was brought from Bavaria State Research Center for Agriculture Institute of Agronomy and Plant Breeding Hop Research Centre in Hüll. The dried cones were ground to pass 1mm sieve by using electrical cereal grinder

3.3 Laboratory analysis

Measuring of the pH value

PH value was measured with the measuring electrode Sen Tix 41(WTW) after homogenizing each of 10 g of samples with 100 deionized water for a period of 20 min and table 3 presents the results of pH of the used materials.

Dry matter % (DM) determination

For the dry matter determination, 10 g of each sample was taken separately in aluminum foil containers and dried at105˚C in a forced dried air oven (WTB-Binder, Tuttinglen, Germany) for 24 hours, and then it was cooled in a desiccator and reweighted again. The dry matter content (%) was calculated as recommended by the AOAC (1984) as cited in Williams' book "Official methods of analysis of the

Association of Official Analytical Chemists1984) and table 3 presents the results of dry matter content of the used materials.

Ash content

The dried samples of the material that their dry matters determined already were put in porcelain crucibles and incinerated in a muffle oven (model N11, Nebertherm GmbH, Lilienthal, Germany) for overnight at 600 C, then it cooled in desiccators and weighed. The ash content calculated as follows:

% Ash = {(ash mass in g)/ (dry mass in g)}* 100 and table 3 presents the results of the ash content of the used materials

Organic dry matter content

The (O.DM) content of the samples determinations was calculated as follows:

% O.DM = {(dry matter in g-ash mass in g)/sample mass in g}*100

or %O.DM = %100-% Ash (dry matter basis).

And table 3 presents the results of organic dry matter contents of the used materials.

Total nitrogen content

The total nitrogen content of the tested materials was determined using an element analyzer (Thermo/Fisons EA 1110 CHNS/O Analyzer CE instruments GmbH). Elements analyzer is operating at the principle of catalytical combustion under the supply of oxygen and the high temperatures. Elements analyzing were conducted according to the DUMAS method. The total protein content was calculated by multiplying the total nitrogen percent by factor 6.25 and table 2 presents the results of the total nitrogen content of the used materials.

Total carbon content

The total carbon content was detected by the Dumas method using elements analyzer (Thermo/Fisons EA 1110 CHNS/O Analyzer CE instruments GmbH). The idea is combusting the sample of known mass in a high temperature (about 900°C) chamber in the presence of oxygen. This leads to the release of carbon dioxide and water. The gases are then passed over special columns that absorb the carbon

dioxide and water and table 3 presents the results of the total carbon content of the used materials.

The acid detergent fiber (ADF)

The acid detergent fiber (ADF) contents of the tested samples was determined according to the Van Soest method (Van Soest, 1963) by using a Tecator Fibertec system (Tecator part No.10001217, Foss Deutschland GmbH, Hamburg, Germany) as outlined in the application note of animal nutrition laboratory. Samples were ground to pass a one-mm sieve. One gram weighed in a filter crucible and placed in a hot extraction unit of the Fibertec system. The extraction was carried out with 100 ml of acid detergent solution for one hour after boiling commenced. This was followed by cold extraction with acetone on the cold extraction unit of the system. The samples were dried at 100°C over night and ashed at 550 °C for three hours and table 3 presents the results of the acid detergent fibre content of the used materials.

$\%\ ADF = \{(W_1\ (g)-W_2\ (g))/W_0\ (g)\}*100$

Where W_1 = dry mass of sample after ADF extraction

W_2= mass of ash

W_0= sample mass

Lignin content of acid detergent fiber (ADL)

Acid detergent lignin was determined in the manner outlined in the application notes of the animal nutrition institute using the Tecator Fibertec system. The samples were prepared with the ADF procedure as outlined in (ADF) preparation but not ashed. A sequential extraction with 72% sulpheric acid was carried out for three hours. The sample, which remaining after filtration was washed with hot water, dried overnight, weighed (W_1) and ashed in muffle furnace at 550 °C for three hours. The residue was then cooled in a desiccator and weighed (W_2).

% ADL was calculated as

$$\%\ ADL = \{(W_1-W_2)/W_0\ (sample\ mass)\}*100$$

and table 3 presents the results of the lignin content of the used materials

Neutral detergent fibre (NDF) content of dry matter

Neutral detergent fibre contents of test samples were determined using the same apparatus in (ADL) except that neutral detergent solution was used (Van Soest and Wine, 1967).

$$\%NDF = \{(W_1\ (g)-W_2\ (g))/W_0\ (g)\}*100$$

Where W_1= dry mass of sample after NDS extraction

W_2= mass of ash

W_0= sample mass

Cellulose was calculated as: % cellulose = ADF%- ADL%

Hemicellulose was calculated as: % Hemicellulose = NDF% - ADF% and table 3 presents the results of neutral detergent fibre content of the used materials.

Mineral analysis (Ca, Mg and P)

A sample was ground to pass a one mm sieve and (1 g) was digested in a block digester at 230 °C using the wet digestion technique and the results are expressed either as a percentage (%) or in parts per million (ppm) [as (mg/kg) on a dry basis]-
The total concentrations of calcium and magnesium were then determined on Perkin Elmer 2380 Atomic absorption spectrophotometer (Lengen, Germany). Calcium concentration was determined at a wave length of 422.7 nm and a slit setting of 0.7 nm using a hollow cathode tube.
The total concentration of magnesium (Mg) was determined at a wave length of 285.2 nm and slit setting of 0.7 nm using a similar lamp. An air acetylene flame was employed in both determinations of calcium and magnesium.
The total concentration of phosphorus was determined by Spectrophotometer (Optima 3300 DV ICP emission spectrophotometer; Perkin Elmer Corporation; Shelton, CT) at 410 nm wave length and the concentration determined from a calibration curve and table 2 presents the results of Ca, Mg and P contents of the used materials.

Crude fat detection

Soxhlet method of fats extraction was used to measure the oil content of the tested samples (Jensen, 2007). Tested samples (20 g) were ground with an electric spices and nuts grinder (MX 32, Pfeulfer, Kitzingen, Germany), and dried in an air forced oven (WTB binder, Tuttinglen, Germany) for 3 hours at 105°C. Then 5 g were weighed from this oven dried material in cellulose extraction thimble a small piece of cotton wool was put at the bottom of the cellulose thimble before taking the sample and the top of thimble was covered with the cotton wool after the sample to prevent the floating. A predried flat bottom flask was weighed with boiling chips. The thimble was placed in an extraction chamber which was suspended above a flask containing of 200 ml hexane.

Extraction chamber with thimble, hexane and boiling chips containing flask were connected with a condenser (300 mm, Kühler Rettberg, Göttingen, Germany). The flow of cold water in the condenser was opened before start heating at 69°C when the flask was heated and the hexane evaporated and it moved up into the condenser where it was converted into a liquid that trickles into the extraction chamber containing the sample. The extraction chamber was designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. This process of boiling was continued for 8 hours. At the end the flask containing oil and small amount of hexane was separated and put into the air drying oven for 2 hours at 95°C. After that, flask which contains extracted oil and boiling chips was weighed. Calculate the percent of oil in the original sample as given below;

Mass of oil = (Wt of flask + extracted oil + boiling chips) - (Wt of flask + boiling chips

% Fat = mass of extracted fats (g)/mass of the sample (g)}*100.

Table 3 presents the results of the total fat content of the used materials.

Table 3 Composition of maize, fennel, hops and oregano used as co-substrates in biogas batch tests

	Maize	Fennel	Hops	Oregano
DM %	88.0	88.8	87.0	90.0
O.DM %	96.0	79.4	83.1	81.8
Carbon tot.%	48.7	51.2	50.6	41.2
N total%	1.8	2.9	2.5	1.8
C/N	27.1	17.7	20.2	22.9
Protein %	11.3	18.3	15.6	11.1
Fat%	3.1	27.5	9.2	4.0
pH value	n.a.	6.6	n.a.	6.0
K (mg/g)	97.8	96.0	215.0	212.0
Na (ppm)	1.4	2.4	4.1	3.2
Mg (mg/g)	5.2	5.2	26.0	26.0
Ca (mg/g)	8.0	8.4	41.5	42.0
P (mg/g)	10.6	15.0	23.0	19.5

n.a. not analyzed

Analysis of essential oils

The powdered materials of dried leaves of *O. vulgare*, (20 g), seeds of *F. vulgare* and *H. lupulus* (10 g each) were hydrodistilled in a Clevenger's type apparatus (Meinhardt, Labor und Maßgeräte GmbH Stützbach, Ilmenau, Germany) for 2 h and the crude oil of each material was dried and collected in sterilized clean closed dark brown glass and kept at 4°C in a refrigerator until it was quantified by Gas Chromatography (GC-FID). The essential oils of oregano and fennel were further analyzed using gas chromatography with flame ionization detector (GC-FID) on

Varian GC-CP-3800 (Frankfurt, Germany). The operating conditions were as follows: capillary column (DB-5) has size 30 m × 0.25 mm. Coating thickness was 0.25 µm. The carrier gas was Helium with flow rate 1.1 ml/min. Injector and detector temperatures were 260°C and 280°C, respectively. Split ratio was 1:50. The column temperature was held at 60°C for 5 min, and then raised to 250°C at 5°C/min for 10 min. 1 µl of the sample is injected by an auto sampler (Varian 8200CX). The identification of essential oil components was by comparison of their retention times with standard samples of pure chemical components (from Roth, karlsruh, Germany). The essential oil contents of detected oregano leaves, fennel seeds and hop cones were 1%, 6.9% and 0.2% respectively. Essential oil of oregano was characterized by 1.6 *p*-cymene, 2.1% *γ*-terpenene, 30% carvacrol, 38% thymol, and 0.2% limonene whereas fennel seeds essential oil contains 14.6% fenchone and 82% trans-anethole. The composition of essential oil of hops was not analyzed but by other authors the following compounds were found in hops: 12-17% alpha acids, 4-4.5% beta-acids, 3-4% polyphenols, 30-50% Myrcene, 0.3-0.8% linalool, 30-45% humulene, 0.3-0.8% beta–selinene, 0.3-0.8% alpha-selinene and the moisture content of the dry cones is 9-11% (Schattenhofer, 1989)**.** Our results of the essential oil of oregano are in line with Azizi *et al*. (2009). In other hand our results of the essential oil of fennel are consistent with those of Chatzopoulou *et al*. (2006) and also with Cetin *et al*. (2010).

Batch tests anaerobic digesters

Mesophilic batch parallel laboratory digesters were used in this study at the Research station of Rauischholzhausen. The number of digesters is 46 and no connection between them each one is individual unit. Each digester is made up of low pressure polyethylene PE (Speidel Tank und Behälterbau GmbH). All digesters are located in circulated heated water baths. The temperature of the water is 37°C. The water baths' bodies are made up from staiNless steel and are isolated from all sides with 30 mm insulation boards (XPS). The water baths are supplied by 14 heaters (MP heater 300 watt). Each two heaters were put into one water pool. Each water pool was also supplied by a thermostat cable (thermometer) to control the methophilic temperature. Also each water pool was supplied with a small pump (Sera submersible pump P400) to mix and to circulate the heated water, also to keep it in one level within the pool. The total volume of the digester is 20 Liters but the actual

working volume was 15 l in case of the activity tests the digesters were inoculated once with cattle liquid manure obtained from a second anaerobic digester of private biogas plant in Hersfeld (Germany). Versus in the inhibition tests we added cattle liquid manure mixed with a reference material, in our case we added 100 g of fine powder of standard cellulose (according to VDI rules). The cattle' liquid manure used as the main substrate as well as the source of micro-organisms: The digester was stirred or mixed mechanically with anchor at interval of 15 minutes each hour by using a geared DC motors (DO 1113763300).The mixers are programmed with electrical timers. The gas was collected in tight aluminum plastic gas sampling bag (TECOBAG for gas analysis 60 liter). The gas sampling bag is connected with an overflow tube inside the reactor that passes through the plastic capped open of the container. The reactor was fed manually once before starting the experiment through the substrate feeding port (cap). The gas sampling bags were taken 2-3 times per week (depending on the quantity of the gas). The produced biogas was measured volumetrically with the use of gas meter (Ritter drum- type, Bochum, Germany). The methane gas was determined by the use of infra red gas analyzer (Infra-red analyzer GS IRM 100, GS Messtechnik GmbH, Ratingen, Germany). The gas analyzer apparatus has ability to determine the CH_4 and the H_2S content of the biogas at the same time.

Biogas production is given in norm liter per kg of organic dry matter (Nl/ (kg O.DM), i.e. the volume of biogas production is based on standard conditions (T_0= 273° K, and P_0 =1013 mbar. Biogas quality (CH_4) was analyzed 7 times in course of the 4 weeks digestion. Biogas production from manure where no addition from other substances (control) was measured as well and each of the biogas and the methane were corrected by subtracting the production volume of each co-substrate from the volume that produced by the control.

Fig. 7 Mesophilic laboratory digester in Rauischholzhausen (own photo, 2012)

Fig. 8 Measuring the methane content in biogas production by using infra-red analyzer GS IRM 100 (own photo, 2012)

Fig. 9 Biogas collecting bag in a position of measuring connected with a tube that to connect with the measuring biogas meter (own photo, 2012)

Fig. 10 Biogas measuring meter Ritter drum gas type (own photo, 2012)

Table 4 Theoretical calculated biogas and methane (in Nl/kg O. DM) for each of oregano treatments used in the experiments following Baserga formula.

Oregano treatments in gram	Biogas Nl/kg O.DM	Methane Nl/kg O.DM
62.5 g	400	210
125	800	420
250	1600	840
500	3200	1680

Table 5 Theoretical calculated biogas and methane (in (Nl/kg O. DM) for each of fennel treatments used in the experiments following Baserga formula.

Fennel treatments in gram	Biogas Nl/kg O.DM	Methane Nl/kg O.DM
25 g	750	500
50g	1500	1000
100g	3000	2000
200g	6000	4000
400g	12000	8000

3. 4 Statistical data analysis

Statistical data analysis was carried out with the software package SPSS, (version 17, Chicago, Illinois U.S.A). Each treatment was measured in three replicates. In a first step, the data were analyzed by descriptive statistics. Mean standard error and frequency distributions of the data were determined. Differences among treatments were tested with comparative statistics. Variance analysis methods were applied to find significant differences in the means. The following tests and procedures were used: ANOVA and the one factorial post hoc tests ‘‘Tukey HSD test’’ and ‘‘Scheffe’’. Homogenity of Variances was analyzed with the significant level (α= 0.05)

4. Results

4.1. Effect of oregano extracts and its main chemical components on methane produced by *Methanosarcina barkeri*

4.1.1. Effect of oregano extracts

In this study, the methane rates produced by *Methanosarcina barkeri* pure cultures in batch mode tests at mesophilic temperature were measured under the influence of oregano extracts at doses 1.5, 2.0, 3.0 and 4.0 ml per 50 ml of media (Fig.11 and 12).

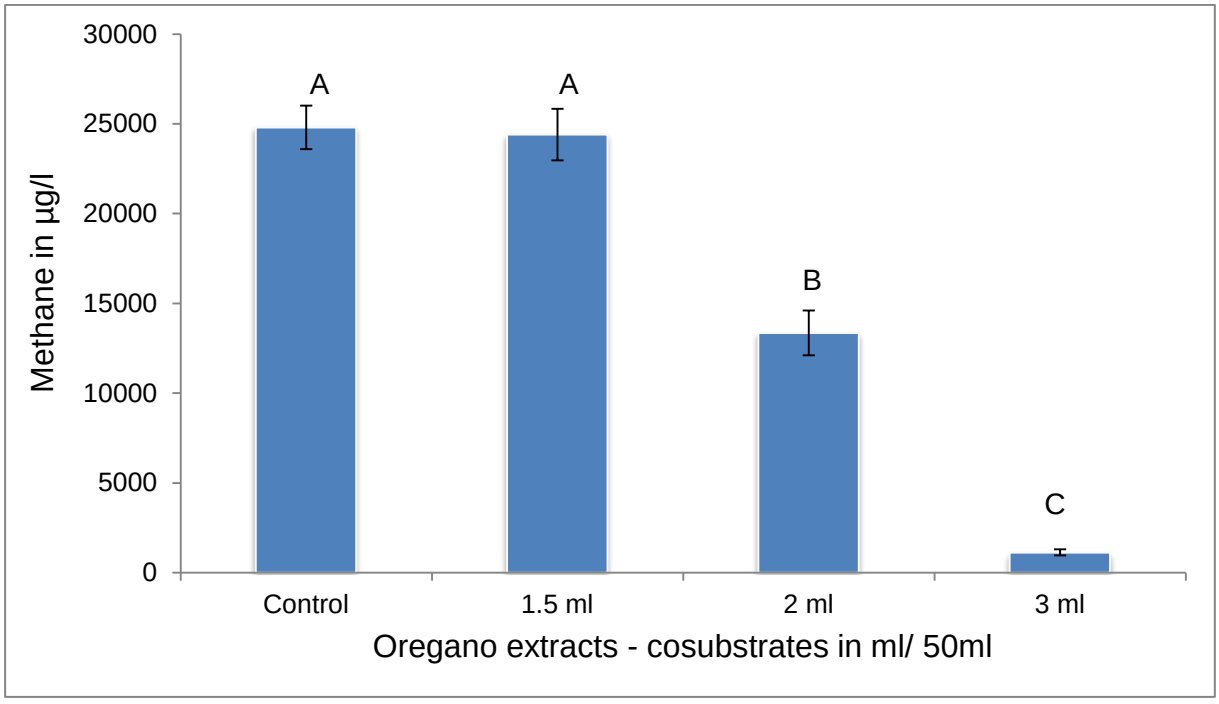

Fig. 11 Effect of *O.vulgare* extracts on methane yield (in $\mu g l^{-1}$) produced by *Methanosarcina barkeri* in a laboratory test after 17 days in mesophilic temperature T= standard error, different letters indicate significant differences between the averages (ANOVA and the one factorial post hoc tests ‘‘Tukey HSD test’’) ($P< 0.05$) Laboratory experiment (Working group “General and Soil Microbiology” 2009).

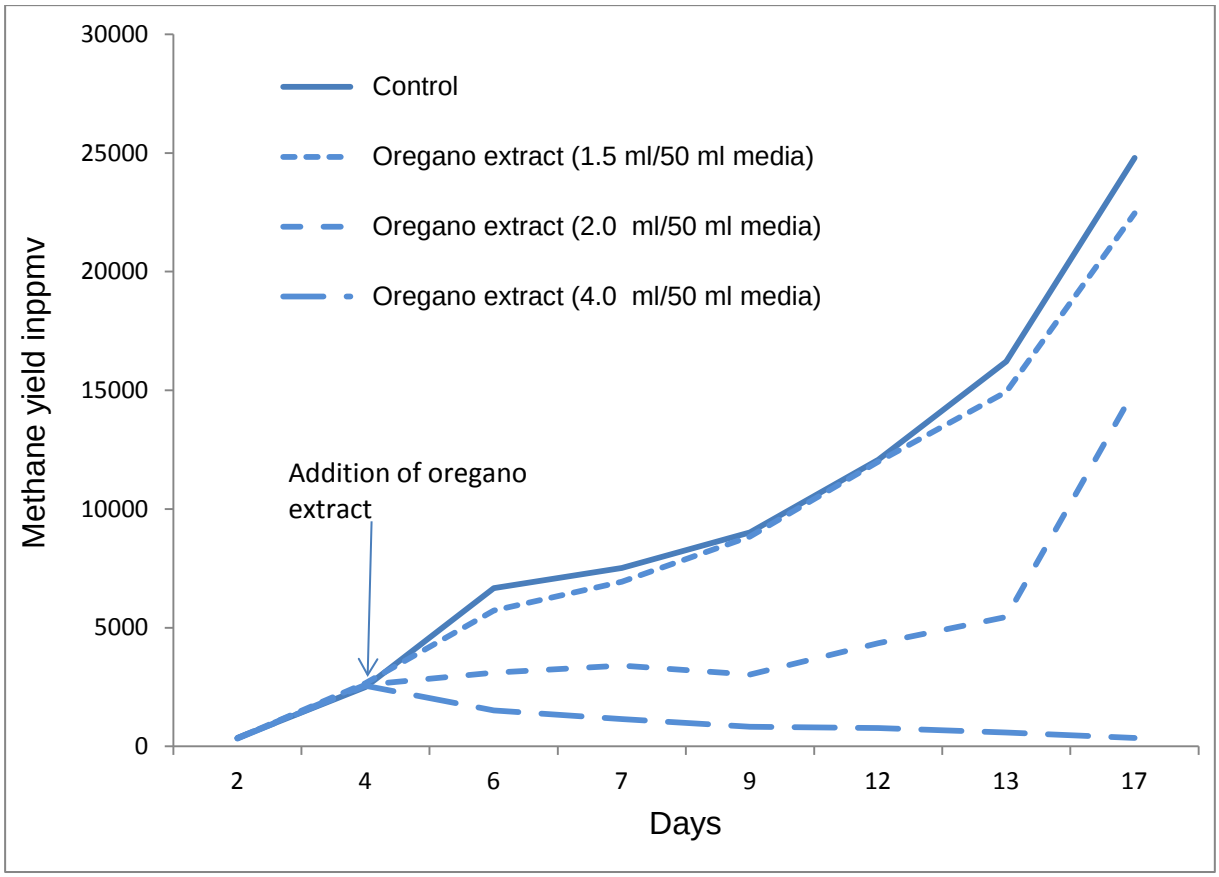

Fig. 12 Effect of different dosages of *O. vulgare* extracts on methane yield (ppmv) produced by *Methanosarcina barkeri* in a laboratory test over 17 days under mesophilic temperature. Extract of oregano was added on the 4th day of the inoculation of *M. barkeri*. Conditions are laboratory experiments (Working group "General and Soil Microbiology "2010).

Figure11 shows the results of the effect of *Origanum vulgare* extract, on methane produced by *M. barkeri* as an end product of series of metabolic reactions**.** The graph shows the methane produced during the incubation of *Methanosarcina barkeri* pure cultures under mesophilic temperature (36°C) in the dark with methanol as a source of carbon and addition of *Origanum vulgare* extracts. A dosage depending effect of the oregano extracts on methane production of the microbes could be shown (Fig.11). Low dosage of 1.5 ml of oregano extract did not modify the methane yield where as the increased concentration of 2.0 and 3.0 ml of oregano extracts per 50 ml significantly reduced the methane production by the microbes ($p < 0.05$). The increase of oregano extract concentration from 1.0 ml to 2.0 ml led to halved methane yield from around 25000 to around 13000 µg/l (Fig.11). Doubling the oregano concentration from 1.5 ml to 3.0 ml per 50 ml caused a strong inhibition of

the methane production on a level of around 1500 µg/l. It can be concluded that a dosage of 1.5 ml of oregano extract was tolerated by the microbes where as 2.0 ml and particularly 3.0 ml oregano extracts can be characterized as toxic. Generally, it is visible that the amount of methane produced decreases as the concentration of the extract dose rises.

Figure12 shows the effect on the methane formation of a pure *Methanosarcina barkeri* culture after addition of 1.5 ml, 2.0 ml and 4.0 ml of oregano extract. The extracts were added on the fourth day after inoculation of the medium with the *Methanosarcina* strain.The laboratory tests were carried out over a time period of 17 days to analyze the dynamic of the methane production during that period. It could be observed that in the control as well as in 1.5 ml extract treatment a steady increase of methane contents was measured. Generally it can be stated that both curves (control and 1.5 ml extract) running parallel. Furthermore, it was found that during the last four days of the experiment the methane production increased strongly. For that reason it can be suggested that the continuation of the experiment over 17 days would lead to higher level of methane content. The application of 2.0 ml of oregano extract caused a lower level of methane production during the period between 4^{th} and 17^{th} day but the development (dynamic) of the curve was similar to control and 1.5 ml extract curves (Fig.12). Totally diverged from the first three curves (control, 1.5 ml and 2.0 ml) was the run of the 4^{th} curve representing the effect of 1.0 ml of oregano extract. It was found that during the whole period from the 4^{th} until the 17^{th} day a steady decrease of methane production was defected. It seems that the oregano extract dosage of 4.0 ml had a toxic effect on *Methanosarcina barkeri* over the whole period.

4.1.2. Effect of carvacrol

In this study two treatments of carvacrol (standard solution from Carl Roth GmbH, Karlsruhe, Germany) were used. The experiment was conducted by addition of carvacrol at concentrations of 0.1 ml and 0.3 ml. The cumulative methane produced by *Methanosarcina barkeri* after addition of carvacrol after the 4^{th} day was measured. A clear dosage depending effect of carvacrol on the microbes that produce methane was observed in this study (Fig.13). The two dosages of 0.1 ml and 0.3 ml of carvacrol per 50 ml were modified the methane yield and they were drastically reduced the methane production by the microbes. The 0.1 ml of carvacrol

concentration led to halved methane yield from around 350000 µg/l (by the control where no addition of carvacrol) to around 170000 µg/l (Fig. 13). Increasing the carvacrol concentration from 0.1 ml to 0.3 ml per 50 ml caused a strong inhibition of the methane production on a level of around 110000 µg/l. It can be concluded that the two dosages of carvacrol added were not tolerated by the microbes and they can be characterized as toxic materials for *M. barkeri*.

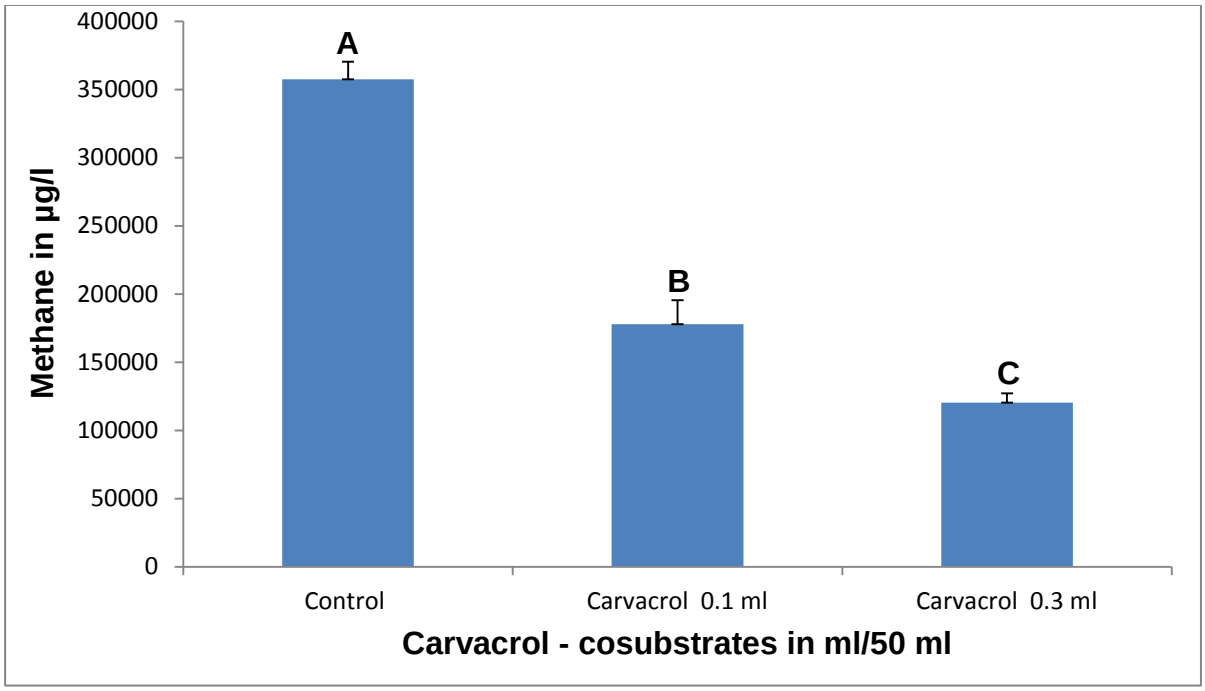

Fig. 13 Effect of pure carvacrol on methane yield (in $\mu g l^{-1}$) produced by *Methanosarcina barkeri* after 17 days under mesophilic temperature, T= standard error, different letters indicate significant differences between the averages (ANOVA and the one factorial post hoc tests ‘‘Tukey HSD test’’) ($p< 0.05$).Laboratory experiment (Working group “General and Soil Microbiology “2009)

Figure 14 presents the results of pure cultures of *M. barkeri* exposed to two different treatments of carvacrol 0.1 ml and 0.3 ml during their growth in artificial reduced buffered mineral media in serum bottles. The laboratory tests were carried out over a time of 17 days to analyze the dynamic of the methane production during that period. The application of 0.1 ml of carvacrol caused a lower level of methane production during the period between 4th and 17th day but the development (dynamic) of the curve was similar to control curve (Fig. 14). Totally diverged from the first two curves (control and0.1 ml) was the run of the 3rd curve representing the effect of 0.3 ml of

carvacrol. It was found that during the whole period from the 4th until the 17th day a steady decrease of methane production was detected. It seems that the carvacrol dosage of 0.3 ml had a toxic effect on *Methanosarcina barkeri* over the whole period.

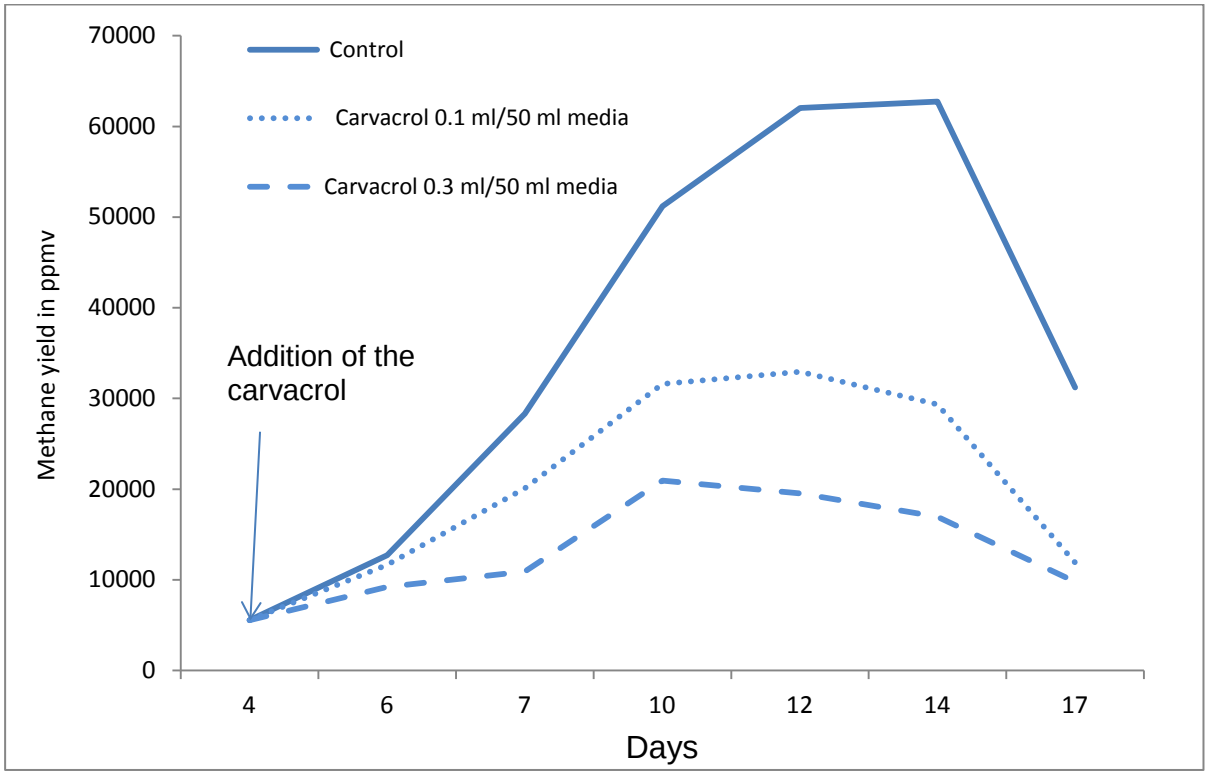

Fig. 14 Effect of different dosage of pure carvacrol on methane yield (in ppmv) produced by *Methanosarcina barkeri*over 17 days under mesophilic temperature. Carvacrol was added on the 4th day of the inoculation of *M. barkeri* (Working group "General and Soil Microbiology "2010).

4.1.3. Effect of thymol

The effect of two different concentrations 0.1 ml and 0.3 ml of pure thymol (Carl Roth GmbH, Karlsruhe, Germany) added to *M. barkeri* was studied. The control (no addition of thymol) received 0.03 ml more methanol because in each treated serum bottle there was 0.03 ml more methanol. The cumulative methane production is shown in figures 15 and 16. A dosage depending effect of thymol on the methane production of the microbes was found (Fig. 15). The two dosages of 0.1 ml and 0.3 ml of thymol per 50 ml modified the methane yield by drastically reduction of methane production by the microbes. The 0.1 ml of thymol concentration led to halved (or less than the half) methane yield from around 350000 µg/l (by the control) to around 150000 µg/l (Fig.15). Increasing the thymol concentration from 0.1 ml to 0.3 ml per 50 ml caused a strong inhibition of the methane production on a level of

around 89000 µg/l. It can be concluded that the two dosages of thymol added were not tolerated by the microbes. For that reason the used thymol compound can be characterized as toxic materials for *M. barkeri*.

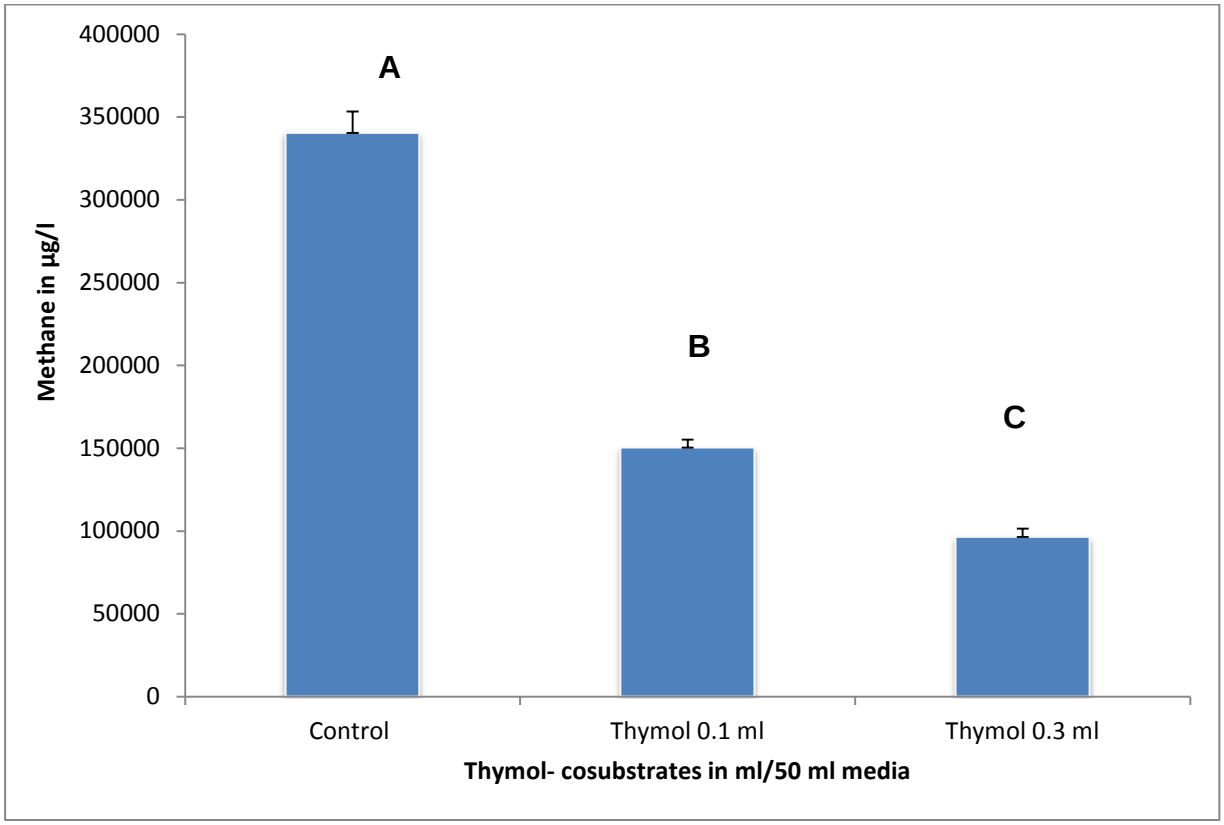

Fig. 15 Effect of pure thymol on methane yield (in μgl^{-1}) produced by *Methanosarcina barkeri* after 17 days under mesophilic temperature, T= standard error, different letters indicate significant differences between the averages (ANOVA and the one factorial post hoc tests ‘‘Tukey HSD test’’) (p=0.05) (Working group “General and Soil Microbiology “2009).

Figure 16 shows the result of the two concentrations of thymol 0.1 ml and 0.3 ml on the methane produced by the cultures of *Methanosarcina barkeri*. In all experiments *Methanosarcina barker* used methanol as carbon and energy source. The laboratory tests were carried out over a time of 17 days to analyze the dynamic of the methane production during that period. The application of 0.1 ml of thymol caused a lower level of methane production during the period between 4^{th} and 17^{th} day but the development (dynamic) of the curve was similar to control curve (Fig. 16). Totally diverged from the first two curves (control and 0.1 ml) was the run of the 3^{rd} curve representing the effect of 0.3 ml of thymol. It was found that during the whole period from the 4^{th} until the 17^{th} day a steady decrease of methane production was

detected. It seems that the thymol dosage of 0.3 ml had a toxic effect on *Methanosarcina barkeri* over the whole period.

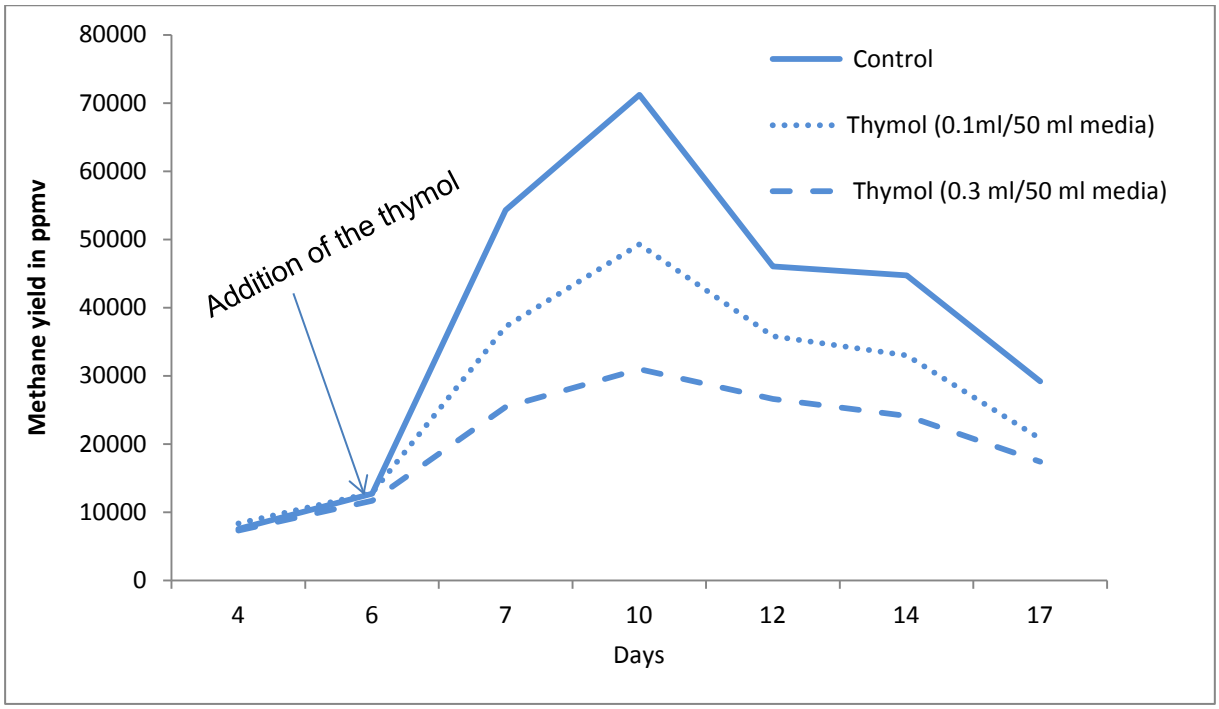

Fig. 16 Effect of pure thymol on methane yield (in ppmv) produced by *Methanosarcina barkeri* over 17 days under mesophilic temperature. Thymol was added on the 4th day of the inoculation of *M. barkeri* (Working group "General and Soil Microbiology "2010).

4.1.4. Effect of the mixture of carvacrol and thymol

Figure 17 shows the effect of the mixture of carvacrol and thymol at the treatment doses of 0.1 ml and 0.3 ml on the methane produced by *M. barkeri*under the mesophilic temperature (36°C). A dosage depending effect of carvacrol and thymol mix on the methane production of the microbes was observed (Fig. 17). The two dosages of 0.1 ml and 0.3 ml of the mix per 50 ml modified the methane yield by drastically reduction the methane production by the microbes. The 0.1 ml of the mix concentration led to a severe reduction of the methane yield from around 350000 µg/l (by the control) to around less than 100000 µg/l (Fig. 17). Increasing the mix concentration from 0.1 ml to 0.3 ml per 50 ml caused a strong inhibition of the methane production on a level of around 50000 µg/l. It can be concluded that the two dosages of the mix added were not tolerated by the microbes and they can be characterized as toxic materials for *M. barkeri*.

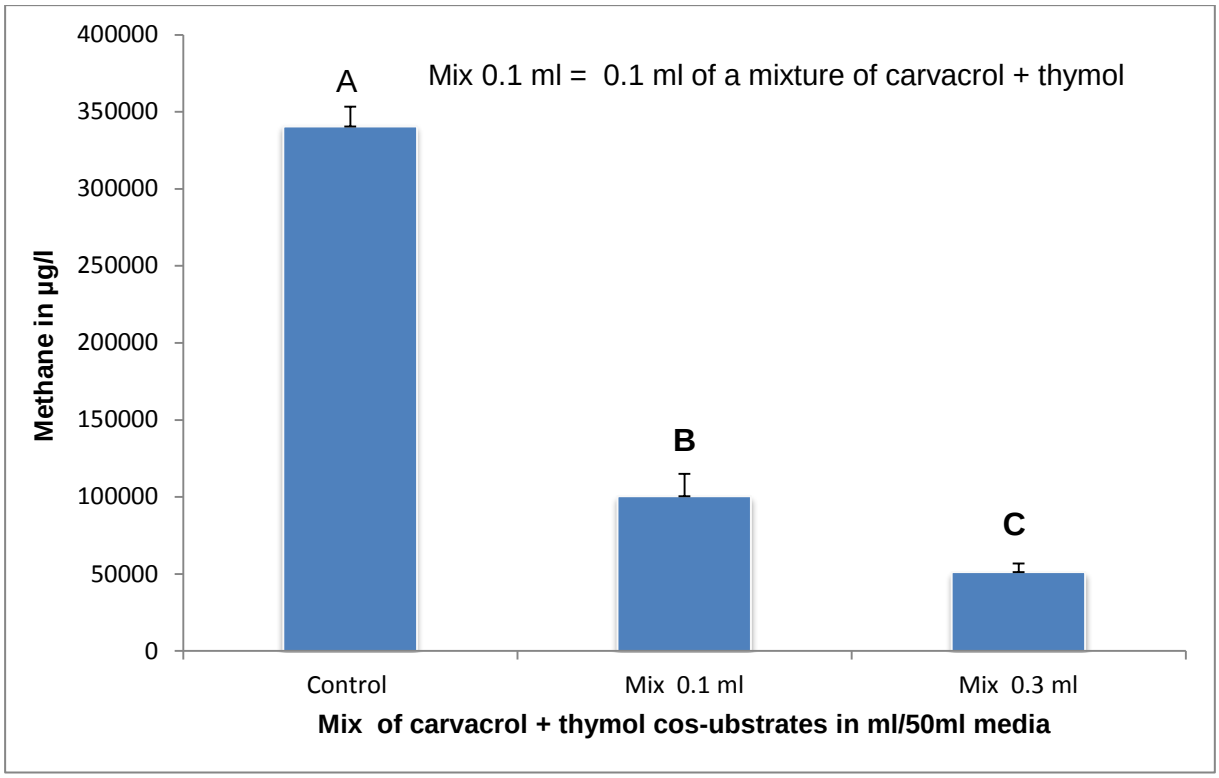

Fig.17 Effect of the mixture of carvacrol + thymol (Mix 0.1 ml/50 ml and 0.3 ml/ 50ml of media) on methane yield (in μgl^{-1}) produced by *Methanosarcina barker* after 17 days under mesophilic temperature, T= standard error, different letters indicate significant differences between the averages (ANOVA and the one factorial post hoc tests ‘‘Tukey HSD test’’) ($p< 0.05$) (Working group “General and Soil Microbiology “2009)

Figure 18 shows the results of an experiment conducted in 2010 to investigate the effect of two doses of carvacrol and thymol mixture to pure cultures of *M. barkeri.* The laboratory tests were carried out over a time of 17 days to analyze the dynamic of the methane production during that period. Methane yield was detected seven times. The application of 0.1 ml of the mixture of thymol and carvacrol caused a lower level of methane production during the period between the 6th and the 17th day but the development (dynamic) of the curve was similar to control curve (Fig. 18). Totally diverged from the first two curves (control and 0.1 ml) was the run of the 3rdcurve representing the effect of 0.3 ml of the mixture of thymol and carvacrol. It was found that during the whole period from the 6th until the 17th day a steady decrease of methane production was detected. It seems that the mixture of thymol and carvacrol dosage of 0.3 ml had a toxic effect on *Methanosarcina barkeri* over the whole period.

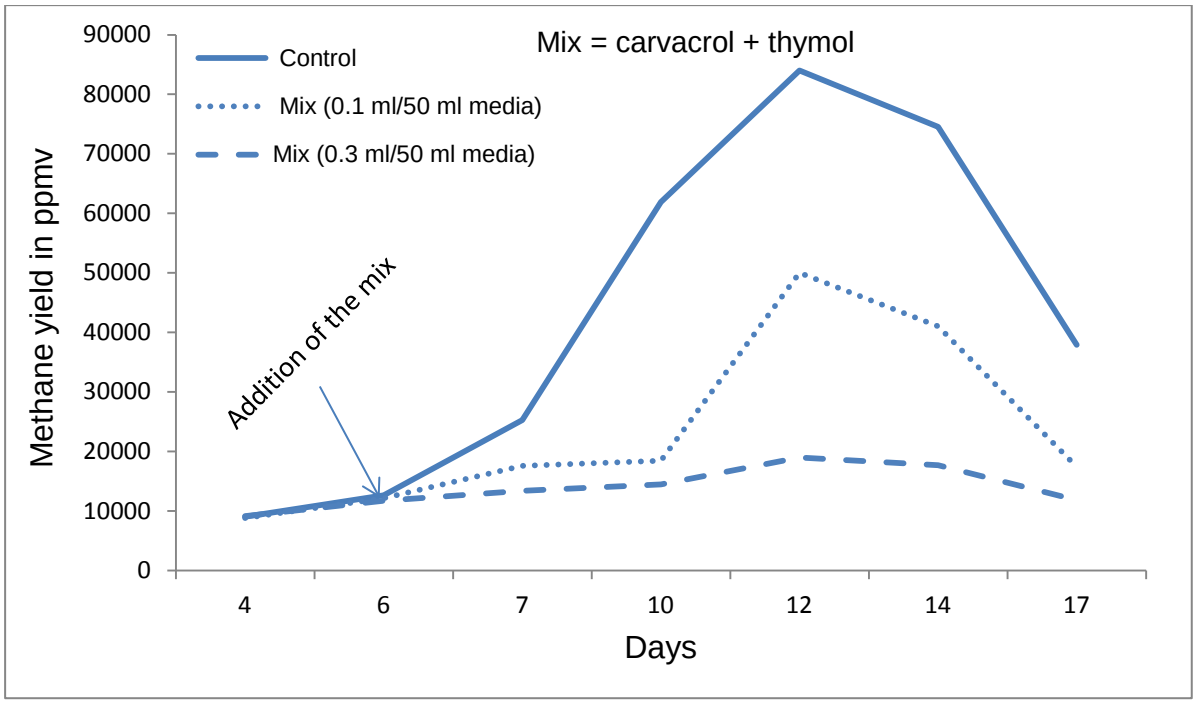

Fig. 18 Effect of the mixture of carvacrol + thymol (Mix 0.1 ml/50 ml and 0.3 ml/ 50ml of media) on methane yield (in ppmv) produced by *Methanosarcina barkeri* over 17 days under mesophilic temperature. The mixture of carvacrol and thymol was added on the 6th day of the inoculation of *M. barkeri* (Working group “General and Soil Microbiology “2010”).

4.2 Results of biogas experiments

4.2.1. Effect of oregano herbs (*Origanum vulgare*)

The value of the pH of the cattle liquid manure before adding the doses of oregano was 7.4 and after adding them and at the end of the experiment it was 6.5 where a reduction of biogas, therefore it means that there was accumulation of volatile fatty acid lead to slight drop in the pH value.

The addition of oregano treatments to the cattle liquid manure as co-substrates in batch digesters mode were evaluated by monitoring the biogas and methane production (Fig.19). In oregano treatments when there was inhibition effect, it could be clearly seen in the kinetic of the biogas and the methane formation compare to that of the control experiments in which no oregano leaves were added.

Effect of oregano on total biogas

Figure 19 shows the means of the real results of cumulative amounts of biogas obtained by the co-digestion of each of *O. vulgare* treatment that was added to the cattle liquid manure in the mesophilic anaerobic laboratory digester after subtracting the biogas obtained by the cattle liquid manure. The biogas is expressed in normal liter per kg of organic dry matter under the standard conditions i.e. T_0 = 273°K and P_0 = 1013 mbar

As can be seen, the production of biogas from *Origanum vulgare* treatments is different according to the amount and the concentration of *O. vulgare* used. Oregano treatments gave biogas ranging between 389 to 109 Nl/kg O.DM compare to the control (only cattle liquid manure without additions substrates), which produced 247 Nl/kg O.DM. The biogas obtained by the treatment 62.5 g was significantly higher than the control ($P< 0.05$), but it is less and inconsistent with the calculated theoretical biogas by the same treatment, which is 400 Nl/kg O.DM (Table 4) thus the theoretical is much more when all the organic dry matter content was expected to convert to biogas. The biogas that was obtained from the treatment 125 g was 375 Nl/kg O.DM, which is higher than the biogas that produced from the control and statistically it is significantly different ($P< 0.05$), but however, it is not inconsistent with biogas that calculated theoretically from the same treatment (800 Nl/kg O.DM).

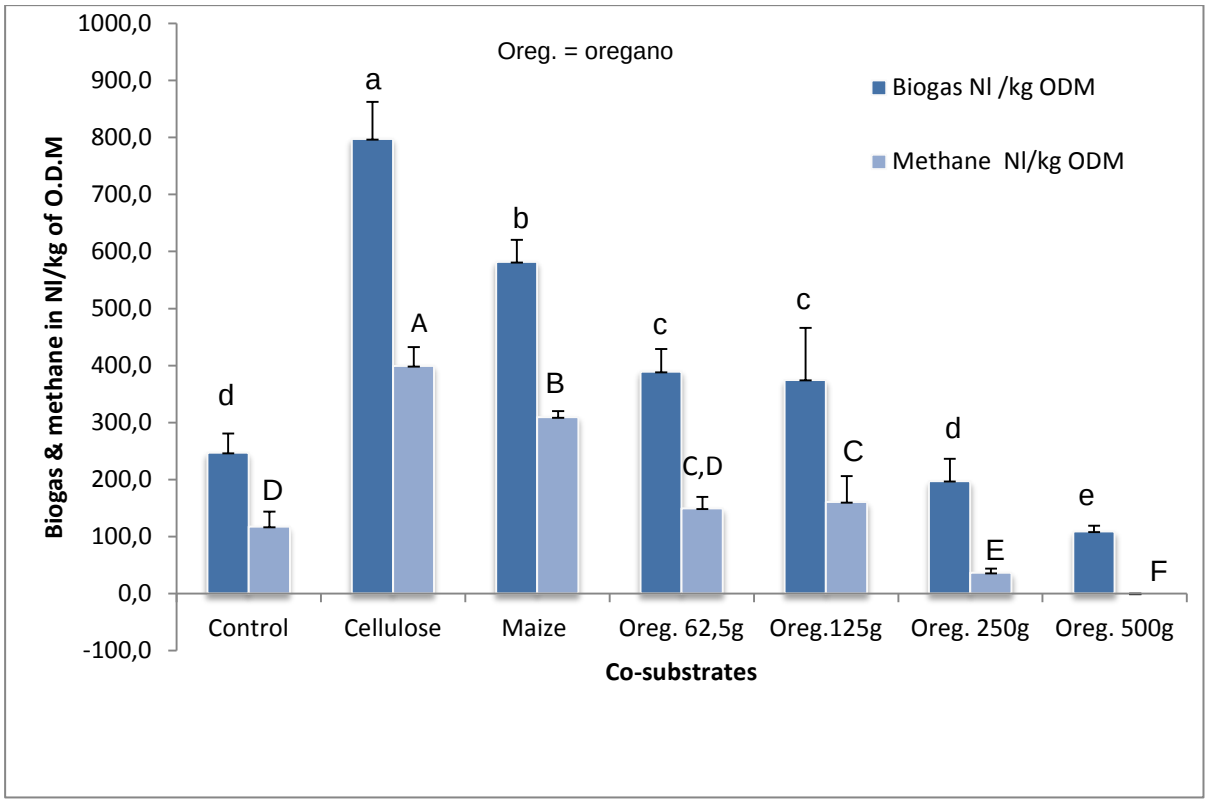

Fig. 19. Biogas and methane production (Nl/kg O DM) of different plant co-substrates with cellulose and cattle liquid manure after subtracting the biogas produced by the control alone from the batch values are expressed as means. Letters over the bars indicate the statistically significant difference of production between oregano-treatments and that of cellulose and maize as a reference group. Error bars indicate the standard error n= 3 (biogas experiment Rauischholzhausen “August 2011).

Although, the biogas obtained by the treatment 250 g and 500 g is 197.3 and 109 Nl/kg O.DM respectively and each amount of them is lower than that of the control and statistically it is significantly different from that of control ($P< 0.05$). The biogas obtained by the treatments 250 and 500g is strikingly lower than the calculated theoretical biogas that produced by each of the two treatments, which is 1600 Nl/kg O.DM and 3200 Nl/kg O.DM respectively (Table 4).

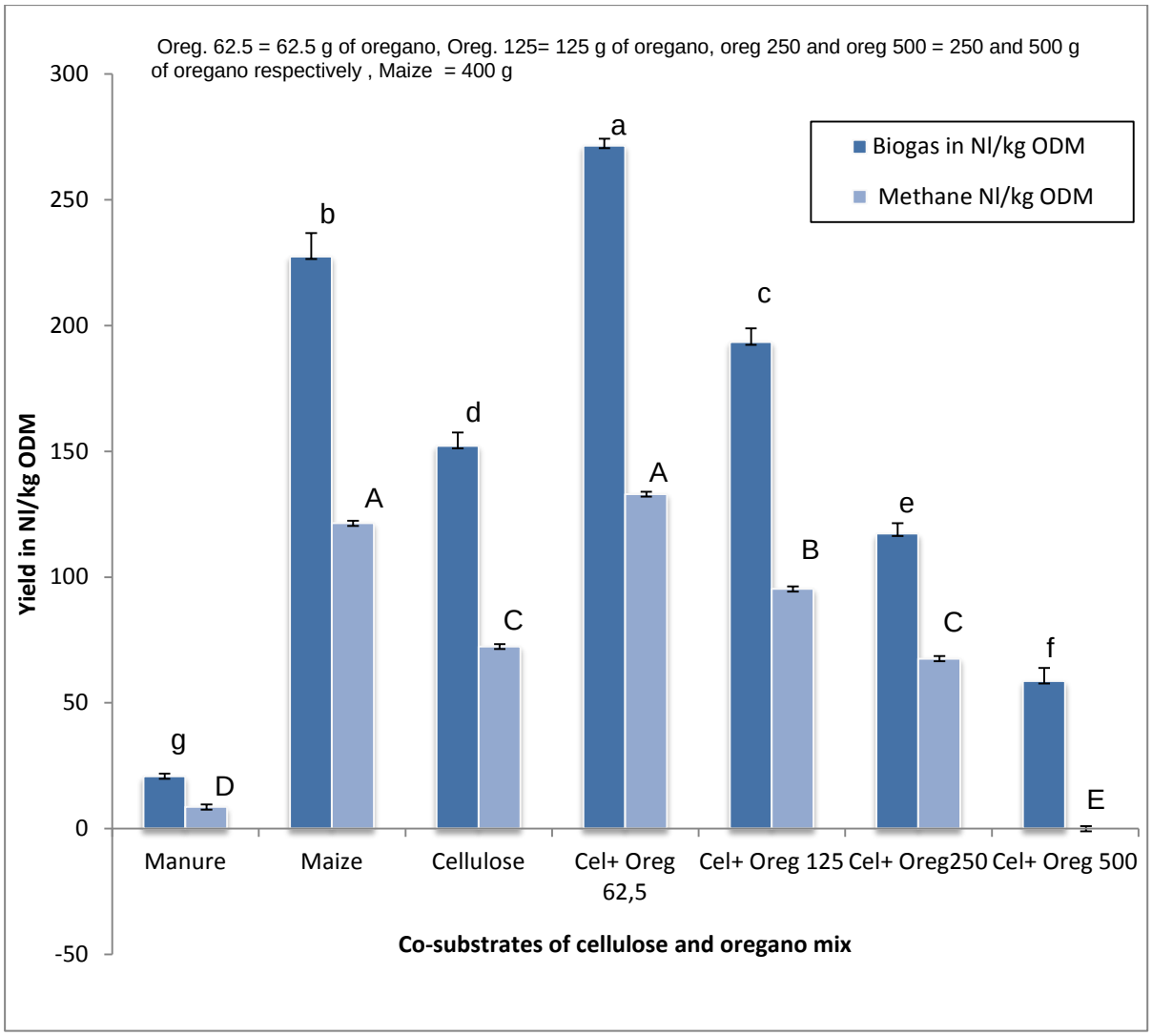

Fig 20 Biogas and methane production (Nl/kg O.DM) of different plant co-substrates with cellulose and cattle liquid manure after subtracting the biogas produced by the control alone from the batch values are expressed as means. Letters over the bars indicate the statistically significant difference of production between oregano-treatments and that of cellulose and maize as a reference group. Error bars indicate the standard error n= 3 (biogas experiment Rauischholzhausen “June 2012).

Fig. 20 shows the result of the experiment that conducted in June 2012 and the same procedures that had been done in the previous experiment were repeated here except in each digester standard cellulose and cattle liquid manure were added to the tested doses of oregano leaves powder and they gave biogas range between 271.5 and 58.7 Nl/kg O.DM. Dose 62.5 g of oregano gave 271.5 Nl/kg O.DM which is the highest biogas of all the co-substrate materials used and it is more than the biogas that produced by the cellulose and manure together, which is equal to 173

Nl/kg O.DM and statistically it is significantly different from the biogas that produced by each of manure and cellulose ($P< 0.05$). Then the biogas production starts to decline for instance doses 125 g, 250 g and 500 g of oregano leaves when each was mixed with the standard cellulose and manure they gave 193.4, 117.3 and 58.7 Nl/kg O.DM respectively and statistically they are all significantly different from the biogas that was produced by the cellulose alone ($P< 0.05$). Maize which was dried powder in this experiment produced 227.5 Nl/kg O.DM and it was higher yield than all the other co-substrate treatments except dose 62.5 g of oregano when mixed with cellulose it yielded 271.5 Nl/kg O.DM. Cattle liquid manure in this experiment gave 20.8 Nl/kg O.DM, which is the lowest amount of biogas and this may be attributed to the low amount of dry matter, which was 3%. Generally the results of biogas from all co-substrate material are lower than the two other experiment (first and the last one). In December 2012 the third and the last experiment of oregano treatments doses were mixed also, with manure and standard cellulose in each digester and they produced biogas ranged between 1603.5 to 301.8 Nl/kg O.DM. The treatment dose 62.5g of oregano produced 1603.5 Nl/kg O.DM of biogas, which is the highest amount of all co-substrate materials and statistically it is significant different from that produced by standard cellulose alone ($p<0.05\%$). Then in this experiment the biogas started to decrease from 921.3 to 253 by treatment doses 250 and 500 g respectively ($p< 0.05$), (Fig. 21).

In this study it can be observed that the microorganisms of biogas production can able to degrade 4.2-8.33 g/kg of cattle liquid manure from *O. vulgare* but more quantity than that will cause a reduction in the biogas production. Also it is observed that the 100 g of fine powder of crystalline cellulose, which used as a reference in this study, produced considerably higher biogas than did maize which is also used as reference and that is due to the maize was not all totally degraded by microorganisms that share in biogas production.

Nevertheless, these results were obtained by using the batch test mode to evaluate the effect of different rates of *O. vulgare*. Also, it may provide more sensitive information for assessing the impact of biogas production, management strategies. Be aware from the thread of toxic materials found in plants tissue. Also, do not neglect their effects.

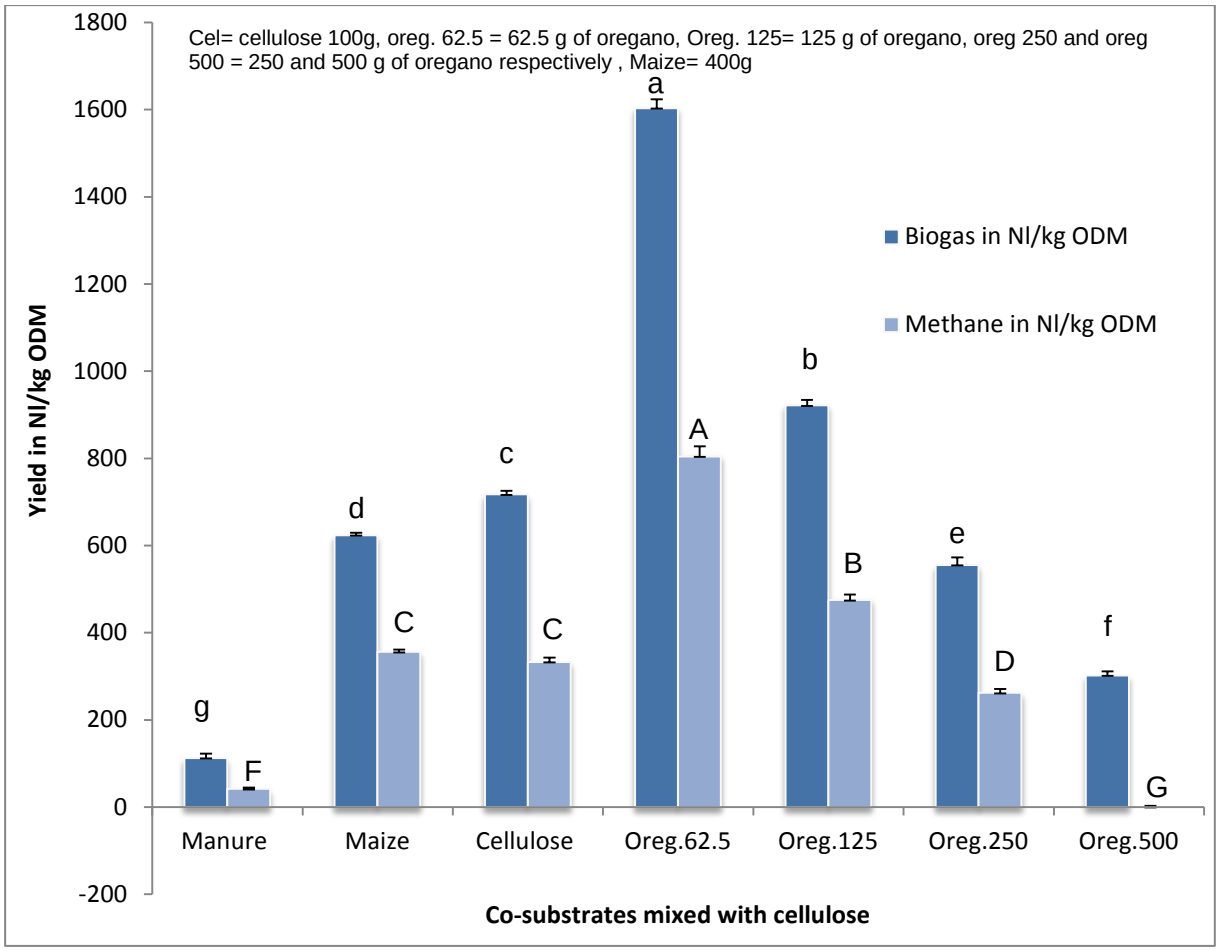

Fig. 21 Biogas and methane production (in Nl/kg O DM) of different plant co-substrates with cellulose and cattle liquid manure after subtracting the biogas produced by the control alone from the batch values are expressed as means. Letters over the bars indicate the statistically significant difference of production between oregano-treatments and that of cellulose and maize as a reference group. Error bars indicate the standard error n=3 (biogas experiment Rauischholzhausen “December 2012).

Effect of oregano on methane production

Data presented in Figures19, 20 and 21 shows the effect of oregano treatments on the methane production. The addition of cellulose to manure led to increase the methane production compared to manure (without co-substrate) from 110 to 400 Nl/kg of methane (Fig.19). The methane content in cellulose treatment was around 45%. It was observed that the application of maize as co-substrate increased the methane content to 54%. The addition of powdered oregano leaves (oreg.62.5) in combination with manure induced effective increase of methane. The detected methane yield of the four treatments of oregano was ranged from 150 to (0) Nl/kg

O.DM (Fig. 19). The combination of oreg.125 treatment and manure produced 160.5 Nl/kg O.DM of methane, which is the highest value compared to the other three treatments of *Origanum vulgare* and statistically it is significantly different from the control ($P< 0.05$) and the theoretical estimated methane too (420 Nl/kg O.DM). Treatment oreg. 250 produced 37Nl/kg O.DM of methane, which is lower than the control and statistically it is significantly different from the control ($P< 0.05$) and less than the theoretical calculated methane, which is 840 Nl/kg O.DM (Table 4). Digesters applied with the maximum dosage of 500 g of *Origanum vulgare* had produced zero level of methane yield as compared with manure (without co-substrates).

The addition of maize to manure increased the methane production compared with the control (only manure) from less than 20 Nl/kg of O.DM to 120 Nl/kg of O.DM (Fig. 20). The methane content of maize treatment was around 54% (Fig. 20). It was found that the application of cellulose as a co-substrate caused an increasing in methane production as maize level. The addition of ground oregano leaves (org.62.5) in combination with cellulose induced strong increase of methane yield. The measured methane produced by oregano treatments after combination of cellulose and manure in each digester was ranged from zero to 133 Nl/kg of O.DM (Fig. 20). Doubling the doses of the ground leaves of oregano from 125 g, 250 g and 500 g (in combination with cellulose) resulted in clear reduction in methane production (Fig. 20). Figure 19 shows the result of the last experiment where the cellulose and manure were combined together and used with each digester of oregano treatments. Combination of maize with manure increased the methane production compared with manure (without co-substrate) from 50 Nl/kg of O.DM to around 360 Nl/kg of O.DM (Fig. 21). The maize treatment gave 56% methane content. It was found that in the same experiment using of cellulose as co-substrate with manure cause the same level of methane as observed in maize. The addition of ground leaves of oregano (oreg.62.5) with combination with cellulose significantly increases the methane production. The detected methane yield was ranging from zero to around 790 Nl/kg of O.DM (Fig. 21). The combination of oreg.62.5 and cellulose led to maximum values compared to the other three treatments of oregano. Doubling the treatments of ground oregano leaves from 62.2 g to 125 g, 250 g and 500 g resulted in clear reduction of methane yield (Fig. 21). Digesters applied with maximum concentration of 500 g of oregano gave zero Nl methane/kg of O.DM compared with manure (without co-substrate).

4.2.2. Effect of fennel seeds (*Foeniculum vulgare* L.)

Effect of fennel on the total biogas production

Figure 22 summarizes the volumes of biogas obtained from the co-digestion of *Foeniculum vulgare* treatments with cattle liquid manure in an anaerobic digester. As can be seen, the production of biogas is different according to the amount of chemical content of *Foeniculum vulgare* used. The biogas produced by treatment 25 g was 375.3 norm liter, which is significantly higher than the control ($p<0.05$), which produced only 247 norm liter. Also it is less than the theoretical calculated biogas table 5 (750 Nl/kg O.DM). The second treatment 50 g of fennel seeds produced 379.92 norm liter, it is higher than the control but not significantly different from the dose 25 g ($p > 0.05$). Then the volume of the biogas obtained start to increase and it decrease again at dose 200 g of fennel seed and it obtained 275.8Nl/kg O.DM, which is not significant different from that of the control ($p > 0.05\%$). The volume of the biogas obtained by dose 400 g was 102.1 Nl/kg O.DM which is the lowest amount of biogas and significantly different from all other used materials. The biogas obtained from all fennel treatment is inconsistent with the calculated theoretical biogas by the same treatments thus the theoretical is much more (Table 5). In this study it can be observed that the microorganisms of biogas production degrade 1.7 g to 6.7 g of *Foeniculum vulgare* per kg of cattle liquid manure. Nevertheless, these results obtained using a batch test mode to evaluate the effect of different rates of *Foeniculum vulgare* may provide more sensitive information for assessing the impact of biogas production management strategies to be aware of the thread of toxic chemical materials that found in the plants tissue and not neglect their effects. In December 2012 the last experiment of the fennel treatments was conducted with combining manure and standard cellulose in each digester.

The addition of maize led to increase the biogas production compared to manure (without co-substrate) from less than 100 Nl/kg of O.DM to 620 Nl/kg of O.DM (Fig. 23). The methane content of maize treatment was around 54% (Fig. 23). It was found that the application of cellulose as a co-substrate caused the same level of biogas and methane yield as found with maize (Fig. 23). The addition of ground fennel seeds (F50) in combination with cellulose induced strong increase of biogas production. The detected biogas yield was 2300 Nl/kg of O.DM and 1100 Nl/kg of

O.DM (Fig. 23). The combination of F50 + cellulose led to maximum values compared to the other treatments of the experiment. Rising treatments of ground fennel seeds from F50 to F100, F200 and F400 (in combination with cellulose) resulted in clear reduction of biogas yield (Fig. 23). Digesters applied with maximum treatment of 400 g of fennel seeds had the same level of biogas yield as measured with manure (without co-substrate).

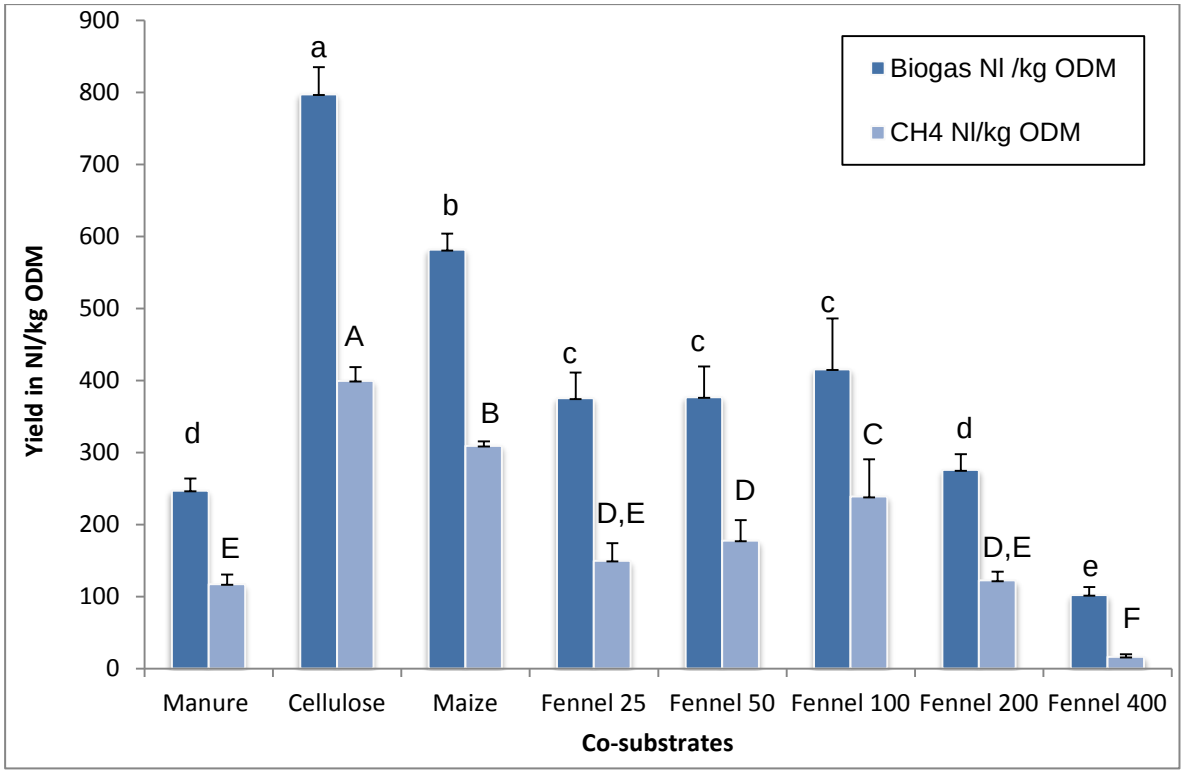

Fig 22 Biogas production (in Nl/kg O DM) of different plant substrates after subtracting the biogas produced by the control alone from the batch Values are expressed as means. Letters over the bars indicate the statistically significant difference of production between fennel-treatments and that of cellulose and maize as a reference group. Error bars indicate standard error n=3 (biogas experiment Rauischholzhausen “August 2011).

Effect of Fennel on methane production

Fig 22 displays the effect of fennel treatments on the methane production. The treatments of fennel produced methane ranged between 238.8 to 16.5 Nl/kg O.DM.The fennel seeds 25 g treatment produced 149.6 Nl/kg O.DM of methane, which is statistically not significant different from the control ($P > 0.05$). Dose 50 g treatment produced 117.6 Nl/kg O.DM of methane, which is higher than that of the control and statistically it is significantly different from the control ($P < 0.05$). Dose 100 g treatment produced 238.8 of methane; which is the highest amount and

statistically it is significant different ($P < 0.05$). Then the amount of methane produced start to decrease to 122.4 and 16.5 norm liter by treatments 200 g and 400 g respectively. In the last experiment the methane was 1189, 471.1, 290 and 17.8 norm liter by doses 50, 100, 200 and 400 g respectively and it is significantly different from the production that by the cellulose ($P < 0.05$). Again it can be observed that the methane produced by each dose treatment of fennel seeds is inconsistence with the theoretical calculated methane using Baserga formula (Table 5).

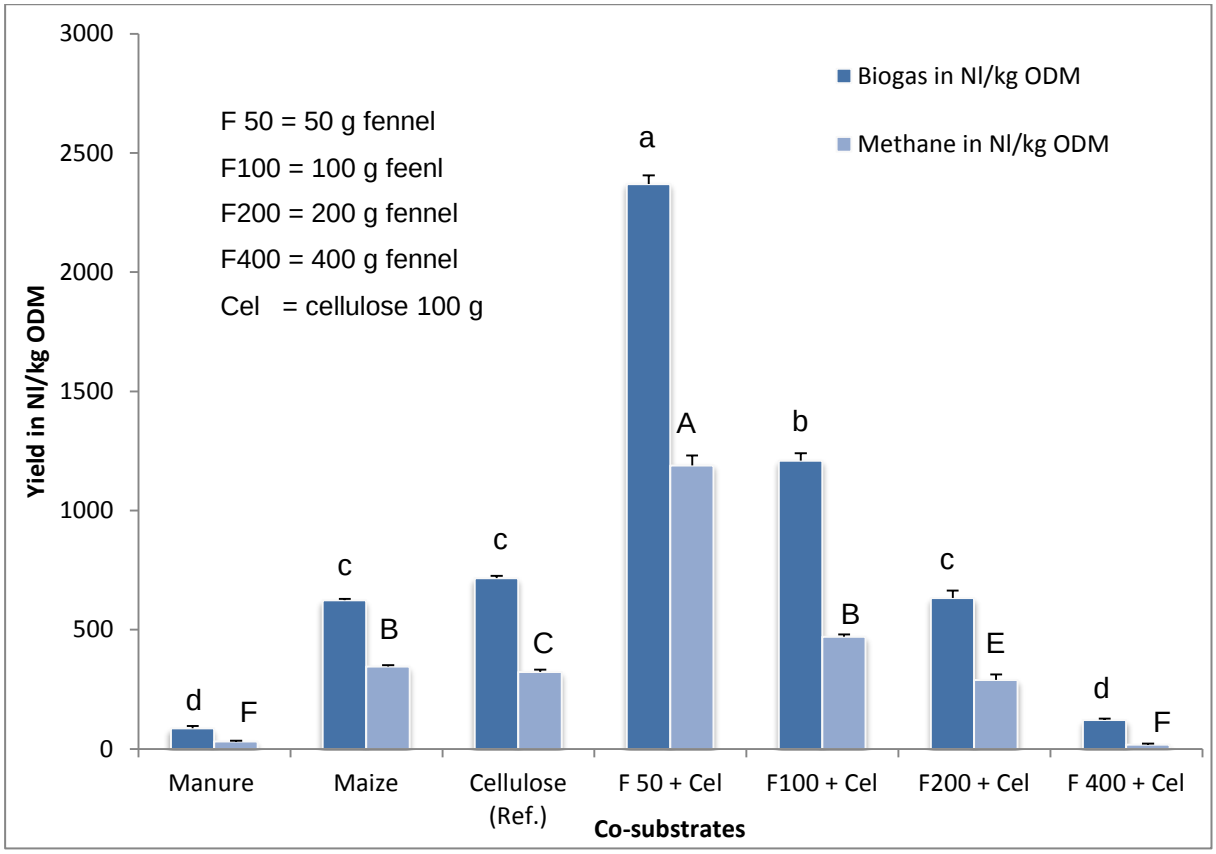

Fig 23 Biogas and methane production (Nl (kg O DM)-1) of different plant substrates after subtracting the biogas and methane produced by the control alone from the yield of other substrates. Values are expressed as means. Letters over the bars indicate the statistically significant difference of production between fennel-treatments and that of cellulose and maize as a reference group. Error bars indicate standard error n=3 (biogas experiment Rauischholzhausen December 2012).

The addition of maize led to increase the methane yield compared to manure (without co-substrate) from less than 50 Nl of biogas/kg of O.DM to around 320 Nl of methane /kg of O.DM (Fig. 23). The methane content in maize treatment was around

55%. It was found that the application of cellulose as a co-substrate caused the same level of methane yield as found with maize. The addition of ground fennel seeds (F50) in combination with cellulose induced strong increase of methane production. The detected methane yield was 1200 Nl/kg of O.DM and less than 20 Nl/kg of O.DM (Fig. 23).The combination of F50 + cellulose led to maximum values compared to the other treatments of the experiment. Rising doses of ground fennel seeds from F50 to F100, F200 and F400 (in combination with cellulose) resulted in clear reduction of methane yield (Fig. 23). Digesters applied with maximum dosage of 400 g of fennel had the same level of methane yield as measured with manure (without co-substrate).

4.2.3. Effect of hop cones (*Humulus lupulus* L.)

Effect of hop cones on biogas and methane production

Figures 24 and 25 show the results of experiments conducted in mesophilic laboratory digester to investigate the effect of four treatments of *Humulus lupulus* cones with the combination of the cellulose and cattle liquid manure. The addition of maize to manure led to increase the biogas (from 15 Nl/kg of O.DM to 220 Nl/kg of O.DM) and methane (from around 12 Nl/kg of O.DM to 125 Nl/kg of O.DM) production compared to manure without co-substrate (Fig. 24). The methane content in maize treatment was around 54% (Fig. 24). It was observed that the application of cellulose as a co-substrate with manure resulted in the half level of biogas and methane yield as found with maize. Furthermore, the addition of fine powder of hope cones (H50) in combination of cellulose caused severe reduction in biogas and methane production. The detected biogas yield of H50 was zero Nl/kg of O.DM (Fig. 24). By doubling the concentration of hop cones from H50 to H100, H200 or H400 resulted in obvioussevere reduction of biogas and methane yield (Fig. 24). Moreover, the other rising treatments of hop had the same result of H50 biogas and methane production. Thus hop cones can be characterized as toxic and dangerous substrates for biogas and methane producing microbes.

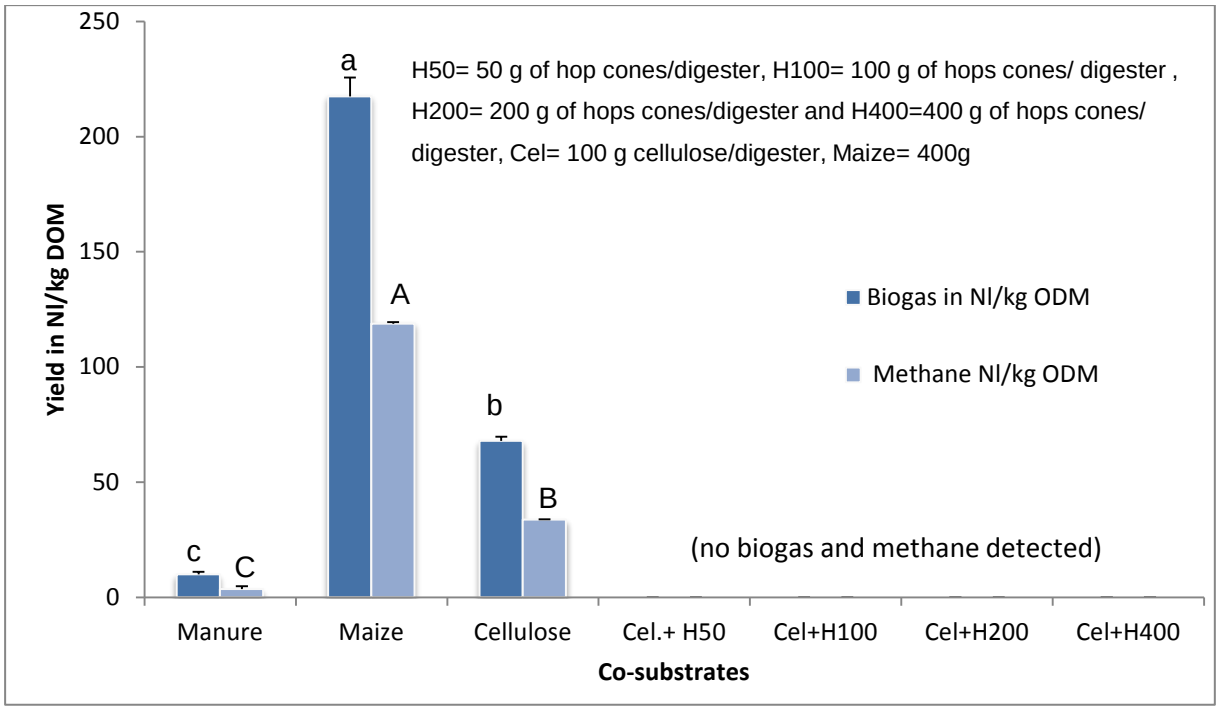

Fig 24 Biogas and methane production (Nl/kg O DM) of different plant co-substrates with cellulose and cattle liquid manure after subtracting the biogas produced by the control alone from the batch values are expressed as means. Letters over the bars indicate the statistically significant difference of production between hops treatments and that of cellulose and maize as a reference group. Error bars indicate the standard error n=3 (biogas experiment Rauischholzhausen June 2012).

In figure 25 the mixture of cellulose and manure increases the biogas (from120 Nl/kg of O.DM to around 720 Nl/kg of O.DM) and methane (from 32 Nl/kg of O.DM to 340 Nl/kg of O.DM) production compared to manure without co-substrate. The methane content in cellulose treatment was around 44%. It was estimated that the application of maize as a co-substrate with manure produced 640 Nl/kg of O.DM biogas and 340 Nl/kg of O.DM of methane compared with the control manure without co-substrate. Moreover, the methane content of maize treatment was around 54% (Fig.25). The addition of fine ground hop cones (H50) in combination with cellulose significantly caused a huge reduction of biogas and methane production (Fig. 25). The combination of H50 with cellulose resulted in zero Nl/kg of O.DM of biogas and methane compared with the control (only manure). By increasing the concentration of the fine powder of hop cones to H100, H200 or H400 resulted in clear reduction of biogas and methane equal to the production of (H50). Hop cones showed the highest

toxicity to the methane producing microorganisms when comparing with the other co-substrates in the experiment. As a consequence, the results of hop cones reveal that they may contain toxic chemicals severely affecting the biogas producing microorganisms. It is highly recommended to avoid using hop cones in biogas production.

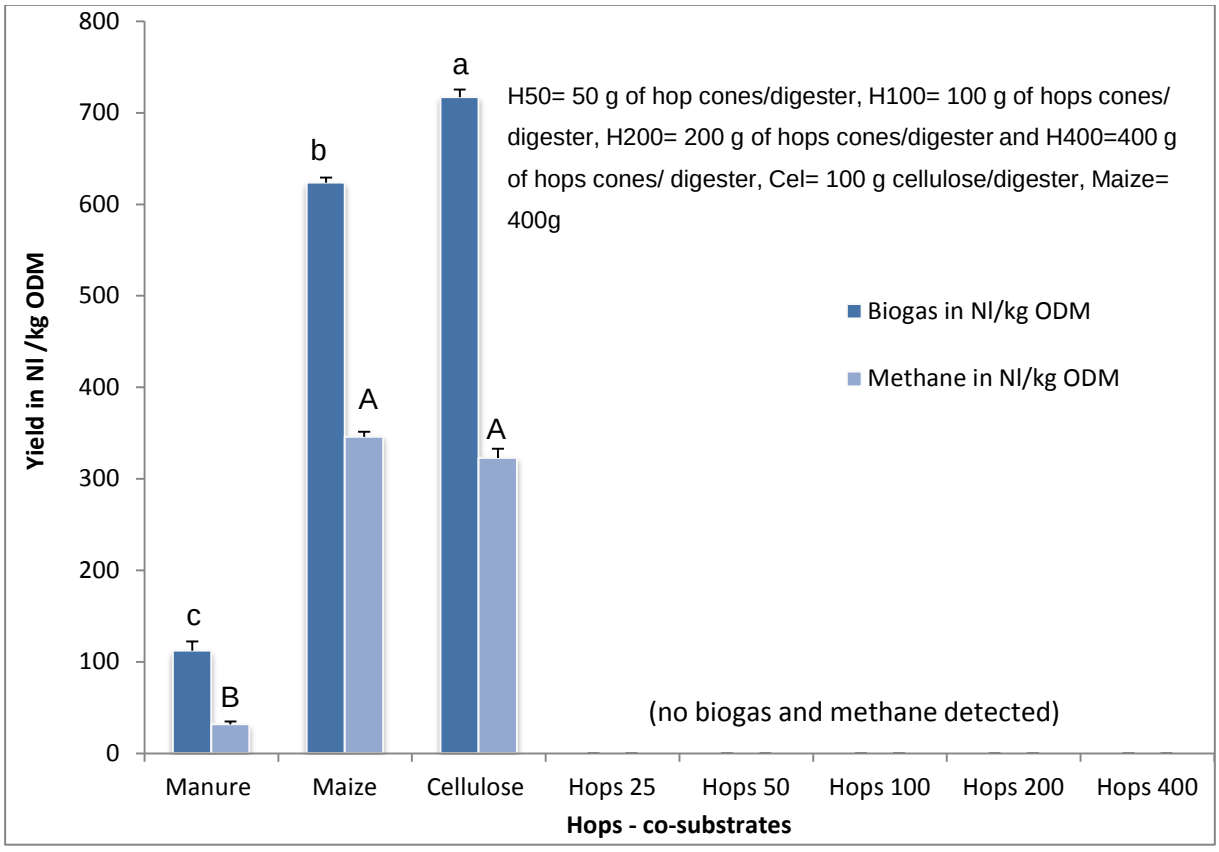

Fig 25 Biogas and methane production (in Nl/kg O.DM) of different plant co-substrates with cellulose and cattle liquid manure after subtracting the biogas produced by the control alone from the batch values are expressed as means. Letters over the bars indicate the statistically significant difference of production between hops-treatments and that of cellulose and maize as a reference group. Error bars indicate the standard error n=3 (biogas experiment Rauischholzhausen December 2012).

5. Discussion

5.1. Effect of oregano extracts and its main chemical components on methane production of *Methanosarcina barkeri*

5.1.1. Effect of oregano extracts

In the conducted experiments, the addition of *Origanum vulgare* methanol extract to the culture media at doses of 2.0 and 3.0 ml/50ml reduced clearly the methane production by *Methanosarcina barkeri*. This effect was observed during 17 days of incubation period under the conditions of mesophilic temperature of around 36°C (Fig.11&12). It is suggested that this impact was mainly caused by the antimicrobial activity of the secondary metabolites of oregano (Hart et. al., 2008). To be precise, comparing the methane produced by the control (no addition of extracts but has the same amount of methanol added to the other treated bottles) with that produced by treated serum bottles (2 and 3 ml of an oxic extracts) was significantly reduced. Consequently, there was a direct correlation between the concentration of the extract and the effect on the methane production, therefore the effect of extract is a dose depending effect. These results indicate that an oxic extracts of oregano leaves may altered the substrates in the culture media to favour methane production as end metabolic product. In fact, these secondary compounds are constituted from a mixture of chemical molecules that can exert antimicrobial activities by multiple mechanisms of action. It seems that these compounds can inhibit a broad variety of both gram-positive and gram-negative bacteria and other microorganisms (Calsamiglia *et al*., 2007). It can be suggested that inhibitory effects of the oregano compounds are also related to the *Archaea Methanosarcina barkeri.*

In the conducted microbial experiments extracts of common oregano (*Origanum vulgare* ssp. *vulgare*) were tested. It needs to point out that there are several species, subspecies and variations of spice plants called as oregano found in the market. Not all of them are *belong* to the botanical genus *Origanum.* Examples for non-origanum spices are plants belonging to the genus Lippia called as Mexican oregano.

The genus *Lippia*, belongs to the family Verbenaceae, comprises approximately 200 species. The species are mainly distributed throughout the South and Central America countries, and Tropical Africa territories (Terblanché and Kornelius, 1996). Most of the *Lippia spp*. are traditionally utilized as gastrointestinal and respiratory

remedies (Morton, 1981). Some *Lippia* species have shown antimalarial (Gasquet, 1993), antiviral (Abad *et al*., 1999) and cytostatic activities.
One of the most widely used species is *Lippia graveolens* Kunth known as Mexican oregano, an aromatic plant native of Southern North America, México, Guatemala, Nicaragua and Honduras (Martínez-Rocha *et al*., 2008). The chemical composition of the essential oil of *L. graveolens*, being carvacrol, thymol and *p*-cymene the major constituents. In addition, the essential oil demonstrated significant antimicrobial and antioxidant activity (Salgueiro, *et al*., 2003, Martínez-Rocha *et al*., 2008). Examples of other *Lippia* species are *Lippia aristata Schau, Lippia canescens* Kunth, *Lippia chamaedrifolia* Steud, *Lippia reptans and Lippia sellowii.*

Another example for a non-origanum spice plant is Spanish oregano (*Thymus capitatus*). It is a perennial, herbaceous shrub commonly used as a spicy herb and in some countries is known under the common name “zaâtar” (Bounatirou *et al*., 2007). It is often used to flavor meats, soups and stews. Furthermore, its essential oils were reported to have antimicrobial activities (Reddy et al., 1998). Most of which are mediated by thymol and carvacrol, as the phenolic components of the oil. Spasmolytic as well as antioxidant activities were also reported for the phenolic oil extract of the plant (Miguel *et al*., 2004; Sacchetti et al., 2005).

Economical important and wide used spice plants belonging to the genus origanum are Greek oregano (Origanum vulgareL. ssp. *hirtum*), Turkish oregano (*Origanum onites and O. minutiflorum*), marjoram (*Lippia graveolens* Kunth) and Spanish oregano (*Thymus capitatus*).

The composition of the secondary metabolites of oregano depends on genotype (subspecies), growing conditions and extraction methods. Greek oregano is characterized by its main components thymol (40%), carvacrol (25%) and *p*-cymene (17%). In addition small amounts of y-terpinene, borneol and terpinene-4-ol were found in Greek oregano (Milos *et al*., 2000). Both extracts as well as essential oil of Greek oregano showed significant growth inhibitory effect against *Escherichia coli*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Aspergillus niger* at concentration of 0.05% and 0.1% (v/v) and for extracts at concentration of 0.1% (Mohácsi-Farkas, *et al*., 2001).

Turkish oregano (*Origanum onites*) contains 62% carvacrol, 6% y-terpinene and 5% *p*-cymene and in addition small quantities of other compounds (Biondi *et al*., 1993; Kokkini *et al*., 2004). Lambert (2001) was tested Turkish oregano against *Penicillium sp*. Moreover he found that among the tested spices only Turkish oregano significantly inhibited the fungal growth. In another study Akgül and Kivanc (1988) investigated the anti-fungal activity of Turkish oregano (*Origanum onites)* powder at concentrations of 1.0, 1.5, and 2.0 % (wt/v) and its essential oil at concentrations of 0.05 % and 0.025% (v/v). Their results showed significant inhibitory effect against the fungi *Aspergillus flavus* and *Aspergillus niger.*

Turkish oregano (*O. minutiflorum*) is characterized by its major components 68% carvacrol, 12%, *p*-cymene, 8%, y-terpinene, and 3% *β*-caryophyllene (Dadalioglu and Evrendilek, 2004). Dadalioglu and Evrendilek (2004) investigated the essential oil of Turkish oregano (*O. minutiflorum*), against *Listeria monocytogenes* count at concentration of 5, 10, 20, 30, 40, 50, and 80 µl/ml doses. Their results show signification reduction of the initial count from 7.23 log cfu/mL to 4.00, 3.45, 3.54, 2.41, 0.23, 0.12 and 0.00 log cfu/ml, respectively. In another study, Dadaliglu (2004) investigated the essential oil of the Turkish oregano (*O. minutiflorum*) at concentration of 5 µl/ml against *Salmonella typhimurium.* Furthermore*,* they concluded that the count levels of 6.95 log cfu/mL were reduced to 2.90 log cfu/mL.

Spanish oregano (*Thymus capitatus*), is belong to the genus thymus, which is a member of the same botanically family *Lamiaceae.* The main components of the Spanish oregano essential oil were carvacrol (62–83%), *p*-cymene (5 –17%), y-terpinene (2–14%) and β-caryophyllene (1– 4%) (Bounatirou *et al*., 2007).

Generally, the essentials oils of most *Origanum* genus members found in the market contain (93 – 97%) monoterpenes and 3% sesquiterpenes. It can be stated that around 66 to 86% of the monoterpenes are monoterpenoids (oxygen-containing terpenes) (Bounatirou *et al*., 2007).

The essential oil of tested extracts of common oregano (*Origanum vulgare* L. ssp. *vulgare*) is characterized mainly by carvacrol and thymol. There are different chemo-types of common oregano: on one hand a carvacrol rich type (82% carvacrol found in Croatia) and on the other hand a thymol rich type (75% thymol found in Italy and Israel). Additionally, limonene, *p*-cymene, α- and β-pinene, are found in lower

quantities (Pascual *et al*., 2001, Figuérédo *et al*., 2006, Azizi *et al*., 2009, Grevsen *et al*., 2009).

The impact of the essential oils of different *Origanum vulgare* subspecies and chemo-types on methanogenes can be due to one of the major components or to the synergetic action (or interaction) of all components of the plant. Furthermore it should be considered that plants belonging to the family *Lamiaceae* such as oregano contain phenolic compounds with varied structure and large size of molecules. The main phenolic compounds detected in *Lamiaceae* are phenolic acids such as rosmarinic acid, caffeic acid and salvianolic acid (Zheng and Wang, 2001, Chatzopoulou *et al*, 2010). These phenolic acids are characterized by several phenolic rings and hydroxyl groups resulting in high antioxidative capacity of the crop. Due to the antimicrobial activity of phenolic acids found in oregano they may inhibit or modify the activity of microorganisms *(*Zheng and Wang, 2001, Mueller *et al.*, 2008).

The observations from the conducted experiments are comparable to the same remarks have been found in animal science noted by several authors. In some investigations the inhibition effect of ruminal methanogenesis by addition of individual or blends of essential oils was found. For example Patra (2012) reported that Spanish oregano (*Thymus capitatus*) essential oil significantly reduced the methane that produced in an in vitro cultivation. He attributed that to the presence of the content of carvacrol, which is characterized as a phenolic monoterpene. Furthermore the phenolic nature of Spanish oregano (86% carvacrol) might explain its high potency in inhibiting methanogens involved in the methane production. The outcomes of our study were supported by some earlier results of methanogenesis inhibition in the rumens of the ruminant animals.

The results of the conducted study were consistent with in vitro results of some studies. Hossein Jahani-Azizabadi (2011) used an in vitro rumen microbial fermentation in batch tests. He introduced 50 ml of buffered rumen fluid (1:2; rumen fluid: buffersolution) in 125 ml serum bottles containing 500 mg of 80:20 alfalfa hay to concentrate as basal diet and added 1 µ/ml of *Origanum vulgare* essential oil. They concluded that essential oil of oregano (*Origanum vulgare*) caused a significant decrease of total methane gas production.

Tekippe *et al*. (2011) fed eight primiparous and multiparous Holstein lactating cattle (6 of which were ruminally cannulated) in a crossover design trial with two 21-day periods. Cattle were fed once daily. The oregano material was top-dressed and mixed with a portion of the total mixed ration. He gave each cow 500 g/day of oregano (*Origanum vulgare*) leaves for 3 weeks and he found that methane gas produced by the stomach of the cow was reduced by 40% after 8 hours from feeding. The results of this conducted study are consistent with those of Machebeuf *et al*. (2008), who used an in vitro 16 h at 39°C incubation batch ruminal cultures to determine the effect of two chemotypes of *Origanum vulgare* (extracts contains 890g/kg carvacrol and 50g/kg thymol) at doses 3 and 5 mM, and the other thymol chemotype of *O. vulgare* (extract contains 210 g/kg carvacrol and 350 g/kg thymol) at doses 2 and 3 mM on enteric methane production. Furthermore, he recorded 63% and 97% inhibition respectively for the first chemotype and 60% and 95% respectively for the second chemotype of *Origanum vulgare*.

In another study Chaves *et al*. (2008a) conducted an in vitro experiment to evaluate the effect of *p*-cymene which is a monoterpene found in *Origanum vulgare*. Furthermore, he used 20 mg per liter, 99% pure *p*-cymene and incubated it for 6 h with 10 ml of ruminal content in phosphate buffered media at 39°C. He found that methane gas production was reduced by 30% and he attributed this effect to the decrease of methanogenic activity of ruminal *Archaea*. A further investigation was done by Forgács (2012) who investigated the effect of limonene (found in big quantity in citrus peel but also found in small amount in *O. vulgare*), added to the organic municipal solid waste in 5 l continuously stirred reactor for 21 days. He found that the methane production started decreasing after 15 days of anaerobic digestion operation. Busquet *et al*.(2006) noted that addition of carvacrol, and oregano oil (at 3mg/ml) reduced total volatile fatty acids (VFA) and increased the pH value after 24 h of fermentation. Similarly, Castillejos *et al*. (2006) reported that thymol at concentration of 5 mg/ml increased the final pH value and decreased the total volatile fatty acids (VFA) concentration in 24 h *in vitro* batch culture. In another research study Macheboeuf (2008) reported that thymol at in vitro tests caused a suppression of CH_4 to the extent of 99% at 6 mM whereas carvacrol reduced CH_4 production by 98.4% at 5 mM. The increase in pH value is an indication of a reduction in the total volatile fatty acids (VFA) concentration or a high liberation of carbonate (HCO_3) ions from the buffer solution.

The mechanisms by which *Origanum vulgare* and the chemical constituents of its essential oil are affecting methanogens are not completely known. But there are some suggestions and explanations, which determine the way by which methanogens are affected by *Origanum vulgare.* Bodas *et al*. (2012) reported that carvacrol acts as the trans-membrane carrier for the monovalent cations and exchanging its hydroxyl proton by cations such as K^+, and these resulting in reducing the energy ATPs, which cause the cell death. Further on he reported that it can reduce the peptidolytic activity of the rumen bacteria. Another suggestion is reported by Helander *et al*. (1998) who attributed the inhibitory effect to the lipophilic character of the active principles. Helander *et al*. (1998) emphasized that compounds with lipophilic character permeate the cell membranes of the microorganisms. This mechanism results in the inhibition of the membrane bound electron to flow and there with the energy metabolism. This leads to a collapse of the proton pump and draining of the ATP pool. Moreover high concentrations of essential oils may lead to lysis of the cell membranes and denaturation of cytoplasmic proteins (Helander *et al*., 1998). Ultee *et al*. (1999) reported that carvacrol, found in Spanish oregano (*Thymus capitatus*) essential oil can been considered as a biocidal, which results inbacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death.

5.1.2. Effect of carvacrol

Carvacrol is a phenolic monoterpene normally found in high quantities in the essential oil of common oregano (*O. vulgare ssp. vulgare)* (Biondi *et al*., 1993, Kokkini *et al*., 2004, Azizi *et al*., 2009) and *Thymus vulgaris* (Cosentino *et al*., 1999). In the conducted study it was found that the compound carvacrol was inhibitory compared to the tested oregano extract. These results suggest that carvacrol is the main active compound with antimicrobial character in oregano essential oil. This observation is in agree with a previous study of Macheboeuf *et al*. (2008). In his investigation the toxicity of carvacrol to methanogenic activity in anaerobic pure culture was evaluated in standardized assays.

In the conducted study the addition of 0.1 ml (98 mg) and 0.3 ml (294 mg) of pure carvacrol to pure cultures of *M. barkeri* caused reduction in methane about 59.5% and 68.3%, respectively. These results indicate that carvacrol has a negative effect on the activity of the microbes to produce methane. Macheboeuf *et al*. (2008)

reported that concentrations of 1.5, 2.0, 3.0 and 5.0 mM of carvacrol which was incubated for 16 h in an in vitro batch ruminal culture, the methane was reduced by about 13%, 32%, 85% and 98% respectively. Moreover he noticed that the acetate-propionate proportion ratio was decreased too. Although when the acetate and propionate ratio reduced this suggests that methanogens shall not find enough acetate to convert it to methane. Macheboeuf (2008) in another study observed a linear decrease of methane production when carvacrol was supplied at 225 mg/l, 300 mg/l, 450 mg/l and 750 mg/l in batch culture. Moreover it resulted in a reduction in methane about 13%, 32%, 85% and 98% respectively. Contradictory, in the same study methane production was not affected by addition of *O. vulgare* essential oil (890 g/kg carvacrol and 50 g/kg thymol) at concentration of 150 mg/l. While at 450 mg/l and 750 mg/l methane was markedly inhibited by about 63% and 97% respectively. Benchaar *et al*. (2011) observed that inhibition of methanogensis with common oregano and its main component carvacrol occurred concomitantly with a reduction in acetate, propionate and total volatile fatty acids (VFA) concentration.

In another study conducted by Ultee *et al*. (1998) the effect of 0.75 mmol per liter of carvacrol on food borne pathogens *Bacillus cereus* at 30°C in brain heart infusion media was investigated. He concluded that the growth of the *Bacillus cereus* was totally inhibited. Additionally, the effect of thymol and carvacrol on bacteria was analyzed by Sivropoulou *et al*. (1996) in vitro test. He investigated the effect of three oregano essential oils, such as *Origanum vulgare* ssp. *hirtum*, *Origanum dictamnus*, and commercially available oregano oil on eight strains of gram-positive and gram-negative bacteria. It was found that all three essential oils exhibited high levels of antimicrobial activity. Furthermore, among the major components of the three oils, carvacrol and thymol exhibited the highest levels of antimicrobial activity. In the same study he observed that the *y*-terpinene and *p*-cymene were inactive.

Another study was carried out by Paster *et al*. (1995). He investigated the effect of oregano oil for 24 h as fumigants against the mycelia and spores of *Aspergillus ochraceus*, *Aspergillus flavus* and *Aspergillus niger*. He found that 2.0 µl/l was the minimum concentration inhibiting the growth of the mycelia of the fungi, while concentrations of 2.0 to 2.5 µl/l were eradicating spores.

Results from this research study revealed that the phenolics (i.e, carvacrol) exhibited clearly antimicrobial activities in vitro. Phenolic compounds such as carvacrol, have

been shown to have high antimicrobial activity attributed to the presence of a hydroxyl group in the phenolic structure (Burt, 2004). Compounds with phenolic structures have a broad spectrum of activity against a variety of both gram-positive and gram-negative bacteria (Lambert *et al*., 2001). The mechanism by which phenolic compounds are thought to exert their antimicrobial activity is through the disturbance of the cytoplasmic membrane (Burt, 2004). Helander *et al*. (1998) showed that carvacrol decreased the intracellular ATP pool and increased the extracellular ATP concentration of *Esherichia coli*. Carvacrol has been considered biocidal agent, which resulting in a bacterial membrane perturbations that led to leakage of intracellular ATP and potassium ions and ultimately cell death (Ultee *et al*., 2000). The addition of carvacrol (400 mg l^{-1}) in vitro resulted in an increase of pH value. Recently, Busquet *et al*. (2006) investigated the effects of the phenolic compound carvacrol at concentrations of 3, 30, 300, and 3000 mg/ L in 24-h in vitro batch culture incubations. In his study, the final pH value and the molar proportion of butyrate were increased, and the molar proportion of propionate was decreased when this phenolic compound was supplied at high concentrations (i.e., 300 mg/ l for carvacrol).

Stewart *et al*. (1991) reported that in the rumen, gram-positive bacteria are generally acetate- and butyrate-producing bacteria, while gram-negative bacteria are generally propionate-producing bacteria. Burt (2004) suggested that gram-positive bacteria appear to be more susceptible to the antibacterial properties of oregano essential oil than gram-negative bacteria. However, Helander *et al*. (1998) reported that two phenolic compounds, thymol and caravacrol, also inhibited the growth of gram-negative bacteria by disrupting the outer cell membrane.

5.1.3. Effect of thymol

Thymol is a phenolic monoterpene found in the essential oils of common oregano and thyme. Carvacrol is a naturally occuring isomer of thymol. Thymol has been shown to have a broad spectrum of activity against a variety of gram-positive and gram-negative bacteria (Lambert *et al*., 2001; Walsh *et al*., 2003).

The novelty of the present study is to investigate the effect of pure thymol on the methanogens that grown in an anaerobic artificial media at mesophilic temperature condition. Several studies have investigated the effect of thymol or of essential oil with high thymol content on rumen fermentation (Benchaar *et al*., 2007; Castillejos *et*

al., 2008): The treatments of 0.1 ml (equal 86.4 mg), and 0.3 ml (equal 259.2 mg) of pure thymol used in the own conducted study caused around 61% and 75% inhibition of methane production respectively (Fig.15) compared to the methane gas produced by the control.

Chaves *et al*. (2012) investigated in an in vitro ruminal batch culture that was incubated for 6, 12 and 24 h in serum bottles. He observed a slight reduction in the methane production in the three different treatments. Furthermore Macheboeuf *et al*. (2008) found a reduction of methane about 84% and 99% in an in vitro batch test incubation for 16 h and at concentration of 3 mM and 6 mM respectively. Evens and Martin (2000) examined the effects of increasing concentrations of thymol. The authors analyzed concentrations of 50 mg/l, 100 mg/l, 200 mg/l and 400 mg/l of culture fluid on in vitro fermentation as incubated for 24h by mixed rumen bacteria in batch culture system. Moreover, methane concentration was not affected, when thymol was supplied at concentrations of 50 mg/l, 100 mg/l, and 200 mg/l of fluid culture. However, at 400 mg/l methane concentration was drastically decreased to 94%. Furthermore, they observed that the pH value of the medium was increased along with the reduction of acetate and propionate concentration by 44% and 78% respectively. Higher pH value and a reduction in the total volatile fatty acids (VFA) are an indication of an overall inhibition of rumen microbial fermentation (Benchaar *et al* 2011). Benchaar *et al*. (2007) reported that concentration of 500 mg/l thymol increased the pH value of the media in vitro. In another research study from Macheboeuf *et al*. (2008) was observed that essential oil from *Origanum vulgare* and its component, thymol caused a suppression of the methane to an extend of 99% at a concentration of 6mM.

In the study of Castillejos *et al*. (2006), they were observed that a concentration of 500 mg/l of thymol increased the final pH value in incubation for 24h in vitro batch culture. However, Helander*et al*. (1998) reported that two phenolic compounds, thymol and caravacrol, also inhibited the growth of gram-negative bacteria by disrupting the outer cell membrane.

5.1.4. Effect of the mixture of carvacrol and thymol

Both Carvacrol and thymol compounds have strong antimicrobial activity against wide range of bacteria as mentioned, due to the presence of the hydroxyl group in their chemical structures. The mixture of carvacrol and thymol induced significantly

higher toxicity to the methanogenic microbe *Methanosarcina barkeri* than each individual of carvacrol or thymol. In the conducted experiments the treatments 0.1 ml and 0.3 ml of the mixture caused reductions of methane about 70.5% and 83.8% respectively. Benchaar *et al*. (2007) reported that the phenolic monoterpenes carvacrol and thymol were exhibited broad spectrum of antimicrobial activity in vitro. It is visible that carvacrol and thymol work together as synergist. Moreover the volume of the methane in all microbiological investigations decreases as the concentration of the inhibitors doses increases (i.e. it is dose response).

5.2. Effect of Mesophilic laboratory digestions

5.2.1. Effects of oregano leaves

The results from the conducted experiments confirm that oregano at doses of 250 g and 500 g significantly reduced the total biogas and methane production by the methanogenic *Archaea*. But at lower oregano concentrations of 62.5 g and 125 g the biogas and methane production were not affected. These findings suggest that the effect of oregano on biogas production is a dose response dependent action. The activity of oregano essential oil would be expected to relate to the respective composition of the plant essential oils, the structural configuration of the constituent components of the volatileoils and their functional groups and possible synergistic interactions between components.(Dorman and Deans, 2000, Patra and Yu, 2012).

In general, compounds with phenolic structure such as thymol and carvacrol, are more affective as antimicrobials in comparison to the other non-phenolic structural compounds. The importance of the hydroxyl group in the phenolic carvacrol itself is not essential for the antimicrobial activity. But it indicates special features in the antimicrobial mode of action of carvacrol due to the hydroxyl group(Veldhuizen *et al*., 2006). The importance of the hydroxyl group in the phenolic structure was set in terms of activity when carvacrol was compared to its methyl ether, which is less active than carvacrol (Dorman and Deans, 2000). Furthermore, the relative position of the hydroxyl group exerted an influence upon the components effectiveness (Dorman and Deans, 2000). Evans and Martin (2000) observed that when thymol at 400 µg/ml was added to in vitro incubation with ruminal fluid it is significantly reduced the concentration of ruminal methane, along with a reduction in acetate and propionate concentration.

Skandamis and Nychas (2000) inoculated eggplant salad with *Escherichia coli* supplemented with different concentrations of *Origanum vulgare* essential oil in agar media for overnight at 37°C. He observed that the addition of oregano essential oil increased the death rate of *E. coli*.

According to Pannzi *et al*. (1993) and also to Helander *et al*. (1998) the antimicrobial activity of the essential oil of oregano plants is attributed to the terpenes and phenolic compounds. As well as the type and the number of the functional groups that contained in the essential oil. *Origanum vulgare* contains in addition to thymol and carvacrol other mono and sesquiterpenes such as myrcene, α-terpinen, β-pinene, α-pinene, *p*-cymene limonene γ-terpinen and γ-cadinene (Pascual *et al*., 2001, Figuérédo *et al*., 2006, Grevsen *et al*., 2009, Azizi *et al*., 2009).

The reduction of biogas and methane by *Origanum vulgare* treatments are extending those findings of Patra and Yu (2012), who tested the essential oil of *Thymus capitatus* L.Hoffmanns and Link in vitro tests at 3 different doses of 0.25 g, 0.50 g, and 1.0 g/liter for their effect on methane production, fermentation, and selected groups of ruminal microbes, including total bacteria, cellulolytic bacteria, *Archaea*, and protozoa. The essential oil significantly reduced the methane production with increasing doses. With reductions of 87%, for oregano oil, at dose of 1.0 g/l in contrast to the control. The concentrations of total volatile fatty acids (VFA) were altered linearly by the oregano essential oil. The oregano essential oil also altered the molar proportions of acetate, propionate, and butyrate. Furthermore, the oregano essential oil decreased the abundance of *archaea*, protozoa, and major cellulolytic bacteria (i.e., *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *R. albus*) linearly with increasing essential oil doses.

The results of the conducted experiments are agree with the findings of Macheboeuf *et al*. (2008), who conducted an in vitro batch incubation of ruminal culture for 16 h incubation using *Origanum vulgare* (890 g/kg carvacrol, 50 g/kg thymol) and (210 g/kg carvacrol, 350 g/kg thymol). He observed that methane is reduced by 97% and 95% respectively. In literature some studies in animal nutrition proved that when oregano used in animal feeding rations the total produced methane gas is significantly reduced. Tekippe *et al*. (2011) fed cattle with 500 g of common oregano (*Origanum vulgare*) leaves, for each cow per day for three weeks, and he found that the methane produced by the animal rumen was reduced by 40%. Contradictory

Chaves *et al*. (2012) investigated the effect of 120 mg of common oregano (*Oreganum vulgare*) per kg of whole barley silage plant in an in vitro batch ruminal culture. He observed no reduction in methane and no change in volatile fatty acids concentration (VFA). This suggests that the concentration was too low that the microorganisms can tolerate and degrade it.

Hristov *et al*. (2013) investigated the effects of dietary supplementation of *Origanum vulgare* L. leaf material on rumen fermentation, production, and milk fatty acid composition in dairy cattle. His experimental design was a replicated 4 × 4 Latin square with 8 rumen-cannulated Holstein cattle and 20-d experimental periods. Treatments were control (no oregano supplementation), 250 g/cattle per day, 500 g/d and 750 g/d oregano. They concluded that oregano supplementation had no effect on rumen pH value, and volatile fatty acid concentrations, but decreased ammonia concentration and linearly decreased methane production per unit of dry matter intake (DMI) compared with the unsupplemented control.

The reduction and inhibition of the total biogas and methane production noted in this conducted study are not due to the total solid, the total volatile solids, and the C/N ratio insufficiently or with the effect of lignin content, but it is due to the chemical constituents that found in the oregano plant. The mode of action of these chemical components is beyond this research study, but some theories in the literature explain the mode of action. For instance Helander *et al*. (1998) explained that the component of essential oil of oregano disrupt the cell membrane and decreasing the intracellular ATP pool and increasing the extracellular ATP pool. Another theory said that the addition of oregano plant to animal diets as a supplementary stuff increases the total volatile fatty acids (VFA) concentration and decreases the acetate propionate ratio (Benchaar *et al*., 2007). Thus increasing the volatile fatty acids in the media affect the activities of methanogens. Although, decreasing the acetate propionate ratio suggest that methanogens will not find enough substrates to convert them to methane. Other theory says that essential oil inhibits the protozoa and thus decreases the methane production because protozoan provide a habitat for some methanogens that live on and within them (Ando *et al*., 2003).

Generally, it can be concluded that the mode of actions of thymol and carvacrol can be characterized as follows:

- Carvacrol and thymol are membrane permeabilizer.
- Both compounds acting independently.
- Oregano essential oil creates membrane permeability problems for the microorganisms.
- Both compounds cause structural damage of cell membrane.

The physical and the chemical characteristics of the organic material for biogas production are very important information for designing and operating anaerobic digesters because they affect biogas production (Fisher *et al*., 1986 as cited by Zhang *et al*., 2007). The measured value of organic matter and dry organic matter of the used materials are in the range of the values that reported in the literature. Amon et al. (2007) reported that maize has more than 80% organic dry matter and it produced average of 337 Nl of methane per kg O.DM. Contradictory to that oregano used in this experiment has about 82% organic dry matter but produced methane ranged between zero and 105 and Nl per kg of O.DM. Macro and micro-nutrients were balanced for the biogas microorganisms. (Ca, K, Mg, P, Na). A balanced availability of nutrients for the growth of the microorganisms in biogas digesters is important for the process performance, i.e. stability and substrate utilization (Takashima and Speece, 1989). The availability of certain trace elements has also been shown to strongly impact the biogas production. Trace elements known to be crucial for the activity of enzymes in methanogenic systems are cobalt (Co), nickel (Ni), iron (Fe), zinc (Zn), molybdenum (Mo) and/or tungsten (W) (Takashima and Speece, 1989). The C/N ratio of the *Origanum vulgare* was 23.3. Where as the optimum ratio of C/N in literature is between 15 and 30 (Weiland, 2010).

5.2.2. Effects of fennel seeds

Foeniculum vulgare possess strong antimicrobial activities against wide range of microbes (Bakkali *et al*., 2008). *Foeniculum vulgare* essential oil contains mainly trans-anethol, limonene, estragol and fenchone.

Trans-anethole is a flavoring agent present in the essential oils of anise, fennel and star anise. Chemically it is an alkenylbenzene identified as (1-methoxy-4-(1-propenyl) benzene or para-propenylanisole, (Cosentino *et al*., 1999; Khan and Abourashed, 2011). *trans*-Anethole-containing oils are widely used in the food and liquor industries (Newberne *et al*., 1999).

Estragole (*p*-allylanisole, methyl chavicol) is a phenylpropane, found in essential oils of basil (*Ocimum basilicum*), bay leaves (*Laurus nobilis*,), fennel and anise. Its chemical structure consists of a benzene ring substituted with a methoxy group and a propenyl group. It is an isomer of anethole, differing with respect to the location of the double bond. It is used as an additive and flavouring agent. Estragol has antimicrobial activity towards *Shigella* sp.(Bagamboula *et al*., 2004).

Fenchone is a ketonic monoterpene, a colorless oily liquid, a constituent of the essential oils of fennel and lavender (*Lavandula angustifolia*). Fenchone is used as a flavor in foods and in perfumery and exhibited a very strong antimicrobial activity against *Staphylococcus aureus* (Dadalioglu and Evrendilek, 2004, Bouzouita *et al*., 2005; Karakaya *et al*., 2011).

D-limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a monocyclic monoterpene with a lemon-like odor and is a major constituent in several citrus oils (orange, lemon, mandarin, lime and grape fruit) and in less quantities in oregano and fennel. Because of its pleasant citrus fragrance, d-limonene is widely used as a flavor and fragrance additive in perfumes, soaps, foods, chewing gum, and beverages. Limonene showed antimicrobial activities against *Micrococcus luteus,Streptococcus epidermidis, S. aureus* and *Salmonella typhimurium* (Dorman and Deans, 2000; Ran\vcić *et al*., 2003)

It was found that trans-anethole and fenchone each has strong antimicrobial activities against *Aspergillus versicolor* and *Aspergillus flavus* at minimum inhibition concentration of 7.0 to 15.0 µl/ml and 1.3 to 2.2 µl/ml respectively (Bakkali *et al*., 2008). Anethole has high antimicrobial activity which related to the ether group on its aromatic ring (Davidson and Naidu, 2010). Furthermore *F. vulgare* has antimicrobial activities against two mycopathogenic species (Lo Cantore *et al*., 2004). The antimethanogenic activity of *F. vulgare* is being attributed to the chemical constituents of its essential oil. However Chaves *et al*. (2007) observed the effect of 20 mg/l trans-anethole in an in vitro incubation for 6 h with batch ruminal culture in Bellco tubes. Moreover he observed that methane was reduced by 13.3%. Also Patra *et al*. (2010) reported the effect of methanol and ethanol extracts of fennel incubated for 24 h in an in vitro batch ruminal culture. He found that methane was reduced up to 61%. The mechanism by which essential oil of fennel effect methanogens is unknown. But Hook *et al*. (2010) attributed that from animal nutritional and

microbiological point of view to the antimicrobial activity and the reduction of hydrogen availability in the ruminal stomach of the animal. Martin *et al*. (2010) reported that to produce biogas from citrus peel and citrus waste, itis essential to extract D-limonene from the peel for anaerobic digestion processes to take place. Because he observed that the samples without limonene extracted from, were significantly affected. Limonene, which is almost found in citrus peels and in small amount in fennel seeds, has negative effects on methanogens.

Ruberto *et al*. (2010) investigated the antibacterial activities of the essential oil of fennel against 25 different genera of bacteria including animal and plant pathogens, food poisoning and spoilage bacteria. He concluded that fennel showed a higher degree of inhibition compared to the blank samples. Furthermore, in another study by Gulfraz *et al*. (2010) fennel oil showed inhibition against *Bacillus cereus*, *Bacillus magaterium*, *Bacillus pumilus*, *Bacillus substilis*, *Eschericha coli*, *Klebsiella pneumonia*, *Micrococcus lutus, Pseudomonos pupida*, *Pseudomonos syringae*, and *Candida albicans.*

In general, *Foeniculum vulgar* contains monoterpenes and phenylpropanes. In the conducted experiments, it was found virtually that the biogas produced by *Foeniculum vulgare* was significantly reduced when compared with the control especially in the treatments with 200 g and 400 g.

In addition, the inhibition and the reduction of total biogas and methane gas production noted in this conducted experiment cannot be attributed to the total solid, the total volatile solids, the C/N ratio or to the effect of lignin content (Chen *et al*., 2008). But it is attributed to the chemical constituents of the fennel.

Amon *et al*. (2007) reported that maize has more than 80% organic dry matter and it produced average of 337 Nl of methane per kg O.DM. Contradictory to that fennel used in this experiment has about 79% organic dry matter. But it produced methane ranged between 155 and 20 Nl per kg of O.DM. Macro and micro-nutrients were balanced for the biogas microorganisms. The C/N ratio of the *Foeniculum vulgare* was about 18. Where as the optimum ratio of C/N in the literature is between 15 and 30 (Weiland, 2010). Also, the contents of the various nutrient elements in the tested material are shown. Macro and micro-nutrients were balanced for the microorganisms that produce biogas.

5.2.3. Effects of hop cones

Hop cones are rich in secondary metabolites classified as resinous bitter acids (α- and β-acids), essential oils (α-humulene, β-caryophyllene, myrcene and the linalool, geraniol) (Bernotienë *et al*., 2004). Furthermore, a wide range of phenolic acids, and flavonoid glycosides are found (Moir, 2000; Van Cleemput *et al*., 2009). All of these compounds possess antimicrobial activities against wide range of microbes (Siragusa *et al*., 2008). Although α- and ß-acids seem to account for the majority with β-acids having greater antimicrobial activity than the α-acids (Narvaez *et al*., 2011). Therefore, results obtained from this research study represent the combined effects of all these bioactive compounds. In the conducted study it was observed that all treatments of hop cones added to the mixture of cattle liquid manure and the standard cellulose produced neither biogas nor methane. Theoretically the greatest rate of biogas suppose to be produced from the high concentrations of hop cones (400 g) because it contains approximately 400 g/kg DM of fiber (cellulose, hemicellulose and lignin), 50.6% total carbon, nitrogen 2.5%, fat 9.2 and 15.6% of protein. Also it contains 21.4 mg/g of K^{+1}, 4.19 mg/g P^{+3}, Mg^{+2} 2.6 mg/g and Ca^{+2} 41.5 mg/g. But unfortunately as seen in the figures 19 and 20 there is neither biogas nor methane produced in all hops treatments. The explanation for these results can be that hop cones might contain compounds that specially inhibit the methane production in the digester. Narvaez *et al*. (2011) reported that hop cones in ruminant animals when added to the diet rations as alternative supplements for antibiotics (now was banned to be used in Europe) it reduced the methane gas production and increased the volatile fatty acid (VFA) production add to that it reduce the acetate propionate ratio. Again as mentioned increasing the volatile fatty acids leads to the inhibition of the methanogens. Additionally decreasing the acetate propionate ratio limit the substrates for the aceticlastic methanogen thus it suffer to survive. Narvaez *et al*. (2012) reported that the addition of three hop varieties (Cascade, Millennium and Teamaker) powder to an artificial rumen system (rusitec). Furthermore he reported that the relative abundance of 16S rRNA copies associated with methanogens was significantly reduced by Cascade and Millennium compared with the control. Furthermore, the extracts of hop cones cause an increase in the propionate to acetate ratio. Narvaez *et al*. (2012) reported that all varieties of hops he tested in an in vitro batch incubation ruminal culture reduced the methane gas by 20 to 21%. Moreover he reported that addition of 800 mg/l was changed the number of

ruminal microbes and reduced the total gas and that is correlated to the inhibition of methanogens. Mc Allister *et al*. (2008) reported two theories by which hops reduce the methane production in the ruminant animals the first theory is the direct inhibition of methanogens with necessary reduction of the H_2 into other alternative products (as increasing volatile fatty acids). The second theory is reducing the supply of metabolic H_2 to methanogens (the methane gas is formed from H_2 and CO_2 in the stomach of ruminant animals).

The purpose of using hops cone mixed with cattle liquid manure as a co-substrate in this research study was to define the effects of hops on total biogas and methane production in a mesophilic laboratory digester. We consider the comparing effects of hops biogas and methane is perhaps more meaningful in the real world for farmers of hops who use the rest and waste of it after harvesting.

All these results suggest that hops exert a greater favorable effect on biogas and methane microorganism producers compared to those of control and reference substances and characterized as toxic material for biogas producer microbes.

It needs to be pointed out that although chemical analysis of the essential oil of hop cones was not performed in this conducted research study. But the chemical composition of the essential oil of hop cones was taken from the literature.

Medicinal plants characterized by the presence of chemical components, which have high antimicrobial effect. After the banning of antibiotic usage in animal nutrition in European countries (regulation 1831/2003/EC) animal nutritionist and microbiologists benefit from the medicinal plants (Castanon, 2007).

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6 Conclusions

This dissertation has investigated in mesophilic conditions the effect of plant containing secondary metabolites (oregano herb, fennel fruits and hop cones) mainly terpenes on the biogas and methane production via anaerobic digestion.

Generally, from this present study it can be concluded that: Methanol extracts of oregano leaves in high doses and the active components of the essential oil at different levels inhibit both biogas and methane content produced by *Methanosarcina barkeri* or in the biodigester. Fennel has adverse effect on biogas and methane production at different levels. But hop cones have the worst effect on both biogas and methane production compare with the blank samples. The findings of this study suggest that the effect increased with the increasing dose of the added material and vice versa. Furthermore, this effect cannot be attributed only to the presence of terpenes in those plants, but may be other secondary compounds found in these plants have negative effect too The effect of oregano, fennel and hop cones that we have identified therefore assists in our understanding of the role of the plant secondary metabolites in biogas production. Although the current study is based on few samples of medicinal plants, the findings suggest that more plants needed for further study to evaluate the effect of other secondary metabolites in the three plants in order to determine the optimal level of active components and their effect on biogas microbes. In general, it can be concluded that medicinal herbs, their essential oils and components have the potential to be consider as alternative to antibiotic and growth promoters after the inhibition of antibiotics usage in animal nutrition in European countries.

The findings in this study are subjected to at least three limitations. First, due to limited resources only one type of methanogens was tested. Second because of limited resources only oregano extract and two active main components of its essential oil carvacrol and thymol were investigated. Third, this effect cannot only attributed to terpenes, may be the other secondary metabolites contained in the three plants exert negative effect on biogas microbes too.

Nevertheless, there still further studies in the future to investigate and evaluate the effect of the other secondary metabolites on biogas production. In addition,

laboratory work to analyze the volatile fatty acids, pH value and proportion of acetate to propionate in the biodigester.

7 Summary

A high demand for agricultural biomass production in Germany was attributed to the increasing number of biogas plants every year. The value of a crop as a substrate for biogas production via anaerobic digestion depends on its biomass yield capacity compared to the effort for cultivation and on its ability to produce biogas with high methane content.

After the EEG 2012 amendment which determined the amount of maize that should be used in biogas production farmers searching for alternative substrates for biogas production. These alternatives can be cereal crops, vegetables, house hold wastes, grasses or farm residuals. Few farmers grow spices as marginal crops. The rests and residues of spices after harvesting can be used in the biogas plants. These spices contain secondary metabolites such as terpenes, flavenoids and other phenolic compounds. In the literature not enough studies can be found that focused on the effect of plant secondary metabolites on biogas and methane production through anaerobic digestion.

The influence of plant secondary metabolites mainly terpenes in oregano leaves, fennel seeds and hop cones on biogas and methane production was investigated in this study.

Two types of experiments were conducted. First two in vitro experiments were carried out using *Methanosarcina barker* as model for methanogens. It was cultivated in artificial bicarbonate buffered and sulfide reduced mineral media supplied with vitamins and trace minerals in serum bottles at mesophilic conditions. Oregano methanolic extract, carvacrol and thymol as main components of its essential oil were added at doses of 1.5 ml, 2.0 ml and 3.0 ml/50 ml of media. Carvacrol, thymol and their mixture were added at 0.1 and 0.3 ml/50 ml of media.

Essential oil of oregano contains mainly carvacrol and thymol, *p*-cymene, limonene and α- and β-pinene in small quantities. The essential oil of fennel contains mainly trans-anethol, fenchone and estragol. The essential oil of hops contains mono and sesquiterpenes in addition to resins, flavonoids, glycosides and phenolic acids.

It was found that the extracts of oregano at the concentration of 2.0 ml and 3.0 ml have clear negative effect on the activity of *Methanocarcina barkeri* to produce

methane. Nevertheless the dose of 1.5 ml of oregano extract has no negative effect. All doses of carvacrol, thymol and their mixture showed negative effect on the methane. An increase in concentration of each of tested material was associated with a decrease in the methane production.

In second type of experiments 46 parallel running 20 liter biogas digesters were fed with 15 kg cattle liquid manure 3-5% dry matter. The cattle liquid manure was brought from a second phase running biogas plant. Oregano was supplied at 62.5 g, 125 g, 250 g and 500 g/digester. Fennel seeds were added at 25 g, 50 g, 100 g, 200 g, and 400 g/digester. Hop cones were applied at 25 g, 50 g, 100 g, 200 g and 400 g/digester. In case of the inhibition test standard cellulose at 100 g/digester was mixed with the cattle liquid manure. Maize and standard cellulose were used as reference materials at concentration of 450 g and 100 g/digester respectively.

Oregano in batch digesters at 250 g (16.7 g/kg manure) and 500 g (33.3 g/kg of manure) doses showed negative effect on the biogas and methane production and no methane was produced by the 500 g (33.3 g/kg manure) dose. Contrary the doses 62.5 g and 125 g showed positive effect. Fennel seeds at doses 200 g (13.3 g/kg manure) and 400 g (26.7 g/kg manure) have clear negative effect on biogas and methane production. Neither methane nor biogas was produced by all hop cones concentrations.

The findings of the conducted study added valuable information about the inhibition of biogas by plant secondary metabolites found in oregano, fennel and hop cones.

Zusammenfassung

Die hohe Nachfrage nach landwirtschaftlicher Biomasse in Deutschland wird der wachsenden Zahl an Nutzpflanzen für die Biogasproduktion pro Jahr zugeschrieben. Die Wertigkeit der Kultur als Substrat für die Biogasproduktion mittels anaerober Gärung ist abhängig vom Ertrag im Vergleich zum Arbeitsaufwand der Kultivierung und seiner Eignung Biogas mit hohem Methangehalt zu produzieren.

Aufgrund der Änderung des EEG 2012 besteht ein verstärktes Interesse der Landwirte alternative Substrate für die Biogasproduktion zu finden, da der Einsatz von Mais limitiert wurde. Alternative Kulturen zur Herstellung von Biogas sind u.a. Getreide, Gemüse, Haushaltsabfälle, Gräser und Abfälle aus der Landwirtschaft. Die Überreste und Abfälle der landwirtschaftlichen Gewürzpflanzenproduktion können der Biogasproduktion zugeführt werden. Ebenfalls diese Gewürzpflanzen enthalten sogenannte sekundäre Pflanzeninhaltsstoffe wie Terpene, Flavonoide und andere phenolische Komponenten. In der Literatur existieren nicht ausreichend Studien, die den Effekt sekundärer Pflanzeninhaltstoffe auf die Biogas- und Methanproduktion mittels anaerober Gärung untersuchen.

Der Einfluss sekundärer Pflanzeninhaltstoffe, vorwiegend Terpene, aus Oregano-Blättern, Fenchelsamen und Hopfenzapfen auf die Biogas- und Methanproduktion wurden in dieser Arbeit untersucht.

Zwei verschiedene Experimente wurden durchgeführt worden. Als erstes wurden zwei *in vitro* Versuche mit *Methanosarcina barkeri* als Modell für Methanogene durchgeführt. Die Kultivierung wurde in künstlichen bikarbonatgepufferten und sulfitreduzierten Mineralmedium, angereichert mit Vitaminen und SL 10 Spurenelementen, in Serumflaschen unter mesophilen Bedingungen durchgeführt. Das methanolische Oreganoextrakt enthält als Hauptkomponenten des ätherischen Öls Thymol und Carvacrol. Die Extrakte wurden in folgenden Konzentrationen dem Medium zugeführt; 1,5 ml, 2,0 ml und 3,0 ml zu je 50 ml medium. Carvacrol, Thymol und deren Gemisch wurden bei 0,1 und 0,3 ml pro 50 ml Medium zugegeben.

Das ätherische Öl des Oregano enthält überwiegend Carvacrol und Thymol, sowie *p*- Cymen, Limonen und α- andβ-Pinen in Spuren. Das ätherische Öl des Fenchels enthält vorwiegend *trans*-Anethol, Fenchon und Estragol. Im ätherischen Öl des

Hopfens sind Mono- und Sesquiterpene, Harz, Flavonoide, Glykoside und Phenolsäuren enthalten.

Die Untersuchung von 2,0 ml und 3.0 ml Extrakt aus Oregano weist einen deutlich negativen Effekt auf die Methanproduktion von *Methanocarcina barkeri* auf. Die Dosis von 1,5 ml Oreganoextrakt weist keinen negativen Effekt auf die Methanproduktion auf. Carvacrol, Thymol und ihr Gemisch zeigen bei allen Konzentrationen einen negativen Effekt auf die Methanproduktion. Die Anhebung der Konzentrationen jedes getesteten Materials ist mit einer Abnahme der Methanproduktion assoziiert.

In einem zweiten Biogasexperiment sind 46 Batchbehälter (20Liter)mit 15 kg flüssigerRingergülle gefüllt worden. Die Rindergüllestammt aus einer zwei-phasigen Biogasanlage.Oregano ist in Dosen von 62,5 g, 125 g. 250 g und 500 g pro Fermenter hinzugefügt worden. Fenchelsamen sind mit je 25 g, 50 g, 100 g, 200 g und 400 g pro Fermenter zugegeben worden. Hopfenzapfen sind mit 25 g, 50 g, 100 g, 200 g und 400 g pro Fermenter zugegeben worden. Für den Fall einer Enzymhemmung ist standardmäßig 100 g Zellulose beigegeben worden. Mais und Zellulose sind als Referenzsubstanzen (450 g und 100 g pro Fermenter) genutzt worden.

Die Verwendung von 250 g (16.7 g/kg Rindergülle) und 500g (33.3 g/kg of Rindergülle) Oregano weist einen negativen Effekt auf die Biogas- und Methanproduktion auf. Bei 500 g wird kein Methan mehr produziert. Im Gegensatz dazu führen Konzentrationen von 62,5 g und 125 g zu einem verstärkenden Effekt auf die Biogas- und Methanproduktion. Die Zugabe von 200 g (13.3 g/kg Rindergülle) und 400 g (26.7 g/kg Rindergülle) Fenchelsamen resultiert in einem deutlich negativen Effekt auf die Biogas- und Methanproduktion. Weder Methan noch Biogas konnte von den Hopfenzapfen produziert werden.

Die Ergebnisse dieser Arbeit geben wertvolle Informationen zur Inhibierung der Biogasproduktion durch sekundäre Pflanzeninhaltsstoffe, wie sie in Oregano, Fenchel und Hopfen ermittelt werden.

References

Abad, M. J., Bermejo, P., Palomino, S. S., Carrasco, L. and Chiriboga, X. (1999). Antiviral activity of some South American medicinal plants. Phytotherapy Research, 13(2), 142–146.

Abdallah, N., El-Gengaihi, S and Sedrak, E. (1978). The effect of fertilizer treatments on yield of seed and volatile oil of fennel (*Foeniculum vulgare Mill*.). Die Pharmazie, 33(9), 607.

Adam, K., Sivropoulou, A., Kokkini, S., Lanaras, T and Arsenakis, M. (1998). Antifungal activities of *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa* Essential oils against human pathogenic fungi. Journal of Agricultural and Food Chemistry, 46(5), 1739–1745.

Ahmadi, L., Mirza, M and Shahmir, F. (2002). The volatile constituents of *Artemisia marschaliana Sprengel* and its secretory elements. Flavour and Fragrance Journal, 17(2), 141–143.

Ahring, B. (2003). Perspectives for anaerobic digestion. Advances in Biochemical Engineering/Biotechnology, 81, 1–30.

Akassou, M., Kaanane, A., Crolla, A. and Kinsley, C. (2010). Statistical modelling of the impact of some polyphenols on the efficiency of anaerobic digestion and the co-digestion of the wine distillery waste water with dairy cattle manure and cheese whey. Water Science and Technology: a Journal of the International Association on Water Pollution Research, 62(3), 475–483.

Akgül, A and Kivanc, M. (1988). Inhibitory effects of selected Turkish spices and oregano components on some foodborne fungi. International Journal of Food Microbiology, 6(3), 263–268.

Albers, S.-V and Meyer, B. H. (2011). The archaeal cell envelope. Nature Reviews Microbiology, 9(6), 414–426.

Aligiannis, N., Kalpoutzakis, E., Mitaku, S. and Chinou, I. B. (2001). Composition and antimicrobial activity of the essential oils of two *Origanum* Species. Journal of Agricultural and Food Chemistry, 49(9), 4168–4170.

Amon, T., Amon, B., Kryvoruchko, V., Zollitsch, W., Mayer, K. and Gruber, L. (2007). Biogas production from maize and dairy cattle manure: Influence of biomass composition on the methane yield. Agriculture, Ecosystems and Environment, 118(1), 173–182.

Ando, S., Nishida, T., Ishida, M., Hosoda, K. and Bayaru, E. (2003). Effect of peppermint feeding on the digestibility, ruminal fermentation and protozoa. Livestock Production Science, 82(2), 245–248.

Angel, R., Conrad, R., 2013. Elucidating the microbial resuscitation cascade in biological soil crusts following a simulated rain event. Environmental Microbiolology. vol 15 (10): 2799-2815

Antoni, D., Zverlov, V.V., Schwarz, W.H., 2007. Biofuels from microbes. Applied Microbiolology and Biotechnolgy, 77(1): 23–35.

Arsova, L., 2010. Anaerobic digestion of food waste: Current status, problems and an alternative product. Columbia University.

Aschenbach, K., Conrad, R., Rehakova, K., Dolezal, J., Janatkova, K., Angel, R., 2013. Methanogens at the top of the world: occurrence and potential activity of methanogens in newly deglaciated soils in high-altitude cold deserts in the Western Himalayas. Frontiers in. Microbiolology . 4:358

Azizi, A., Yan, F and Honermeier, B. (2009). Herbage yield, essential oil content and composition of three oregano (*Origanum vulgare* L.) populations as affected by soil moisture regimes and nitrogen supply. Industrial Crops and Products, 29 (2–3), 554–561.

Bagamboula, C. F., Uyttendaele, M. and Debevere, J. (2004). Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and *p*-cymene. Food microbiology, 21(1), 33–42.Bagamboula, C. F., Uyttendaele, M. and Debevere, J. (2004). Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and *p*-cymene. Food microbiology, 21(1), 33–42.

Bakkali, F., Averbeck, S., Averbeck, D and Idaomar, M. (2008). Biological effects of essential oils–a review. Food and Chemical Toxicology, 46 (2), 446–475.

Baratta, M. T., Dorman, H. J., Deans, S. G., Figueiredo, A. C., Barroso, J. G and Ruberto, G. (1998). Antimicrobial and antioxidant properties of some commercial essential oils. *Flavour and Fragrance Journal*, *13*(4), 235–244.

Barazani, O., Cohen, Y., Fait, A., Diminshtein, S., Dudai, N., Ravid, U., Friedman, J. (2002). Chemotypic differentiation in indigenous populations of *Foeniculum vulgare* var. *vulgare* in Israel. Biochemical Systematics and Ecology, 30(8), 721–731.

Barazani, O., Fait, A., Cohen, Y., Diminshtein, S., Ravid, U., Putievsky, E. and Friedman, J. (1999). Chemical variation among indigenous populations of *Foeniculum vulgare* var. *vulgare* in Israel. Planta medica, 65 (5), 486–489.

Baserga, U. (1998). Landwirtschaftliche Co-Vergärungs-Biogasanlagen*:* Biogas aus organischen Reststoffen und Energiegras. FAT.

Benchaar, C., Chaves, A. V., Fraser, G. R., Beauchemin, K. A and McAllister, T. A. (2007). Effects of essential oils and their components on in vitro rumen microbial fermentation. Canadian Journal of Animal Science, 87(3), 413–419.

Benchaar, Chaoukiand Greathead, H. (2011). Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Animal Feed Science and Technology, 166–167, 338–355.

Benjamin, M. M., Woods, S. L and Ferguson, J. F. (1984). Anaerobic toxicity and biodegradability of pulp mill waste constituents. Water Research, 18(5), 601–607.

Bennett, R. N and Wallsgrove, R. M. (1994). Tansley review no. 72. Secondary metabolites in plant defence mechanisms. New Phytologist, 127(4) 617–633.

Bernotienë, G., Nivinshiene, O., Butkienë, R and Mochkute, D. (2004). Chemical composition of essential oils of hops (*Humulus lupulus* L.) growing wild in Aukstaitija. *Chemija*,15 (2), 31–36.

Betoni, J. E. C., Mantovani, R. P., Barbosa, L. N., Di Stasi, L. C and Fernandes Junior, A. (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. Memórias do Instituto Oswaldo Cruz,101(4), 387–390.

Bezić, N., Samanić, I., Dunkić, V., Besendorfer, V and Puizina, J. (2009). Essential oil composition and internal transcribed spacer (ITS) sequence variability of four south-Croatian *Satureja* species (Lamiaceae). Molecules,14(3), 925–938.

Biondi, D., Cianci, P., Geraci, C., Ruberto, G and Piattelli, M. (1993). Antimicrobial activity and chemical composition of essential oils from Sicilian aromatic plants. Flavour and Fragrance Journal, 8(6), 331–337.

Bodas, R., Prieto, N., García-González, R., Andrés, S., Giráldez, F. J and López, S. (2012). Manipulation of rumen fermentation and methane production with plant secondary metabolites. Animal Feed Science and Technology, 176(1–4), 78–93.

Borja, R., Alba, J and Banks, C. J. (1997). Impact of the main phenolic compounds of olive mill wastewater (OMW) on the kinetics of acetoclastic methanogenesis. *Process Biochemistry*, 32(2), 121–133.

Bounatirou, S., Smiti, S., Miguel, M. G., Faleiro, L., Rejeb, M. N., Neffati, M., and Pedro, L. G. (2007). Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. Food Chemistry, 105(1), 146–155.

Bouzouita, N., Kachouri, F., Hamdi, M., Chaabouni, M. M., Aissa, R. B., Zgoulli, S. andLognay, G. C. (2005). Volatile constituents and antimicrobial activity of *Lavandula stoechas* L. Oil from Tunisia. Journal of Essential Oil Research, 17(5), 584–586.

Börjesson, P., Berglund, M., 2007. Environmental systems analysis of biogas systemsPart II: The environmental impact of replacing various reference systems. Biomass Bioenergy, 31, 326–344.

Braun R (2009) Biogas from energy crop digestion. IEA Task 37 Brochure, International Energy Agency, Paris, France

Brown, D. (2002). The royal horticultural society new encyclopedia of herbs and their uses. 2nd edition.London: Dorling Kindersley.

Bryant, M. P. (1979). Microbial methane production—theoretical aspects. Journal of Animal Science, 48(1), 193–201.

Buddrus, J. (2003).Grundlagen der Organischen Chemie. Walter de Gruyter.

Buntain, M and Chung, B. (1994). Effects of irrigation and nitrogen on the yield components of fennel (*Foeniculum vulgare Mill*).Animal ProductionScience*,* 34(6): 845–849.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. International Journal of Food microbiology, 94(3):223–253.

Busquet, M., Calsamiglia, S., Ferret, A and Kamel, C. (2006). Plant extracts affect in vitro rumen microbial fermentation. Journal of Dairy Science, 89(2), 761–771.

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L and Ferret, A. (2007). Invited review: Essential oils as modifiers of rumen microbial fermentation. Journal of Dairy Science, 90(6): 2580–2595.

Castanon, J. I. R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. Poultry Science, 86(11): 2466–2471.

Castillejos, L., Calsamiglia, S and Ferret, A. (2006). Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in In vitro systems. Journal of Dairy Science, 89(7): 2649–2658.

Castillejos, L., Calsamiglia, S., Martín-Tereso, J and Ter Wijlen, H. (2008). In vitro evaluation of effects of ten essential oils at three doses on ruminal fermentation of high concentrate feedlot-type diets. Animal Feed Science and Technology, 145(1–4):259–270.

Cetin, B., Özer, H., Cakir, A., Polat, T., Dursun, A., Mete, E.and Ekinci, M. (2010). Antimicrobial activities of essential oil and hexane extract of florence fennel (*Foeniculum vulgare* var. *azoricum* (Mill.) Thell. against foodborne microorganisms. Journal of Medicinal Food, 13(1): 196–204.

Celiktas, O. Y., Kocabas, E. E., Bedir, E., Sukan, F. V., Ozek, T and Baser, K. H. C. (2007). Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations. Food Chemistry, 100(2): 553–559.

Cervato, G., Carabelli, M., Gervasio, S., Cittera, A., Cazzola, R and Cestaro, B. (2000). Antioxbdant properties of oregano (*Origanum Vulgare*) leaf extracts. Journal of Food Biochemistry, 24(6): 453–465.

Chaban, B., Ng, S.Y., Jarrell, K.F., 2006. Archaeal habitats-from the extreme to the ordinary. Canadian Journal of Microbiology, vol. 52(2): 73-116.

Chadwick, L. R., Pauli, G. F and Farnsworth, N. R. (2006). The pharmacognosy of *Humulus lupulus* L.(hops) with an emphasis on estrogenic properties. Phytomedicine, *13*(1): 119–131.

Chang, S. T., Chen, P. F and Chang, S. C. (2001). Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum.*Journal of Ethnopharmacology, 77(1): 123–127.

Chao, S. C., Young, D. G and Oberg, C. J. (2000). Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. Journal of Essential Oil Research, 12(5): 639–649.

Charles, D. J., Morales, M. R and Simon, J. E. (1993). Essential oil content and chemical composition of finocchio fennel. In: Janick, J., Simon. J.E.(eds),New Cops.NewYork: Wiley, 570–573.

Chatzopoulou, A., Karioti, A., Gousiadou, C., Lax Vivancos, V., Kyriazopoulos, P., Golegou, S and Skaltsa, H. (2010). Depsides and other polar constituents

from *Origanum dictamnus* L. and their in vitro antimicrobial activity in clinical strains. Journal of Agricultural and Food Chemistry, 58(10): 6064–6068.

Chatzopoulou, P. S., Koutsos, T. V and Katsiotis, S. T. (2006). Study of nitrogen fertilization rate on fennel cultivars for essential oil yield and composition. Journal of Vegetable Science, 12(2): 85–93.

Chaves, A. V., He, M. L., Yang, W. Z., Hristov, A. N., McAllister, T. A and Benchaar, C. (2008). Effects of essential oils on proteolytic, deaminative and methanogenic activities of mixed ruminal bacteria. Canadian Journal of Animal Science, 88(1): 117–122.

Chaves, A. V., Stanford, K., Gibson, L. L., McAllister, T. A and Benchaar, C. (2008). Effects of carvacrol and cinnamaldehyde on intake, rumen fermentation, growth performance, and carcass characteristics of growing lambs.AnimalFeed Science and Technology,145(1): 396–408.

Chaves, Alexandre V., Baah, J., Wang, Y., McAllister, T. A and Benchaar, C. (2012). Effects of cinnamon leaf, oregano and sweet orange essential oils on fermentation and aerobic stability of barley silage. Journal of the Science of Food and Agriculture, 92(4): 906–915.

Chen, Y., Cheng, J. J and Creamer, K. S. (2008). Inhibition of anaerobic digestion process: A review. BioresourceTechnology, 99(10): 4044–4064.

Chiej, R. (1984). Encyclopaedia of medicinal plants. A book, London, *McDonald and*Co Ltd. ISBN 0-356-10541-5,4.

Cornelison, J. M., Yan, F., Watkins, S. E., Rigby, L., Segal, J. B and Waldroup, P. W. (2006). Evaluation of hops (*Humulus lupulus*) as an antimicrobial in broiler diets. International Journal of Poultry Science, 5(2): 134–136.

Cos, P., Vlietinck, A. J., Berghe, D. V and Maes, L. (2006). Anti-infective potential of natural products: How to develop a stronger in vitro “proof-of-concept.Journal of Ethnopharmacology, 106(3): 290–302.

Cosentino, S., Tuberoso, C. I. G., Pisano, B., Satta, M., Mascia, V., Arzedi, E and Palmas, F. (1999). In-vitro antimicrobial activity and chemical composition of Sardinian thymus essential oils. Letters in Applied Microbiology, 29(2): 130–135.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clinical Microbiology. Reviews. 12(4): 564–582.

Crane, A., Nelson, W. O and Brown, R. E. (1957). Effects of D-Limonene and α-D-Pinene on in vitro carbohydrate dissimilation and methane formation by rumen bacteria. Journal of Dairy Science, 40(10): 1317–1323.

CSahin, F., Güllüce, M., Daferera, D., Sökmen, A., Sökmen, M., Polissiou, M.,Özer, H. (2004). Biological activities of the essential oils and methanol extract of *Origanum vulgare* ssp. *vulgare* in the Eastern Anatolia region of Turkey. Food Control, 15(7): 549–557.

Curtis, O. F., Shetty, K., Cassagnol, G and Peleg, M. (1996). Comparison of the inhibitory and lethal effects of synthetic versions of plant metabolites (anethole, carvacrol, eugenol, and thymol) on a food spoilage yeast (*Debaromyces hansenii*). Food Biotechnology, 10(1): 55–73.

Dadalioglu, I and Evrendilek, G. A. (2004). Chemical compositions and antibacterial effects of essential oils of Turkish oregano (*Origanum minutiflorum*), bay laurel (*Laurus nobilis*), Spanish lavender (*Lavandula stoechas* L.), and fennel (*Foeniculum vulgare*) on common foodborne pathogens. Journal of Agricultural and Food Chemistry, 52(26): 8255–8260.

Davidsson, Å., Lövstedt, C., la Cour Jansen, J., Gruvberger, C., Aspegren, H.(2008). Co-digestion of grease trap sludge and sewage sludge. Waste Management. 28(6): 986–992.

Dewick, P. M. (2002). The biosynthesis of C5–C25 terpenoid compounds. Natural Product Reports, 19(2), 181–222.

Dorman, H. J. D and Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology, 88(2): 308–316.

Dursun, N., Liman, N., Özyazgan, I., Günes, I and Saraymen, R. (2003). Role of thymus oil in burn wound healing. Journal of Burn Care and Research, 24(6):395–399.

EL-adly, A. A., Abada, E. A and Gharib, F. A. (2007). Antibacterial effects of low power laser light and volatile oil of fennel (*Foeniculum vulga*re var. *dulce*) on Gram-positive and Gram-negative bacteria. International Journal of Agriculture and Biology, 9(1): 22–26.

Evans, J. D and Martin, S. A. (2000). Effects of thymol on ruminal microorganisms. Current microbiology, 41(5): 336–340.

Fan, M and Chen, J. (2001). Studies on antimicrobial activity of extracts from thyme. Wei Sheng Wu Xue Bao Acta Microbiologica Sinica, 41(4): 499.

Fang, C., Boe, K and Angelidaki, I. (2011). Anaerobic co-digestion of desugared molasses with cow manure; focusing on sodium and potassium inhibition. BioresourceTechnology, 102(2):1005–1011.

Figuérédo, G., Cabassu, P., Chalchat, J.-C and Pasquier, B. (2006). Studies of Mediterranean oregano populations. VIII - Chemical composition of essential oils of oreganos of various origins. Flavour and FragranceJournal, 21(1): 134–139.

Forgács, G. (2012). Biogas production from citrus wastes and chickenfeather treatment and co-digestion. Doctoral thesis, Göteborg, Sweden: Chalmers University of Technology.

Friedman, M., Henika, P. R and Mandrell, R. E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes*, and *Salmonella enterica.* Journal of Food Protection, 65(10): 1545–1560.

Garcia-Heras, J.L., 2003. Reactor sizing, process kinetics, and modelling of anaerobic digestion of complex wastes. In Mata-Alvarez, J.(ed) Biomethanization of the organic fraction of Municipal solid wastes. London, UK. International water association, 31-43.

Gasquet, M. (1993). Evaluation in vitro and in vivo of a traditional antimalarial, "Malarial 5." Fitoterapia, vol LXIV,No.5

Gerardi, M. H. (2003). The microbiology of anaerobic digesters (waste water microbiology series). Hoboken, New Jersey: John Wiley and Sons.

Gershenzon, J., Maffei, M and Croteau, R. (1989). Biochemical and histochemical localization of monoterpene biosynthesis in the glandular trichomes of spearmint (*Mentha spicata*). Plant physiology, 89(4): 1351–1357.

Greathead, H. (2003). Plants and plant extracts for improving animal productivity. Proceedings of the Nutrition Society, 62(02): 279–290.

Grevsen, K., Frette, X. C and Christensen, L. P. (2009). Content and composition of volatile terpenes, flavonoids and phenolic acids in Greek oregano (*Origanum vulgare* L. ssp. *hirtum*) at different development stages during cultivation in cool temperate climate. European Journal of Horticultural Science, 74(5): 193.

Guillén, M. D and Manzanos, M. J. (1996). A study of several parts of the plant *Foeniculum vulgare* as a source of compounds with industrial interest. Food Research International, 29(1): 85–88.

Gujer, W and Zehnder, A. J. B. (1983). Conversion processes in anaerobic digestion. Water Science and Technology, 15(8-9): 127–167.

Gulfraz, M., Mehmood, S., Minhas, N., Jabeen, N., Kausar, R., Jabeen, K and Arshad, G. (2010). Composition and antimicrobial properties of essential oil of *Foeniculum vulgare*. African Journal of Biotechnology, 7(24): 4364-4368.

Hadacek, F.(2002). Secondary metabolites as plant traits: current assessment and future perspectives. Critical Reviews in Plant Science. 21 (4): 273–322.

Hart, K. J., Yáñez-Ruiz, D. R., Duval, S. M., McEwan, N. R and Newbold, C. J. (2008). Plant extracts to manipulate rumen fermentation. Animal Feed Science and Technology, 147(1–3): 8–35.

Hartmann, T. (2007). From waste products to ecochemicals: Fifty years research of plant secondary metabolism. Phytochemistry, 68(22): 2831–2846.

Heiermann, M., Ploechl, M., Linke, B., Schelle, H., Herrmann, C. (2009). Biogas crops-part I: specifications and suitability of field crops for anaerobic digestion. Agricultural Engineering International: the CIGR Ejournal, XI (2009): 1-17.

Helander, I. M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J. and von Wright, A. (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry, 46(9): 3590–3595.

Hendriks, A.T.W.M., Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology, 100 (1), 10–18.

Hernandez, J. E and Edyvean, R. G. J. (2008). Inhibition of biogas production and biodegradability by substituted phenolic compounds in anaerobic sludge. Journal of Hazardous Materials, 160(1): 2028.

Hook, S. E., Wright, A.-D. G and McBride, B. W. (2010). Methanogens: methane producers of the rumen and mitigation strategies. Archaea, 2010.(2010): 1-11.

Hossein J. A. (2011). Effect of various medicinal plant essential oils obtained from semi-arid climate on rumen fermentation characteristics of a high forage diet using in vitro batch culture. African Journal of Microbiology Research, *5*(27):4812-4819.

Hristov, A. N., Lee, C., Cassidy, T., Heyler, K., Tekippe, J. A., Varga, G. A., and Brandt, R. C. (2013). Effect of Origanum vulgare L. leaves on rumen fermentation, production, and milk fatty acid composition in lactating dairy cows. Journal of Dairy Science, 96(2): 1189–1202.

Hristov, A. N., Hanigan, M., Cole, A., Todd, R., McAllister, T. A., Ndegwa, P. M and Rotz, A. (2011). Review: Ammonia emissions from dairy farms and beef feedlots. Canadian Journal of Animal Science, 91(1): 1–35.

Humane, R. E. (1969). A roll tube method for cultivation of strict anaerobes. Method in Microbiology, 3B (3): 117-132.

Hungate, R. E and Macy, J. (1973). The roll-tube method for cultivation of strict anaerobes. Bulletins of the Ecological Research Committee, 17:123–126.

Ietswaart, J. H., 1980. A taxonomic revision of the genus *Origanum* (Labiatae). Folia Geobotanica et Phytotaxonomica, 16(4):390.

Jensen, W. B. (2007). The origin of the Soxhlet extractor. Journal of Chemical Education, 84(12): 1913-1914.

Jirovetz, L., Buchbauer, G., Denkova, Z., Stoyanova, A., Murgov, I., Schmidt, E and Geisser, M. (2005). Antimicrobial testings and gas chromatographic analysis of pure oxygenated monoterpenes 1, 8-cineole, α-terpineol, terpinen-4-ol and camphor as well as target compounds in essential oils of pine (*Pinus pinaster*), rosemary (*Rosmarinus officinalis*), tea tree (*Melaleuca alternifolia*). Scientia Pharmaceutica, 73(1): 27–38.

Kalemba, D and Kunicka, A. (2003). Antibacterial and antifungal properties of essential oils. Current Medicinal Chemistry, 10(10), 813–829.

Karakaya, S., El, S. N., Karagözlü, N and Şahin, S. (2011). Antioxidant and antimicrobial activities of essential oils obtained from oregano (*Origanum vulgare* ssp. *hirtum*) by using different extraction methods. Journal of Medicinal Food, 14(6): 645–652.

Khan, I. A and Abourashed, E. A. (2011).Leung's encyclopedia of common natural ingredients: used in food, drugs and cosmetics:3rd edition. Reference Reviews, Vol. 24 (7):42 - 43.

Kimura, M., Anzai, H and Yamaguchi, I. (2001). Microbial toxins in plant-pathogen interactions: Biosynthesis, resistance mechanisms, and significance. The Journal of General and Applied Microbiology, 47(4): 149–160.

Kintzios, S. E. (2002). Profile of the multifaceted prince of the herbs.In Kintzios, S. E(ed) Oregano:The genera *Origanum* and *Lippia*. London: Taylor and Frances.

Klass, D. L. (1984). Methane from anaerobic fermentation. Science *(*Washington*)*, *223*(4640): 1021–1027.

Kokkini, S.,Karousou, R., Hanlidou, E and Lanaras, T. (2004). Essential oil composition of Greek (*Origanum vulgare* ssp. *hirtum*) and Turkish (O. *onites*) oregano: a tool for their distinction. Journal of Essential Oil Research, *16*(4), 334–338.

Kordali, S.,Cakir, A., Ozer, H., Cakmakci, R., Kesdek, M and Mete, E. (2008). Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish *Origanum acutidens*and its three components, carvacrol, thymol and *p*-cymene. Bioresource Technology, 99(18): 8788–8795.

Kotsyurbenko, O. R., 2005. Trophic interactions in the methanogenic microbial community of low-temperature terrestrial ecosystems. FEMS Microbiology Ecology. 53, 3–13.

Kwon, Y. S., Choi, W. G., Kim, W. J., Kyung cKim, W., Kim, M. J., Kang, W. H and Kim, C. M. (2002). Antimicrobial constituents of Foeniculum vulgare. Archives of pharmacal research, 25(2): 154–157.

Lambert, R. J. W., Skandamis, P. N., Coote, P. J and Nychas, G.J. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology, 91(3): 453–462.

Lange, M and Ahring, B. K. (2001). A comprehensive study into the molecular methodology and molecular biology of methanogenic *Archaea*. FEMS MicrobiologyReviews, 25(5): 553–571.

Lange, M., Westermann, P., Ahring, B.K., 2005. *Archaea* in protozoa and metazoa. Applied Microbiology Biotechnology. vol 66(5): 465–474.

Lawrence, B. M. (1984). The botanical and chemical aspects of oregano. Perfumer andFlavorist, 9(5): 41–51.

Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. The Journal of Nutrition, 134(12): 3479S–3485S.

Lo Cantore, P., Iacobellis, N. S., De Marco, A., Capasso, F and Senatore, F. (2004). Antibacterial activity of *Coriandrum sativum* L. and *Foeniculum vulgare* Miller

var. *vulgare* (Miller) essential oils. Journal of Agricultural and Food Chemistry, *52*(26): 7862–7866.

Macheboeuf, D., Morgavi, D. P., Papon, Y., Mousset, J.-L and Arturo-Schaan, M. (2008). Dose–response effects of essential oils on in vitro fermentation activity of the rumen microbial population. Animal Feed Science and Technology*,* 145*(*1–4*):335–350.*

Marques, A., Encarnaccão, S., Pedro, S and Nunes, M. L. (2008). In vitro antimicrobial activity of garlic, oregano and chitosan against *Salmonella enterica*. World Journal of Microbiology and Biotechnol*ogy,*24(10): 2357–2360.

Martín, M. A., Siles, J. A., Chica, A. F and Martín, A. (2010). Biomethanization of orange peel waste. Bioresource technology, 101(23): 8993–8999.

Martínez-Rocha, A., Puga, R., Hernández-Sandoval, L., Loarca-Piña, G and Mendoza, S. (2008). Antioxidant and antimutagenic activities of Mexican oregano (Lippia graveolens Kunth). Plant Foods for Human Nutrition, 63(1): 1–5.

Mata-Alvarez, J., Mace, S. and Llabres, P., 2000. Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Bioresource Technology. 74(1): 3–16.

McAllister, T. A and Newbold, C. J. (2008). Redirecting rumen fermentation to reduce methanogenesis. Animal Production Science, 48(2), 7–13.

McGarvey, D. J and Croteau, R. (1995). Terpenoid metabolism. The Plant Cell, 7(7): 1015- 1026.

McGaw, L. J., Jäger, A. K and Van Staden, J. (2000). Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. Journal of Ethnopharmacology, 72(1): 247–263.

McInerney, M. J., Bryant, M. P and Stafford, D. A. (1980). Metabolic stages and energetics of microbial anaerobic digestion. In*Stafford D. A., Wheatly B.I., and Hughes*,D. E(eds*)*.proceedings of the first International Symposium on Anaerobic Digestion held at University Collage, Cardiff, Wales September 1979. London: Applied Science, 91–98

Miguel, G., Simoes, M., Figueiredo, A. C., Barroso, J. G., Pedro, L. G and Carvalho, L. (2004). Composition and antioxidant activities of the essential oils

ofThymus caespititius, Thymus camphoratusand Thymus mastichina. Food Chemistry, 86(2): 183–188.

Miller, T. L and Wolin, M. J. (1974). A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Applied microbiology, 27(5): 985-987.

Milligan, S., Kalita, J., Pocock, V., Heyerick, A., De Cooman, L., Rong, H and De Keukeleire, D. (2002). Oestrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin. Reproduction, 123(2):235–242.

Milos, M., Mastelic, J and Jerkovic, I. (2000). Chemical composition and antioxidant effect of glycosidically bound volatile compounds from oregano (*Origanum vulgare* L. ssp. *hirtum*). Food Chemistry, 71(1): 79–83.

Mohácsi-Farkas, C., Tulok, M and Balogh, B. (2001). Antimicrobial activity of Greek oregano and winter savory extracts (essential oil and SCFE) investigated by impedimentary. Acta Horticulturae (ISHS),597: 199-204.

Moir, M. (2000). Hops: a millennium review. Journal of the American Society of Brewing Chemists, 58(4): 131–146.

Morton, J. F. (1981). Atlas of medicinal plants of Middle America: Bahamas to Yucatan.Springfield, USA: Charles C Thomas.

Mueller, M., Lukas, B., Novak, J., Simoncini, T., Genazzani, A. R and Jungbauer, A. (2008). Oregano: a source for peroxisome proliferator-activated receptor γ antagonists. Journal of Agricultural and Food Chemistry, 56(24): 11621–11630.

Naidu, A. S. (2010). Natural food antimicrobial systems. a book CRC Press. USA.

Nallathambi, V.,1997. Anaerobic digestion of biomass for methane production: A review. Biomass Bioenergy 13(1-2): 83–114.

Napoli, E. M., Curcuruto, G and Ruberto, G. (2010). Screening the essential oil composition of wild Sicilian fennel. Biochemical Systematics and Ecology, *38*(2): 213–223.

Narvaez, Nelmy, Wang, Y., Xu, Z., Alexander, T., Garden, S and McAllister, T. (2012). Effects of hop varieties on ruminal fermentation and bacterial community in an artificial rumen (rusitec). Journal of the Science of Food and Agriculture, 93(1): 45–52.

Narvaez, N., Wang, Y., Xu, Z and McAllister, T. (2011). Effects of hops on in vitro ruminal fermentation of diets varying in forage content. Livestock Science, 138(1):193–201.

Nevas, M., Korhonen, A.-R., Lindstrom, M., Turkki, P and Korkeala, H. (2004). Antibacterial efficiency of Finnish spice essential oils against pathogenic and spoilage bacteria. Journal of Food Protection, 67(1): 199–202.

Newberne, P., Smith, R. L., Doull, J., GoO.DMan, J. I., Munro, I. C., Portoghese, P. S andAdams, T. B. (1999). The femagrasassessment of trans-anethole used as a flavouring substance. Food and Chemical Toxicology, 37(7): 789–811.

Nickerson, G. B and Van Engel, E. L. (1992). Hop aroma component profile and the aroma unit. Journal of the American Society of Brewing Chemists, 50*: 82-104*.

Olivier, G. W. (1996). The world market of oregano. In Padulosi, S.(ed).*Oregano: proceedings of the IPGRI International workshop on oregano. promoting the conservation and use of underutilized and neglected crops.* Valenzano, Bari, Italy:Vol14: 141–145.

Özkalp, B., Sevgi, F., Özcan, M and Özcan, M. M. (2010). The antibacterial activity of essential oil of oregano (*Origanum vulgare* L.). Journal of Food, Agriculture and Environment, 8(2): 272–274.

Panizzi, L., Flamini, G., Cioni, P. L and Morelli, I. (1993). Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae. Journal of Ethnopharmacology, 39(3): 167–170.

Parawira, W., Murto, M., Read, J. S and Mattiasson, B. (2005). Profile of hydrolases and biogas production during two-stage mesophilic anaerobic digestion of solid potato waste. Process Biochemistry, 40(9): 2945–2952.

Park, S. H and Seong, I. (2010). Antifungal Effects of the Extracts and Essential Oils from Foeniculum vulgare and Illicium verum against Candida albicans. *Korean* Journal of Medical Mycology, 15(4): 157–164.

Parkin, G.F., Owen, W.F.(1986). Fundamentals of anaerobic digestion of wastewater sludges. Journalof Environmental Engineering. 112 (5): 867–920.

Parthasarathy, V. A., Chempakam, B and Zachariah, T. J.(2008). Chemistry of Spices.Oxford shire, UK: CABI International.

Pascual, M. E., Slowing, K., Carretero, E., Sánchez Mata, D and Villar, A. (2001). Lippia: traditional uses, chemistry and pharmacology: a review. Journal of Ethnopharmacology, 76(3): 201–214.

Paster, N., Menasherov, M., Ravid, U and Juven, B. (1995). Antifungal Activity of Oregano and Thyme Essential Oils Applied as Fumigants Against Fungi Attacking Stored Grain. Journal of Food Protection, 58(1): 81–85.

Patra, Amlan K and Yu, Z. (2012). Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. Applied and environmental microbiology, 78(12): 4271–4280.

Patra, Amlan Kumar, Kamra, D. N and Agarwal, N. (2010). Effects of extracts of spices on rumen methanogenesis, enzyme activities and fermentation of feeds in vitro. Journal of the Science of Food and Agriculture, 90(3): 511–520.

Penalver, P., Huerta, B., Borge, C., Astorga, R., Romero, R and Perea, A. (2005). Antimicrobial activity of five essential oils against origin strains of the Enterobacteriaceae family. Apmis, 113(1): 1–6.

Pereira, M. A., Pires, O. C., Mota, M and Alves, M. M. (2005). Anaerobic biodegradation of oleic and palmitic acids: evidence of mass transfer limitations caused by long chain fatty acid accumulation onto the anaerobic sludge. Biotechnology and Bioengineering, 92(1): 15–23.

Piccaglia, R and Marotti, M. (2001). Characterization of some Italian types of wild fennel (Foeniculum vulgare Mill.). Journal of agricultural and food chemistry, 49(1), 239–244.

Prabhudessai, V., Ganguly, A and Mutnuri, S. (2009). Effect of caffeine and saponin on anaerobic digestion of food waste. *Annals of Microbiology*, 59(4), 643–648.

Ramadan, M. F., Asker, S and Mohamed, M. (2009). Atimicrobial and antivirial impact of novel quercetin-enriched lecithin.Journal of Food Biochemistry, 33(4): 557–571.

Ranvcić, A., Soković, M., Van Griensven, L., Vukojević, J., Brkić, D and Ristić, M. S. (2003). Antimicrobial activity of limonene. Matières Médical., 23(XXIII): 83–88.

Reddy, B., Angers, P., Gosselin, A and Arul, J. (1998). Characterization and use of essential oil from *Thymus vulgaris* against *Botrytis cinerea* and *Rhizopus stolonifer* in strawberry fruits. Phytochemistry, 47(8): 1515–1520.

Roberts, M. T., Dufour, J.-P and Lewis, A. C. (2004). Application of comprehensive multidimensional gas chromatography combined with time-of-flight mass spectrometry (GC times GC-TOFMS) for high resolution analysis of hop essential oil. Journal of Separation Science, 27(5-6): 473–478.

Rozzi, A and Remigi, E. (2004). Methods of assessing microbial activity and inhibition under anaerobic conditions: a literature review. Reviews in Environmental Science and Biotechnology, 3(2): 93–115.

Ruberto, G., Baratta, M. T., Deans, S. G and Dorman, H. J. D. (2000). Antioxidant and Antimicrobial Activity of *Foeniculum vulgare* and *Crithmum maritimum* Essential Oils. Planta Medica, 66(8): 687–693.

Sacchetti, G., Maietti, S., Muzzoli, M., Scaglianti, M., Manfredini, S., Radice, M., and Bruni, R. (2005). Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. Food Chemistry, 91(4): 621–632.

Sacchettini, J. C., and Poulter, C. D. (1997). Creating isoprenoid diversity. Science, 277(5333):1788–1789.

Salgueiro, L. R., Cavaleiro, C., Gon\ccalves, M. J., and da Cunha, A. P. (2003). Antimicrobial activity and chemical composition of the essential oil of Lippia graveolens from Guatemala. Planta medica, 69(01): 80–83.

Schattenhofer, M. (1989). Hops from Germany. *CMA Bonn*, 6–9.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and Molecular Biology Reviews, 61(2), 262–280.

Schnürer, A. and Nordberg, A. (2008). Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. Water Science and Technology, 57(5):735 – 740.

Shahat, A. A., Ibrahim, A. Y., Hendawy, S. F., Omer, E. A., Hammouda, F. M., Abdel-Rahman, F. H. and Saleh, M. A. (2011). Chemical composition, antimicrobial and antioxidant activities of essential oils from organically cultivated fennel cultivars. *Molecules*, 16(2): 1366–1377.

Sierra-Alvarez, R., and Lettinga, G. (1990). The methanogenic toxicity of wood resin constituents. Biological Wastes, 33(3): 211–226.

Siragusa, G. R., Haas, G. J., Matthews, P. D., Smith, R. J., Buhr, R. J., Dale, N. M., and Wise, M. G. (2008). Antimicrobial activity of lupulone against Clostridium perfringens in the chicken intestinal tract jejunum and caecum. Journal ofAntimicrobial Chemotherapy, 61(4): 853–858.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T. and Arsenakis, M. (1996a). Antimicrobial and cytotoxic activities of origanum Essential Oils. Journal of Agricultural and Food Chemistry, 44(5):1202–1205.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T. and Arsenakis, M. (1996b). Antimicrobial and cytotoxicactivities of Origanum essential Oils. Journal of Agricultural and Food Chemistry, 44(5):1202–1205.

Skandamis, P. N. and Nychas, G.-J. E. (2000). Development and evaluation of a model predicting the survival of *Escherichia coli* O157: H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentrations. Applied and Environmental Microbiology, 66(4): 1646–1653.

Skoula, M and Harborne, J. B. (2002). 3 The taxonomy and chemistry of *Origanum.* InKintzios,S.E.(ed)Oregano*:The genera Origanum and Lippia*.London:Tylor andFrancis, *25*, 67.

Soylu, S., Yigitbas, H., Soylu, E. m. and Kurt, Ş. (2007). Antifungal effects of essential oils from oregano and fennel on Sclerotinia sclerotiorum. Journal of Applied Microbiology, 103(4): 1021–1030.

Speece, R. E.(1996). Anaerobic biotechnology for industrial wastewaters. Environmental Science and technology, 17(9), 416A-427A.

Stahl-Biskup, E. (2002). Essential oil chemistry of the genus *Thymus.* in Stahl-Biskup, E and Saez, F.(eds)Thyme*:* thegenus *Thymus*. London and New York: Taylor and Francis, 75–124.

Stevens, J. F. and Page, J. E. (2004). Xanthohumol and related prenylflavonoids from hops and beer: to your good health.Phytochemistry, 65(10): 1317–1330.

Stewart, C. S. and Jouany, J. P. (1991). The rumen bacteria. In Jouany*,* J.P.(ed)*.* Rumen microbial metabolism and ruminant digestion*.* Paris INRA:15–26.

Symons, G. E. and Buswell, A. M. (1933). The Methane fermentation of carbohydrates1, 2. Journal of the American Chemical Society, 55(5): 2028–2036.

Tabak, M., Armon, R., Potasman, I and Neeman, I. (1996). In vitro inhibition of *Helicobacter pylori* by extracts of thyme. Journal of Applied Microbiology, 80(6): 667–672.

Takashima, M. and Speece, R. E. (1989). Mineral nutrient requirements for high-rate methane fermentation of acetate at low SRT. Research Journal of the Water Pollution, 16(11/12):1645–1650.

Tekippe, J. A., Hristov, A. N., Heyler, K. S., Cassidy, T. W., Zheljazkov, V. D., Ferreira, J. F. S., Varga, G. A. (2011). Rumen fermentation and production

effects of *Origanum vulgare* L. leaves in lactating dairy cows. Journal of Dairy Science, 94(10): 5065–5079.

Terblanché, F. C. and Kornelius, G. (1996). Essential oil constituents of the genus *Lippia* (Verbenaceae), a literature review. Journal of essential oil research, 8(5): 471–485.

Turner, C. E., Elsohly, M. A. and Boeren, E. G. (1980). Constituents of *Cannabis sativa* L. XVII. A Review of the Natural Constituents. Journal of Natural Products, 43(2): 169–234.

Turner, G., Gershenzon, J., Nielson, E. E., Froehlich, J. E. and Croteau, R. (1999). Limonene synthase, the enzyme responsible for monoterpene biosynthesis in peppermint, is localized to leucoplasts of oil gland secretory cells. Plant Physiology, 120(3): 879–886.

Ultee, A., Kets, E. P. W and Smid, E. J. (1999). Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*.Applied and Environmental Microbiology, 65(10): 4606–4610.

Ultee, A., Slump, R. A., Steging, G and Smid, E. J. (2000). Antimicrobial activity of carvacrol toward Bacillus cereus on rice. Journal of Food Protection, 63(5): 620–624.

Ultee, Gorrisand Smid. (1998). Bactericidal activity of carvacrol towards the food-borne pathogen Bacillus cereus. Journal of Applied Microbiology, 85(2): 211–218.

Van Cleemput, M., Cattoor, K., De Bosscher, K., Haegeman, G., De Keukeleire, D and Heyerick, A. (2009). Hop (*Humulus lupulus*)-derived bitter acids as multipotent bioactive compounds. Journal of natural products, 72(6):1220–1230.

Van Soest, P. J. (1967). Development of a comprehensive system of feed analyses and its application to forages. Journal of animal Science, *26*(1): 119–128.

Van Soest, P. J and Wine, R. H. (1967). Use of detergents in the analysis of fibrous feeds. IV: Determination of plant cell-wall constituents. Journal of the Association of Official Analytical Chemists, 50(50): 5.

Van Soest, Peter J. (1963). Use of detergents in the analyses of fibrous feeds. A rapid method for the determination of fiber and lignin. Journal of the Association of Official Analytical Chemists, 46: 829–835.

Vavilin, V. A., Rytov, S. V and Lokshina, L. Y. (1996). A description of hydrolysis kinetics in anaerobic degradation of particulate organic matter. Bioresource Technology, 56(2): 229–237.

VDI, V. D. I. (2006). *4630:* Fermentation of organic materials*,* characterisation of the substrate, sampling, collection of material data, fermentation tests. Beuth Verlag GmbH, Dusseldorf.

Veeken, A., Kalyuzhnyi, S., Scharff, H and Hamelers, B. (2000). Effect of pH and VFA on hydrolysis of organic solid waste. Journal of environmental engineering, 126(12): 1076–1081.

Veldhuizen, E. J. A., Tjeerdsma-van Bokhoven, J. L. M., Zweijtzer, C., Burt, S. A and Haagsman, H. P. (2006). Structural requirements for the antimicrobial activity of carvacrol. Journal of Agricultural and Food Chemistry, 54(5), 1874–1879.

Verzele, M and De Keukeleire, D. (1991). Chemistry and analysis of hop and beer bitter acids.Amsterdam: Elsevier .

Vindis, P., Mursec, B., Rozman, C., Janzekovic, M and Cus, F. (2008). Biogas production with the use of mini digester. Journal of Achievements in Materials and Manufacturing Engineering, 28(1): 99–102.

Walsh, S. E., Maillard, J.-Y., Russell, A. D., Catrenich, C. E., Charbonneau, D. L and Bartolo, R. G. (2003). Activity and mechanisms of action of selected biocidal agents on Gram-positive and-negative bacteria. Journal of Applied Microbiology, 94(2): 240–247.

Weiland, P. (2010). Biogas production: current state and perspectives. Applied Microbiology and Biotechnology, 85(4): 849–860.

Weiland, P. (2006). Biomass digestion in agriculture: A successful pathway for the energy production and waste treatment in Germany. Engineringin Life Science. 6(3): 302–309.

Weiland, P.(2003). Production and energetic use of biogas from energy crops and wastes in Germany. Applied Biochemistry and Biotechnolology. 109(1-3): 263–274.

Westermann, P. (1996). Temperature regulation of anaerobic degradation of organic matter. *World Journal of Microbiology and Biotechnology*, 12(5): 497–503

Widdel, F and Bak, F. (1992). Gram-negative mesophilic sulfate-reducing bacteria. The Prokaryotes, 4, 3352–3378.

Widdel, F., Kohring, G. W and Mayer, F. (1983). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. Archives of Microbiology, 134(4): 286–294.

Widdel F (1980) Anaerober Abbau von Fettsäuren und Benzoesäure durch neu isolierte Arten Sulfat-reduzierender Bakterien. Dissertation, Universität Göttingen

Williams, S. (1984). Official methods of analysis of the Association of OfficialAnalytical Chemists. Arlington, Virginia: Association of. Official Analytical Chemists, 14:446–447.

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64(1): 3–19.

Yang, W. Z., Benchaar, C., Ametaj, B. N., Chaves, A. V., He, M. L and McAllister, T. A. (2007). Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. Journal of Dairy Science, 90(12): 5671–5681.

Zanoli, P and Zavatti, M. (2008). Pharmacognostic and pharmacological profile of< i> Humulus lupulus</i> L. Journal of Ethnopharmacology, 116(3), 383–396.

Zeng, X., Ma, Y and Ma, L. (2007):Utilization of straw in biomass energy in China. Renewable and Sustainable Energy Reviews, 11(5), 976–987.

Zhang, R., El-Mashad, H. M., Hartman, K., Wang, F., Liu, G., Choate, C and Gamble, P. (2007). Characterization of food waste as feedstock for anaerobic digestion. Bioresource Technology, 98(4):929–935.

Zheng, W and Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. Journal of Agricultural and Food Chemistry, 49(11), 5165–5170.

Zhou, Y. Y., Mao, H. L., Jiang, F., Wang, J. K., Liu, J. X and McSweeney, C. S. (2011). Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep. Animal Feed Science and Technology, 166-167:93–100.

Web sites

Fachagentur Nachwachsende Rohstoffe e.V.: FNR (2013) Retrieved June 10, 2013, from http://www.fnr.de/

Fachagentur Nachwachsende Rohstoffe e.V.: FNR (2012) Retrieved June 10, 2013, from http://www.fnr.de/

DBFZ.(2012.). Deutsche BiomasseForschungszentrum, Report Nr. 12 Monitoring zur Wirkung des Erneuerbare-Energien-Gesetz (EEG) auf die Entwicklung der Stromerzeugung aus
Biomasse. Retrieved fromhttp://www.dbfz.de/web/fileadmin/userupload/ DBFZ Reports /DBFZReport12.pdf

German hop, Retrieved June 2012 and
available at: http://www.deutscherhopfen.de/contentserv/hopfenpflanzerverband.de/data/media/2099/HM-eng-komplett-05.pdf

Bundesministerium für Ernährung,Landwirtschaft und Verbraucherschutz,Retrieved October 2012 and available at:http://www.bmelv.de/SharedDocs/Reden/2012/01-11-BL-Biogas.html

Hopsteiner Guidelines for hop buying. Hop market review and outlook. Steiner, S.S. Inc.,NewYork,http://www.hopsteiner.com/pdf/World%20Crop%20Estimate%202013.pdf

http://www.hopsteiner.com/pdf/Hopfenanbauflachen%202009-2012.pdf

8. Appendix

Declaration/ Erklärung

I declare: this thesis is a result of my own independent work/investigation,which written without anyillegitimate help by any third party and only with materials indicated in thethesis except where otherwise stated. I have indicated in the text where I have used texts from alreadypublished sources, either word for word or in substance, and where I have madestatements based on oral information given to me. At any time during theinvestigations carried out by me and described in the dissertation, I followed theprinciples of good scientific practice as defined in the "Statutes of the Justus LiebigUniversity Giessen for the Safeguarding of Good Scientific Practice".

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubtefremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegebenhabe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriftenentnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sindals solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertationerwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis,wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten."

(Shams eldin Daffallah Yousif Mohamed)

Giessen

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