Microbial ecology of anaerobic reactors for treatment of alcohol industry wastewaters: a review

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Alcohol industries, classified as brewery, winery and distillery, generate huge amounts of acidic, recalcitrant and colored wastewaters with high organic content. Treatment of alcohol production effluents is critical by environmental points of view. Anaerobic treatment is one of the most commonly applied technologies for alcohol industry effluents with a high treatment efficiency and bioenergy recovery. Microbial community structure of bioreactors is the key parameter for efficient removal of organic matter. Microbial diversity of anaerobic reactors treating alcoholic beverage effluents is mainly comprised of hydrolytic *Firmicutes*, acidogenic syntrophic *proteobacteria*, methanogenic *Methanosaeta* spp. and *Methanobacteriales* group. Combined use of molecular tools provided a deeper insight to microbial ecology of bioreactors.

Keywords alcohol industry wastewaters; distillery; winery; brewery; anaerobic treatment; microbial diversity; molecular tools

1. Introduction

Alcoholic beverages can be categorized under three classes as malt beverages or beer, wines and distilled spirits [1]. Variety raw materials such as grains, fruits and vegetables are processed for the production of fermented and distilled alcoholic beverages. The most popular grains include barley, rye, rice and wheat whereas grapes, apple, plum and pomace are most commonly used fruit sources. Potatoes, sugarcane, molasses and agave are among the vegetables that are used in the production of rum, vodka and tequila [2].

Alcohol production processes produce vast amounts of wastewaters with different properties depending on the raw material and product. Treatment of these wastewaters with high volumes is mostly problematic due to acidic, recalcitrant and colored properties and high organic matter content [3-5]. A common approach for treatment of alcohol industry wastewaters is anaerobic treatment technology. It is an advantageous process due to biogas recovery, less energy and nutrient requirements, less sludge production and durability under varying conditions [4,5]. A number of anaerobic reactors such as upflow anaerobic sludge blanket (UASB) reactor [6,7], expanded granular sludge bed (EGSB) reactor [8], upflow anaerobic filter (UAF) [9,10], upflow fixed film column (UFFC) [11], upflow fluidized bed (UFB) [12], down-flow fluidized bed (DFB) [13], anaerobic hybrid reactor [14] and two-stage reactor systems [15,16] have been used for treating alcoholic beverage effluents under different organic loading rates (OLRs) and hydraulic retention times (HRTs).

Diversity of microbial community as well as wastewater characteristics and maintaining desired operating conditions, is a critical component in anaerobic reactors which actually determines the system performance. Treatment efficiency is highly dependent on the activity of bacterial and archaeal populations which are in turn related to the influent wastewater characteristics and environmental/operational conditions such as pH, temperature, shock loadings, etc. Therefore, community analysis is an important practice for monitoring efficiency of biological treatment systems.

This mini-review paper aims to summarize types and main steps in production of alcoholic beverages, their wastewater characterizations, commonly used anaerobic reactor types and molecular methods used for community analysis involved in biodegradation of these wastewaters.

2. Types of alcoholic beverages and production steps

Alcoholic beverages are generally produced dependent on cultural traditions, common local agricultural crops and preferences of majority of consumers. *Raki, Ouzo* and *Grappa* are local spirits of Turkey, Greece and Italy respectively and they are all made of pomace but each has different aromas and production stages. Beer is mostly produced from barley, but rye, corn and wheat are also used as raw materials of beer production. Distillation of grape juice can yield a variety of products such as brandy, cognac, vermouth, etc. upon the production processes.

Alcoholic beverages can be classified under three main groups as malt beverages or beer, wines and distilled spirits. Malt beverages are products of grain fermentation. The production of malt beverages, or beer, comprises four main stages: brew house operations, fermentation, aging or secondary fermentation, and packaging [1].

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Wine is an alcoholic beverage produced by fermentation of sugars in fruit juices, primarily grape juice. As a common practice, the basic steps in vinification (wine production) include harvesting, crushing, pressing, fermentation, clarification, aging, finishing, and bottling [1].

The most commonly produced distilled spirits include whiskies, gins, vodkas, rums, and brandies. Whiskies are produced from fermented grain mashes and aged. Vodkas are produced from fermented grain mashes, but are not aged. Gins generally are produced from the fermented product, grain neutral spirits (GNS), to which either botanical extracts and/or flavors are added to the GNS and bottled, or dried botanicals (e.g., juniper berries) are added to the GNS to extract their oils and then distilled. Rums are made from fermented sugar cane products, such as molasses. Brandy is an alcoholic distillate or mixture of distillates obtained from the fermented juice, mash, or wine from grapes or other fruit (e.g., apples, apricots, peaches, blackberries, or boysenberries) [1]. *Rakı* is a traditional Turkish spirit with 45-50% of alcohol. For production, dry grapes are cut down into small pieces and water is added for fermentation. After that, alcohol content is increased by sugar addition and distillation. Spirit is then incubated for maturation about 1-4 months where anise seeds are added as flavour. [17].

3. Characterization of alcohol production effluents

Distillery effluents are reported as medium to high-strength organic wastewaters. A variety of raw materials used in alcoholic beverage production yields diverse characteristics of wastewaters. In brewery industrial processes, large quantities of wastewaters with high organic content are generated due to dissolved carbohydrates, alcohol from beer wastes, and a high content of suspended solids, e.g. spent maize, malt, and yeast [8,18,19,7]. Biological treatment of wine distillery wastewaters faces problems of both high organic loadings and recalcitrant compounds like caramels, melanoids, tannins etc. [3]. Distilleries that process molasses produce large amounts of dark brown colored effluents with an acidic character [3-5]. High pollution load of the effluents may result in eutrophication which could prevent penetration of sunlight from the surface of water bodies, thus, reduce photosynthesis and lead to anaerobic conditions which is lethal to most aquatic life. The colored nature of wastewaters has almost same impacts as eutrophication by means of light penetration and deoxygenation [4]. Toxic effects of undiluted distillery effluents on freshwater ecosystem have already been reported [9,14,20-23].

pH, biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total organic carbon (TOC), total solid (TS), total volatile solid (TVS), total suspended solid (TSS), total dissolved solids (TDS), chlorides, sulfate, phosphate, total nitrogen (TN), total kjeldahl nitrogen (TKN), total phosphorus (TP) are reported as the most commonly analyzed parameters for wastewater characterization. Table 1 gives a list of characteristics for distillery, brewery and winery wastewaters. Obviously, distillery wastewaters have a high COD content ranging from 15.000 to 190.000 mg/l [15,6,10,24,11,16,25]. Organic matter concentrations for brewery is in the ranges of 300-5.400 mg COD/l [7,8] whereas that of wine distillery varies from 3.100 to 40.000 mg COD/l [5,9]. Distillery wastewaters have an acidic pH between 3-6 [15,6,10,24,11,25]. Wine distillery effluents also show acidic characteristic with a pH of 4-5 [9,13] whereas that of for brewery wastewaters is in the range of 5.6-7.2 [7,8]. Total nitrogen content varies from 350 to 2.180 mg/l due to the production stages and raw material type used in distillery [15,6,10,16,25]. Similarly, total phosphorus ranges from 120 to 690 mg/l for reported distillery effluents [15,6,16,25].

4. Treatment strategies for effluents of alcohol industries

Elimination of high pollution load of alcoholic beverage, mainly distillery effluents is increasingly important by means of environmental aspects. Parallel to the pollution load, treatment costs are generally high. Treatment is generally applied as sequential anaerobic-aerobic and post treatment technologies. In case of aerobic treatment, excess sludge production, inefficient COD removal, aeration cost and sludge bulking are primary problems [26]. Compared to aerobic treatment, use of anaerobic treatment technologies has many advantages by means of less energy input, less sludge production, less nutrient demand and bioenergy recovery [3,27]. Besides the choice of treatment method, the variety of raw materials and seasonal changes of production regime also cause fluctuations in the treatment efficiency [4,5]. Therefore, it is important that the treatment system should be resistant against variations in wastewater characteristics and system shut-downs.

Due to the altering characteristics of wastewaters, a number of treatment approaches for alcoholic beverages can be applied by using combinations of different treatment systems. Anaerobic treatment technologies have been widely adopted for medium-high-strength industrial wastewaters [3,28]. Especially, high rate anaerobic reactors such as UASB and EGSB have been reported to be used for the treatment of brewery wastewaters [8,18,29-31]. These bioreactors, which are based on the principle of suspended growth, use granular sludge and provide an efficient COD removal. Sludge granules are formed by the aggregation of anaerobic bacteria and archaea and self-immobilized in the reactor [32,33]. On the other hand, anaerobic filter (AF) and anaerobic

fluidized bed (AFB) reactors are attached growth systems that include an inert matrix on which the microorganisms accumulate [34]. Hybrid systems are comprised of a sludge blanket and a filter in same reactor volume. The wastewater first passes through a UASB which is followed by a filter medium [28,34]. An advantage of this system is the filter acts as a barrier to prevent the washout of biomass. Two-stage processes employ separate reactors for acidogenic and methanogenic phases of anaerobic treatment. This system provides independent optimization for each phase since the nutritional requirements, pH optima, physiological characteristics; growth and nutrient uptake kinetics and tolerance to environmental factors are specific for the microbial consortia involved in acidogenic and methanogenic phases [35].

Studies carried out on full-scale and lab-scale anaerobic reactors showed that OLRs may vary from 2.5 to 30 kg COD/m^3/day [11,6,13,8,16]. İnce et al [6] studied two different full-scale UASB reactor systems treating raki industry effluents and reported that one had a COD removal of higher than 85% at an OLR of 6-11 kg COD/m^3/day and the other performed 60-80% COD removal efficiency at an OLR of 2.5-8.5 kg COD/m^3/day. Similarly, Diaz et al [7] worked on a full-scale UASB system treating brewery wastewater and obtained 80% COD removal for 11 kg COD/m^3/day at an HRT of 8 hrs. Gao and co-workers obtained 94% COD removal of distiller's grains with a UASB reactor operated at an OLR of 5.6 kg COD/m^3/day with an HRT of 2.5 days [36]. Parawira et al [29] investigated a full-scale UASB reactor treating opaque brewery wastewater which had a low treatment efficiency of 57%. The reactor was operated at an OLR of 6 kg COD/m^3/day and 1 day of HRT [29]. Akkuna and Clark [25] operated a lab-scale granular-bed anaerobic baffled reactor (GRABBR) treating whisky distillery effluent at an OLR of 2.4 kg COD/m^3/day and HRT of 4 days. The reactor was configured as a combination of a baffled system and UASB and performed 92% COD removal efficiency [25]. In another study carried out on a full-scale EGSB reactor treating brewery wastewater, 70-75% COD removal was obtained at OLR of 20 kg COD/m^3/day and HRT of 2 hrs [8]. Two-stage reactors reported for the removal of distillery and malt whisky wastewaters showed the highest COD removal efficiencies, 93% and 96% respectively [15,16]. In a study of two-stage AF-UASB system, AF was operated at an OLR of 2.5-5.1 kg COD/m^3/day and 10 days of HRT whereas OLR for the UASB stage was 0.6-2.5 kg COD/m^3/day and HRT was 20 days [15]. In a study that operated a two-stage UASB-UASB, average OLR was 19.4 kg COD/m^3/day and HRT was 1 day. A lab-scale anaerobic hybrid reactor treating distillery spent wash performed 80% COD removal with an OLR of 8.7 kg COD/m^3/day and 5 h of HRT [24]. A lab-scale upflow fixed film column (UFFC) reactor and an anaerobic fluidized bed (AFB) reactor treating a distillery spent wash and a high-strength distillery wastewater respectively, both performed 80% COD removal efficiency [11,12]. The UFFC reactor was operated at an OLR of 31 kg COD/m^3/day and 6 days HRT [11] whereas that of were 20 kg COD/m^3/day and 1.5-2 days for the AFB reactor [12]. In a study carried out on *awamori* distillery (a traditional Japanese spirit containing 25-30% alcohol and made from rice by distillation) - 80% TOC removal was obtained with an OLR of 18 kg TOC/m^3/day and a HRT 1.6 days [10]. Performance of a lab-scale DFB reactor treating wine distillery effluents was reported to be 85% TOC removal for 4.5 kg TOC/m^3/day and 1.3 h of HRT [13]. In a study that compares treatment performances for white and red wine, same COD removal efficiencies of 90% was obtained for HRTs of 1.5-2 days each and OLRs of 3-4 kg COD/m^3/day and 4-4.5 kg COD/m^3/day, respectively [9]. Venkata-Mohan et al [37] carried out a study for treating distillery effluents in an anaerobic sequencing batch biofilm reactor (AnSBBR). The lab-scale reactor was operated at an OLR of 9.6 kg COD/m^3/day and HRT of 1 day and performed 70% COD removal efficiency.

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Table 1 Wastewater characteristics of alcoholic beverages

Wastewater sources	Parameters														References
	pH	BOD_5 (mg/l)	COD (mg/l)	TS (mg/l)	TVS (mg/l)	TSS (mg/l)	TDS (mg/l)	Chlorides (mg/l)	Sulfate (mg/l)	TN (mg/l)	TKN (mg/l)	TP (mg/l)	Phosphate (mg/l)	TOC (mg/l)	
Distillery	5.2–5.9	24.000–27.000	49.000 – 53.000							975–1.320		613–690			[15]
Distillery	7.3–7.6		30.000-34.000							800-1.000		250-300			[15]
Rakı Distillery[1]	5.5 – 6	12.000–16.000	25.000–33.000						50-100	350–450		150-250			[6]
Rakı Distillery[2]	4 – 6	13.000–15.000	27.000–32.000							500–700		120–150			[6]
Awamori distillery	3.65	50	56							2.180				28.330	[10]
Distillery spent wash	3.3–3.9	45.000–60.000	90.000–130.000	149.000-166.000	45.000–48.000	9000–10.000	70.000–78.000	5.500–6.000	6.000–6.500						[24]
Distillery spent wash	3.0–4.5	50.000–60.000	110.000–190.000	110.000–190.000	80.000–120.000	13.000–15.000	90.000–150.000	8.000–8.500	7.500–9.000	5.000–7.000			2.500–2.700		[11]
Malt whisky		15.600-22.100	37.060-50.700								45.4-71.7	222-665	72.4 – 216.7		[16]
Whisky distillery	3.8	8.900 – 30.000	16.600 – 58.000			232 – 7.810	6.080 - 17.750		40 - 130		500 – 1.200	150 - 600			[25]
Brewery	7.2	2.4	4			1.3					100	15	7.1-45		[7]
Brewery	5.6-6.8		300-5.400			10-1.013			0-120						[8]
Wine distillery	4-5				900-1.600	1.200-1.900								5.500-6.000	[13]
Wine distillery	4.5-5.1		7.000-7.500												[9]

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5. Molecular tools used for microbial community analysis of bioreactors

Natural and engineered environmental systems have a complex microbial structure with a high genetic diversity which is related to process stability. Thus, microbial community is the key factor in biological treatment systems for determination of operational conditions that provide desired reactor performance.

Determination methods of microbial diversity can be divided into two classes namely culture-dependent and culture independent. Traditional methods depend on the cultivation and enumeration of microorganisms. However, the inadequacy of defined media for every type of species limits the applicability of this technique to 0.1% -10% of all microorganisms which underestimates the actual microbial diversity. Slow growth rate and unknown growth requirements of anaerobic microorganisms make their cultivation more difficult. In addition, cultivation methods provide information only about the physical structure of the microorganisms rather than the genotype [38].

Due to the complex nature of the microbial community in biological wastewater treatment processes, culture-independent methods are required for accurate analysis of microbial community. The limitations of cultivation-based techniques are overcome by molecular methods which are based on DNA/RNA analysis. Molecular methods can be classified as fluorescent *in situ* hybridization (FISH), polymerase chain reactions (PCR), cloning-sequencing, nucleic acid fingerprinting methods and quantitative real-time PCR (QPCR), fatty acid methyl ester (FAME) analysis, stable isotope probing (SIP), microarray and metaproteomics, etc [39]. With the introduction of molecular methods and tools, the microbial community dynamics of bioreactors was better understood. However, like all other techniques, there is not a single molecular method that prevails above others since all techniques have strengths and weaknesses as well as biases. DNA dependent methods like cloning or DGGE which indicates presence of species in the community, do not give accurate information about active populations whereas RNA dependent methods do. FISH gives advantage of observing active populations and visual information about communities; however, it can not identify all microbial structure since its boundaries are limited with the probes used in the study. Also some techniques produce different results which are sometimes confusing. Differences of results also indicate biases of molecular techniques which can be circumvented by application of different techniques together [7,18]. The following part summarizes the most common molecular methods used for microbial community analysis in bioreactors treating alcoholic beverage wastewaters reported in literature.

5.1 Fluorescent in situ Hybridization (FISH)

FISH is an r-RNA targeting method which gives information about the active microbial community. FISH is a 4-step process: (i). *Fixation* of the cells and permeabilization of the cell walls; (ii). *Hybridization* of the rRNA with fluorescently-labeled probes. (iii). *Washing* the unbound probes (iv). *Visualization* of the hybridized cells under epifluorescent or confocal scanning laser microscope (CSLM) with appropriate cubes. Probes can be designed to be complementary to species-, group-, or kingdom-specific target sites [38].

5.2 Polymerase Chain Reactions (PCR)

PCR is a method for amplification of specific DNA sequences. Prior to PCR, DNA from the sample is extracted via physical and/or chemical methods. PCR is the first step of most of the DNA-based methods and includes 3-steps : denaturation of the double-strand DNA, annealing of the primers that specify the region of amplification on single strands and extension of newly synthesized DNA strands. PCR is an exponential and cyclic reaction which yields 2^n copies of DNA (n= number of cycle) at the end of the full reaction [39].

5.3 Cloning-Sequencing

Cloning is applied for producing numerous amounts of DNA segments. In environmental samples, cloning provides the identification of each species as a single clone. Cloning can be followed by sequencing of clones to determine the phylogenetic diversity of the environmental or bioreactor sample from which DNA was originally extracted. The sequences can be compared with previously constructed databases helps to determine the species that is the closest phylogenetic relative of the sequence [39].

5.4 Nucleic acid fingerprinting methods

There are several fingerprinting methods such as amplified ribosomal DNA restriction analysis (ARDRA), (automated) ribosomal intergenic spacer analysis ((A)RISA), terminal-restriction fragment length polymorphism (T-RFLP), single-strand conformation polymorphism analysis (SSCP) and Denaturing/Temperature gradient gel electrophoresis (DGGE/TGGE) used for screening clone libraries, comparing diversity of various samples and following the changes in microbial community structure [39]. They are mostly based on similar principles for

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separation and characterization of samples. DGGE and TGGE are among the broadly used fingerprinting methods for community characterization of environmental and bioreactor samples. The PCR products are separated on a polyacrylamide gel containing a linear gradient of a denaturant mixture of urea and formamide or a linear temperature gradient. Separation of the DNA fragments is based on electrophoretic mobility of DNA molecules due to the nucleotide content and composition. DGGE/TGGE can successfully differentiate sequences that differ even in one nucleotide [40].

5.5 Quantitative real time PCR (QPCR)

Quantification of microbial cells in a sample is important by means of determining low levels of populations and abundances of species in total community. QPCR system is based on the detection and quantization of fluorescent signals coming from the amplicons. QPCR makes it possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template [39].

6. Evaluation of the microbial communities treating alcohol industry effluents

In brewery effluents treating anaerobic reactors, granular sludge is commonly used as seed sludge and provides an efficient COD removal. A granule is a consortium of microorganisms where main biodegradation processes take place. The microbial structure of granules was an important aspect of sludge blanket reactors. The granular consortium worked in harmony where substrate was degraded in its journey to the center of the granule. In outer layers, the presence of hydrolytic and fermentative bacteria was observed whereas in the middle parts hydrogen producing syntrophic bacteria and hydrogen utilizing archaea were present and in the core part acetate utilizing archaea existed. Table 2 gives a compilation of microbial ecology of selected anaerobic reactors treating alcoholic beverage wastewaters. In a study, FISH and scanning electron microscopy (SEM) were used to observe spatial distribution of microorganisms in granules [8]. The results showed presence of acetotrophic *Methanosaeta* like cells in cluster zones whereas between clusters presence of hydrogenotrophic archaea, *Methanobacterium*-like, *Methanospirillum*-like, and *Methanococcus*-like were observed. Also these archaea resided close to syntrophic bacteria, *Pelobacter*-like ethanol oxidizer and a propionate oxidizer [8]. Another study gave a focus to microbial ecology of granules by analyzing community with DGGE followed by sequence analysis of excised bands [19]. Bacterial bands were excised and identified as members of *Firmicutes, delta and gamma pretobacteria, Synergistes* and *Cytophaga.* Archaeal bands were identified as *Methanosaeta concillii* and *Methanobacterium formicicum* [19]. The findings of Chan and co-workers [19] were confirmed by the study of Liu et. al. [18]. In this study, several molecular tools were used to identify microbial ecology of granules. Bacterial clone library showed dominance of *Firmicutes* and followed by *gamma* and *delta* subclass of *Proteobacteria*. In the archaeal clone library, only *Methanosaeta concillii* were found; but membrane hybridization indicated that *Methanosaeta concillii* made up only 50% of the archaeal community whereas remaining part consisted groups of *Methanomicrobiales* except the genera *Methanosaeta* and *Methanosarcina*, *Methanobacteriales*, and *Methanococcales*. Membrane hybridization of bacterial cells showed that *Clostridium* spp. and *Desulfovibrio* spp. accounted for 67% and 23%, respectively. FISH results showed that hydrogen utilizing archaea like *Methanobacteriales* cells present mostly in outer layers where acetate using *Methanosaeta* cells resided in center of the granules. Nearly all of *Clostridium* spp. distributed in outer layers of granule where *delta-Proteobacterial* cells evenly distributed inside of the granule. In a study of Leclerc et al, [41] the diversity of archaeal community of 44 anaerobic reactors was investigated by SSCP. Archaeal diversity of the two reactors treating brewery wastewaters, an UASB reactor and a fixed film reactor, consisted of *Methanomicrobiales, Methanobacteriales* and *Methanosaeta* spp. In a recent study [7], microbial community structure of granule was investigated by combined analysis of cloning, DGGE, FISH and SEM. Researchers separated collected granules into three groups (Black, Brown and Gray) according their physical characteristics. FISH results of black and gray granules showed equal fractions of active bacteria and archaea, but active archaea percentage (78%) was much higher than bacteria percentage (22%) in brown granule. It has been showed that *Methanosaeta* was dominant genus (75-95%) in all granules where *Methanosarcina* group was only detected in black and brown granules. DGGE analysis of granules showed presence of *Methanosaeta concillii, Methanosarcina mazei* and *Methanospirillum hungatei* in all granules. DGGE analysis of bacterial diversity showed three different groups; *Firmicutes* (*Clostridium* spp.), *delta-proteobacteria* (*Syntrophobacter* spp.) and *Deferribacteres* (*Synergistes* spp.). Most of the archaeal clones were affiliated with *Methanosaeta concillii* where rest is affiliated with *Methanosarcina mazei* and *Methanospirillum hungatei*. In bacterial clone library half

Table 2 Microbial ecology of selected anaerobic reactors treating alcoholic beverage wastewaters

Reactor type	Wastewater type	Scale	Methodology	Abundance of Archaea	Abundance of Bacteria	References
UASB	Brewery	Full-scale	Cloning	100% *Methanosaeta Concilii*	60% Firmicutes (Clostiridium sporosphaeroides) 20% g-Proteobacteria (Xanthomonas spp.) 20% d-Proteobacteria (Desulfovibrio spp.)	[18]
			Slot-blot hybridization	50% *Methanosaeta Concilii* *50% Methanomicrobiales*, *Methanobacteriale*, *Methanococcales*.	*67% Clostridium* spp. 23% *Desulfovibrio* spp.	[18]
UASB	Brewery	Full-scale	Cloning	70% *Methanosaeta concilii* 25% *Methanosarcina mazei* 5% *Methanospirillum hungatei*	51% *Nitrospira (Magnetobacterium bavaricum)* 18% *Deferribacteres (Synergistes jonesii)* 8% *Firmicutes*	[7]
			DGGE	33% *Methanosaeta concillii* *33%Methanosarcina mazei* *33% Methanospirillum hungatei*	*30% Firmicutes* (*Clostridium* spp.) 30% *d-proteobacteria* (*Syntrophobacter* spp.) 30% *Deferribacteres* (*Synergistes* spp.)	[7]
Fluidized bed	Wine vinasse	Lab-Sclae	Cloning	*16% Methanosarcina barkeri* *16% Methanosarcina frisius* *16% Methanobacterium formicicum* 48% Thermophilic microorganisms Crenarchaea or Thermoplasma sp.	46% *Firmicutes* 16% *Cytophaga-Flexibacter-Bacteroides* group (CFB) 17% *Proteobacteria* 21% high-G-C gram-positive bacteria, green nonsulfur [GNS], *Spirochaetes, Synergistes, Planctomyces-Chlamydia*, and nonaffiliated)	[42]
CSTR	Lees and vinasses	Full-scale	SSCP	*35% Methanobacterium formicicum* *15% Methanoculleus palmaeoli* *15% Methanosarcina frisius* *35% Methanosaeta concillii*		[41]
Fixed Biofilm	Acidogenic vinasse	Full-scale	SSCP	*14% Methanobacterium formicicum* *14% Methanoculleus palmaeoli* *14% Methanosarcina frisius* *28% Methanosaeta concillii* *14% Methanobrevibacter wolinii*		[41]
UASB	Raki effluent	Full-scale	DGGE	*20% Methanosaeta soehngenii* *20% Methanobacterium formicicum* *20% Methanobacterium bryantii*		[43]
Baffled	Whisky effluent	Lab-Scale	SSCP	*25% Methanobacterium formicicum* *13% Methanoculleus palmaeoli* *13% Methanosarcina frisius* *13% Methanobacterium sunterraneum* *13% Methanosaeta concillii*		[41]
UASB	Rum vinasse	Lab-Scale	SSCP	*20% Methanobacterium formicicum* *20% Methanoculleus marisnigiri* *20% Methanosarcina frisius* *20% Methanosaeta concillii*		[41]

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of the clones were related to *Nitrospira* group, rest was related to *Deferribacteres, Firmicutes, alpha-proteobacteria* and *Chloroflexi* group [7].

In our study, microbial ecology of a full-scale EGSB reactor was investigated for one year by DGGE analysis followed by sequencing of excised bands. Parallel to the literature, bacterial cells belonged to *Firmicutes, Proteobacteria, Chloroflexi* whereas the archaeal community was dominated by *Methanosaeta* spp. This data are in agreement with other studies since the archaeal community was dominated by *Methanosaeta* spp. whereas other groups like M*ethanobacteriales and Methanococcales* present as minor groups (unpublished data). Granular sludge of the EGSB reactor was used in another study, as an inoculum for a lab-scale anaerobic sequencing batch reactor (ASBR). Microbial ecology of granules was investigated by FISH and cloning. FISH revealed an active population of microorganisms (80%). Both archaea and bacteria were in equal numbers. The Archaeal subpopulation composed of by genus *Methanosaeta* (35%), *Methanosarcina* (9%), *Methanobacteriales* (17%), *Methanococcales* (13%), and *Methanomicrobiales* (14%). Bacterial clone library grouped phylogeny as 41.5% to *Firmicutes*, 27.5% to *Proteobacteria*, 12% to *Spirocheates* and 10% to *Bacteriodetes,* respectively. Archaeal clone library showed the dominant species as *Methanosaeata Concilii* (63%) and *Methanoculleus marisnigri JR1* (23%) [44].

One of the topics highly discussed about granular structure was the interaction of hydrogen utilizing archaea and hydrogen producing syntrophic bacteria. Especially for the brewery wastewaters, main component was biodegradation of ethanol to acetate and H_2 which are thermodynamically unfavorable under standard conditions. The process can only be performed by syntrophic association of ethanol-acetogenic H_2-producing bacteria (e.g., *Pelobacter* sp. or sulfate-reducing bacteria in the absence of sulfate mainly *delta-proteobacteria*) and both hydrogenotrophic and acetoclastic methanogens [45]. The reaction was H_2 dependent where low concentration of H_2 favors it. Several studies reported the cell-cell contact or substrate exchange between acetogens and hydrogen-utilizing methanogens was essential for syntrophic ethanol or propionate degradation [46-48]. Most dominant archaea in the brewery-degrading granule was the genus *Methanosaeta.* It was also reported that *Methanosaeta* spp. improves granulation and result in more stable reactor performance [49-51].

Microbial ecology of winery effluents was studied by clone library formation of samples from a lab-scale fluidized bed (FB) reactor fed with vinassess [42]. A total number of 579 clones were sequenced and grouped under 146 OTUs. The distribution of bacterial clones was as 46% *Firmicutes,* 16% Cytophaga-Flexibacter-Bacteroides group (CFB), 17% Proteobacteria, 21% minor phyla (high-GC gram-positive bacteria, green nonsulfur [GNS], Spirochaetes, Synergistes, Planctomyces-Chlamydia, and nonaffiliated). Archaeal clones grouped into *Methanosarcina barkeri*, *Methanosarcina frisius*, *Methanobacterium formicicum* and thermophilic *Crenarchaea.* Eukaryotic clones were closely related to yeasts (46%), amoebae (16%), and *Tritrichomonas foetus* (37%) [42]. In the study of Leclerc et al, [41] among 44 reactors, eight were treating vinasses and six of these reactors were full-scale, remaining two were lab- and pilot-scale. Both pilot- and lab-scale reactors were FB reactors. Two of full-scale reactors were fixed film, the other two were continuous stirred-tank reactor (CSTR) and the remaining two were UASB and lagoon types. Both CSTRs were fed with same substrate and had an archaeal diversity similar to each other which composed of *Methanobacterium formicicum*, *Methanomicrobiales* spp., *Methanosaeta* spp. and *Methanosarcina frisus* (only in one reactor). As in CSTRs, two fixed biofilm reactors also showed very similar archaeal community structures to each other: *Methanobacterium formicicum*, *Methanomicrobiales* spp., *Methanosaeta* spp., *Methanosarcina frisus,* (following species were found only in one reactor) *Methanobrevibacter wolinii* and an unidentified archaea. The FB reactors fed with different effluents showed different archaeal community structure as well as two reactors fed with same type effluent but had different reactor types. Pilot-scale FB reactor fed with vinasses had archaeal species of *Methanobacterium formicicum* and *Methanosarcina frisus* as lab-scale reactor fed with wine vinasses had a more diverse archaeal population including *Methanosaeta* spp., *Methanomicrobiales* spp. and *Methanocullues marisnigri.* UASB reactor possessed *Methanosaeta* spp. and *Methanobacterium* spp. opposite to anaerobic lagoon which had *Methanomicrobiales* spp. and *Methanosaeta* spp. The findings showed that microbial diversity of anaerobic reactors fed with same type of substrate was similar to each other [41].

The reactor types used for treating wine distillery wastewaters were quite varying. Several studies reported a link between a stable functioning reactor and diversity of microbial communities. It was previously reported that functional stability in stirred tank reactors was associated with a wide diversity of methanogenic archaea linked to a specific eubacterial flora [52]. It has also been speculated that diversity of archaea was dependent on the type of reactor. The stirred-tank reactors seemed to harbor considerable archaeal diversity whereas UASB granules have less diversity. Adhesion and granulation can be considered as the main selection pressures in the diversity of archaea [41]. In another study, it was reported the microbial communities became very similar, whatever the start-up strategy or the sludge used to seed the bioreactors in fluidized bed reactors [53]. In another study, it has been reported that *Methanosarcinaceae* spp. were sensitive to turbulence and shear associated with high-efficiency reactors therefore opposite to *Methanosaeta* spp. was not present in UASB reactors [54].

Many industries which utilize distillation processes discharge high strength wastewaters which is quite diverse due to diversity of processes and fermented substances which may have higher impact on bacterial diversity

rather less on archaeal one. Also, there is not a unique type of anaerobic reactor as in brewery wastewater treatment [55,4]. In study of Leclerc et al., [41] there were 3 lab-scale reactors treating distillery wastewaters. One was treating whiskey distillery wastewater in an anaerobic baffled type reactor. The other two were treating rum vinasses is a UASB and FB reactor. Both reactors treating rum vinasses showed similar archaeal structures containing *Methanobacterium formicicum*, *Methanosarcina frisius* and *Methanosaeta concillii.* Two unidentified archaeal spp. and *Methanoculleus marisnigiri* were also present but not in both reactors. The baffled reactor contained a much diverse archaeal population of *Methanobacterium formicicum, Methanobacterium subterraneum, Methanomicrobiales* spp., *Methanosaeta concillii* and two unidentified archaeal spp. In another study [56], researchers analyzed aerobic and anaerobic treatment tanks of alcohol manufacturing plant for diversity of *Planctomyces*. Two clone libraries revealed sequences related to anaerobic ammonium oxidizers (ANAMMOX) which suggests the possibility of ANAMMOX reactions in anaerobic reactors treating alcohol distillery wastewaters [56]. Microbial communities of a thermophilic UAF treating *awamori* distillery wastewaters was investigated by FISH and cloning. FISH results showed that the populations of bacteria and archaea were in balance. Cloning experiments showed two different archaeal genera; 19 clones for *Methanoculleus bourgensis* and 3 clones for *Methanosarcina thermophilia* and two different bacterial genera; 10 clones for *Firmicutes* and 5 clones for *Bacteroidetes* [10]. In another study, a thermophilic anaerobic sequencing batch biofilm reactor (ASBBR) was fed with vinasse from sugar cane alcohol production at different OLRs [57]. An increase in sulfate reducing bacteria, specifically *Desulfovibrio* sp, was observed under microscope. Hydrogenotrophic archaea and *Methanosarcina* like spp. dominated over the *Methanosaeta* spp. after start-up which may indicate *Methanosaeta* spp. are more sensitive to variations in OLR [57].

In our study, microbial ecology of two UASB reactors, UASB-1 and UASB-2, treating distillery wastewater of *rakı* industry was investigated. Diversity analysis by DGGE, band excision and sequencing showed members of archaeal community including *Methanosaeta soehngenii*, *Methanobacterium formicicum* and two other archaea related to *Methanobacteriales* group [43]. Following this study, the changes in active archaeal community observed by SMA and FISH technique for two years. The SMA of the reactor was decreased from 344 mL CH_4 / g TVS day to 109 mL CH_4 / g TVS day. Parallel to this data, a decrease in active population of *Archaea* as well as *Methanosaeta* spp. was observed where after two years an increase of *Methanobacteriales* spp. was recorded [17]. In our recent study, microbial diversity of granular sludge from an UASB reactor treating a *rakı* distillery wastewater was investigated by DGGE and sequencing of excised bands. 16 bands were excised for diversity analysis of bacterial cells which showed presence of *Proteobacteria*, *Firmicutes*, *Chloroflexi* and *Actinobacteria* groups. 8 archaeal bands showed relation to known *Methanosaeta*, *Methanosarcina, Methanomicrobiales* species and four unidentified archaeal clones [44].

Spirit distillery wastewater has diverse characteristics due to use of different raw materials for alcohol production like rice for awamori, anise seed for *rakı*, sugar cane juice for rum etc. It has been reported that archaeal community were mostly affected by reactor types whereas bacterial communities were more dependent on composition of wastewater [58-60].

The dominancy of *Methanosaeta* spp. over hydrogen utilizing archaea was a topic which was investigated by many researchers. Study of Kolukirik et al [17] showed that the SMA activity decreased due to a decrease in the presence of active *Methanosaeta* spp. Also, appearance of hydrogen utilizing archaea was observed after the decrease in active *Methanosaeta* population. This finding was previously reported by other researchers [61-66]. It was reported that among the hydrogenotrophic methanogens, *Methanobacteriales* followed by *Methanomicrobiales* were dominant methanogens and *Methanococcales* were almost absent within both full-scale and lab-scale UASB reactors [39]. Presence of *Methanosaeta* spp. was not only important in methane production but also in stabilization of granule structure. Especially FISH studies visualized the location of these archaea in the core of granules [67]. It was also discussed that acetate utilizing archaea had a limited diversity where hydrogen utilizing archaea were quite diverse. In many studies, *Methanosaeta concillii* or other species of the genus appeared as dominant species in archaeal acetate utilizing community. A higher diversity among acetate utilizing archaea was expected since it was reported that carbon flow from acetate to methane made up 60-70% of produced biogas [68]. A possible explanation was that the reactions performed by hydrogenotrophic archaea were energetically more favorable than the reaction [69] and therefore, natural selection pressure was higher on the evolution of hydrogenotrophic archaea rather than acetoclastic ones [70].

7. Conclusion

A variety of raw materials and production processes are involved in alcoholic beverage production. The diversity of grains, fruits and vegetables used as raw materials leads to an assorted characteristics of wastewaters. These medium to high strength organic wastewaters are produced in vast amounts and have an acidic, recalcitrant, and colored nature all of which have been successfully removed by a number of aerobic-anaerobic and post treatment technologies. Suspended growth anaerobic biological systems such as UASB and EGSB reactors are widely used for treatment of alcohol industry wastewaters and provide an efficient COD removal. Furthermore, attached

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growth systems like UFFC, AF, AFB, DFB, AnSBBR reactors are also applied for treatment of alcoholic beverage effluents. Hybrid systems and two-stage processes are among alternatives of anaerobic treatment applications. Several points such as wastewater characteristics, reactor types and operational conditions are important in shaping microbial ecology of anaerobic reactors and optimizing treatment processes. In addition to analysis of wastewater parameters such as COD, BOD_5, nitrogen, phosphorus and solid content, total and active microbial population was investigated by a number of molecular tools; FISH accompanied by SEM, DGGE, cloning, slot-blot hybridization and SSCP. Use of these techniques in combination provides confirmed results about archaeal and bacterial diversity.

Studies are in agreement with each other that different effluents, reactor types and operating characteristics created different microbial consortia. Bacteria are responsible for hydrolysis and biodegradation of organic materials present in effluents; therefore, they are highly affected by the composition of wastewater. Their short doubling time helps them to sustain their lives in different reactor types. On the other hand, *Archaea* which can only utilize end products like acetate, methylated compounds or H_2-CO_2-formate for energy, are quite sensitive to environmental conditions due to their specialized metabolism and long doubling times. Studies also show that archaeal populations are more affected by reactor types instead. It is reported that *Methanosaeta* spp. which is considered as the most important archaeal species for biogas production, since main route for methane production was utilization of acetate, are negatively affected by low OLRs. The low diversity of acetate utilizing archaea is explained by natural selection pressures. Reactions performed by hydrogenotrophic archaea are energetically more favorable than the acetoclastic reaction, therefore natural selection pressure is higher on hydrogenotrophic archaea than acetoclastic ones. Different reactor types allow survival of different groups conducting same reactions. Therefore, it is highly recommended that a diverse population of microorganisms should be present in seed sludge to achieve a stable and efficient reactor performance.

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