

**Microbial diversity and Molecular Biomonitoring for
Efficient Treatment of Tannery Wastewater: Laboratory
and Field- Scale Studies**

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ABSTRACT

Microbial Diversity and Molecular Biomonitoring for Efficient Treatment of Tannery wastewater: Laboratory and Field- Scale Studies

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This dissertation reports on culture- dependent and culture- independent characterization of microorganisms of from tannery wastewater, with special reference to degradation of selected retanning chemicals. In the culture- based study, four bacterial strains isolated from a temporary storage site of composite tannery wastewater were reported to grow and utilize the retanning agents named as Basyantan AN and Retanal MD80 as their carbon and nitrogen sources. Ultra Performance Liquid Chromatography (UPLC) analysis of compounds in the retanning agents from batch cultures over a period of 14 days revealed an average of 18, 26 and 33% degradation of the three detected peaks of Basyantan by isolate B45. The same isolate also showed an average of 34% degradation of Retanal. Kinetic studies conducted on this isolate showed the dependence of growth on the concentration of Retanal as a substrate, with a maximum growth rate (μ_{max}) of 1450 cells ml⁻¹.hr⁻¹ and a half saturation constant (K_s) of 10.16 mg.l⁻¹. On the basis of 16S rRNA gene amplification, sequencing and phylogenetic analysis, the four isolates were identified as *Micrococcus luteus* strain B1R, *Brevibacterium pityocampae* strain B4500R, *Lysinibacillus sphaericus* strain R1902R and *Enterococcus gallinarum* Strain R2801R.

Bench- scale study involving a sequencing batch reactor (SBR) run for 42 days for studying the biodegradation of Basyantan and Retanal revealed a pronounced degradation up to 100% regarding peak 2 of Basyantan. Dynamics of sludge microbial sub- communities as followed by T-RFLP showed the dominance of members of the class Burkholderaceae and a less diverse community in the first five batches of the SBR run; which was then succeeded by members belonging to the phylum Bacteroidetes and classes Rhodocyclaceae, Rhodobacteraceae, Pseudomonadaceae and Bradyrhizobiaceae; with increased diversity towards the end of the run. Flow cytometric analysis of bacterial cells in the sludge was used to create patterns mirroring dynamics of bacterial community over the period of SBR run. The patterns were resolved in 30 sub- clusters eight of which were sorted and members identified. Correlation of the bacterial abundance in the sorted sub- clusters with the measured abiotic data showed the presence of a strong ($p < 0.01$) positive correlation between degradation of Basyantan and Retanal with members of Bacteroidetes and Proteobacteria, indicating the key degradation role played by these members.

Field- scale study on the structure and diversity of the microbial community in the sludge of the Anaerobic- aerobic reactors as well as the root area of a constructed wetland treating tannery effluent located in Modjo tannery was conducted using the 16S ribosomal RNA gene sequencing performed for each of the reactors. The results revealed a total of 31 different phylotypes with the dominant members belonging to *Firmicutes*, *Bacteroidia*, *Proteobacteria*, *Sphingobacteria* and *Synergistia* in the anaerobic and aerobic reactors. In the constructed wetland, members of the group *Cyanobacteria* were also identified in addition to *Firmicutes*, *Bacteroidia* and *Proteobacteria*. The presence of members of groups that are associated with reduction of sulfate

and removal of chromium, nitrate and degradation of synthetic aromatic compounds indicates their implication in the current performance of the treatment system. Furthermore, physico-chemical data (total Nitrogen, total Carbon, pH, Sulphate, Nitrate) have been recorded in the anaerobic, aerobic and constructed wetlands and analyses of these data elucidate the key factor structuring the observed microbial communities.

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1. Introduction

Ethiopia possesses one of the largest populations of livestock in Africa and is 9th in the world, with 41 million cattle, 25 million sheep and 73 million goats (ELIA, 2012). According to the same resource, 2.7 million hides, 8.1 million sheep skins and 7.5 million goat skins are sold on the market every year. The leather industry in Ethiopia has long been traditionally active in exporting hides and skins for the past centuries due to the rich livestock resource in the country. Ethiopia's leather industry has been recognized as the second foreign exchange earner with a total value of export of leather and leather products ranging between 66.9 million and 98.4 million USD in the years 2005 – 2007 (MoTI, 2008).

The conversion of hides and skins into leather generates large amount of liquid wastes of about 30- 35 liter per Kg of raw material processed (Suresh *et al.*, 2001), constituting high salt content, organic load with very high chemical and biological oxygen demands (COD and BOD), inorganic matter, dissolved and suspended solids, ammonia, organic nitrogen, and specific pollutants such as sulfides and chromium (Reemtsma & Jekel, 1997). According to UNIDO report (Ludvick, 2000), among the annual pollution loads of the then 15 tanneries of Ethiopia, total dissolved solids (TDS) takes the lion's share (18,000 ton) followed by COD (12,000 ton), chloride (10,000 ton) and suspended solids (9,000 ton). Likewise, characterization of the wastewater of Modjo Tannery, one of the tanneries in Ethiopia, showed high levels of total dissolved solids (TDS= 6646 mg/l) and chemical oxygen demand (COD= 11123 mg/l), were persistently discharged into Modjo River (Seyoum Leta *et al.*, 2003).

Besides the natural organic contaminants coming from leather and hides, a significant fraction of this group is made up of tanning and retanning chemical agents and surfactants which are used for final stage of the products (Anilin and Fabrik, 2007).

Retanning is one of the chemical-intensive procedures in the post-tanning operation which involves synthetic organic chemicals such as phenol, naphthalene, sulfonated phenols, aromatic ether, melamine-urea and naphthalformaldehyde, which are discharged after being used in the process of retanning and dyeing (Jankauskaite *et al.*, 2012; Reemtsma *et al.*, 1995; Reemtsma and Jekel, 1994).

Some of the compounds like naphthalformaldehyde have been related to endocrine-disrupting effects and some sulfonic acids have been shown to have mildly toxic and irritant properties (Hafida, 2005). Studies on monobenzothiazoles have also shown to be involved in tumor induction, toxic to aquatic organisms at 0.6 $\mu\text{mol. L}^{-1}$ and shown to hamper waste treatment mainly by interfering with the process of nitrification (Gold *et al.*, 1993; De Wever *et al.*, 1994; Czekowski and Roomoor, 1981).

Monobenzothiazoles and dithiobenzothiazoles have been reported as one of the most frequent allergens causing shoe dermatitis (Storrs, 1986; Jung *et al.*, 1988). Study by Hinderer *et al.* (1983) showed that dithiobenzothiazoles induced genetic damage to mammalian cells. Although some of these compounds can be degraded during water treatment, the degradation products may enter the rivers without being transformed into simpler degradable form that contaminate other reservoirs like ground water (Hafida, 2005).

Various techniques involving chemical and/or biological methods have long been applied to treat tannery wastewater as a whole, or individual process streams. These include physico-chemical (Song *et al.*, 2000; Tiglyene *et al.*, 2008), oxidation-based (Paschoal *et al.*, 2009; Preeti *et al.*, 2009), biological and phytological (Durai and Rajasimman, 2011) treatment systems. The hitherto approaches have given more

emphasis to physico- chemical and oxidation systems than to biological treatment methods owing to the high BOD and complex, nature of the tannery wastewater (Espinoza- Quinones *et al.*, 2009; Isaias Tadesse *et al.*, 2004). The employment of the physico- chemical and oxidation methods, however, is costly because of their chemical and energy demanding characters (Khan *et al.*, 2013; Song *et al.*, 2004).

Sequencing batch reactors (SBR) are among the different biological systems used for the removal of carbon and nitrogen from high- strength industry effluents, with the advantage of using minimum space and performing in an all-in-one manner (USEPA, 1999). The SBR has been used since the early 1970's for the biological treatment of wastewater (Irvine and Davis, 1971). Owing to its advances in process control technology, there is renewed interest in the SBR for the treatment of recalcitrant industrial wastewaters (Al- Rekabi *et al.*, 2007). SBR is also characterized by its ability to favor particular microbial groups capable of performing biological processes including nitrification, denitrification and utilization of aromatic compounds in the presence of toxic pressure (Artan *et al.*, 2003; Hafida, 2005).

Constructed wetland is one form of phytoremediation/ rhizoremediation in which, plants (macrophytes) and their associated microbes are used for environmental clean-up. They are simple, cheap, energy- efficient and can be used for secondary and tertiary treatment of municipal or domestic wastewaters, agricultural and industrial wastewaters, usually combined with adequate pre- treatment (Calherios *et al.*, 2006; Klomjek and Nitisoravut, 2005).

The subsurface flow (SF) type of constructed wetland is considered to be advantageous as it has little risk of odors and harboring insect vectors. In addition, the deep porous media in the bed provides greater available surface area for efficient

treatment (USEPA, 2000). Microbial populations the rhizosphere of macrophytes are composed of diverse and synergistic communities with exogenous metabolic activities controlled by plants' root exudates (Hirsch *et al.*, 2001). Such microbial diversity further promotes remediation of wastewater through the degradation of organic and inorganic compounds with their distinctive enzyme systems (Chaudhry *et al.*, 2005).

Stable performance of a biological wastewater treatment system can be achieved by understanding the microbial communities residing in the system in addition to the management of conventional process parameters (Günther *et al.*, 2012). For many decades now, microorganisms responsible for efficient reduction of pollutants in various biological wastewater treatment plants have been investigated (Amann *et al.*, 1998; Blackall *et al.*, 2002; Dabert *et al.*, 2001). Microbial studies on tannery wastewater treatment plants have been successfully conducted with regards to sulphur oxidation (Aguilar *et al.*, 2008), denitrification (Seyoum Leta *et al.*, 2005) and phenol degradation (Paisio *et al.*, 2012). Identified strains of microorganisms such as *Arthrobacter sp.*, *Comamonas sp.* and microbial consortium from activated sludges have been reported to degrade naphthalene-2-sulfonic acid and benzothiazole derivatives in tannery wastewater (Song *et al.*, 2005; Hafida, 2005).

The use of culture-independent molecular techniques has revolutionized the characterization of microbial communities from various habitats (Handelsman, 2004; Martin *et al.* 2006). Microbial diversity analyses of biological reactors employing culture- independent tools are unequivocally more powerful to reveal the complex microbiology of these ecosystems (Domingo *et al.*, 2011; Ravensschlag *et al.*, 2001). Likewise, molecular biomonitoring of biological systems at a single cell and molecular level using cytometric fingerprinting and molecular biological techniques,

is an important tool to investigate the functional role of microbial communities (Günther *et al.*, 2012; Chaudhry *et al.*, 2005; Jussila *et al.*, 2006).

Lefebvre and colleagues (2006) surveyed the diversity of microorganisms in four hypersaline wastewater treatment plants, three of which coming from tanneries, using 16SrRNA gene clone library construction. They reported a high microbial diversity characterized by 14 different bacterial phylotypes. A recent DGGE fingerprinting-based microbial study on tannery effluent- treating constructed wetland systems revealed the presence of diverse and distinct bacterial assemblages inhabiting different macrophytes (Calheiros *et al.*, 2009; Calheiros *et al.*, 2010).

In Ethiopia, currently there are 26 tanning industries in the country producing semi-finished and finished hides and skins (ELIA 2012). Most of these tanneries discharge their wastes without proper treatment resulting in surface and potential ground water pollution (Fisscha Itana, 1998; Seyoum Leta *et al.*, 2003), which needs to be addressed by establishing and maintaining cost-effective wastewater treatment option. A first attempt by Isaias Tadesse *et al.*, (2003) to use a series of pond systems (Advanced Integrated Wastewater Pond System, AIWPS) for the treatment of tannery wastewater has shown the success of this system in the removal of COD (90 – 97%), BOD (94 -99%), sulfates (82 – 95%) and chromium (92- 99%). Following this, culture- based studies in pilot-scale continuous- flow treatment systems for tannery wastewater have been carried out by isolating and characterizing efficient bacteria involved in nitrification and denitrification processes (Seyoum Leta *et al.*, 2004) and sulfide oxidation under denitrifying conditions (Dereje Teshome, 2007). The role of bioaugmentation of isolated bacteria and consortia from alkaline soda lakes into the pilot- scale systems have also been studied with respect to improvement in the overall process parameters and nitrogen removal (Tesfaye Minuta, 2006; Yemiserach

Mulugeta, 2008). Optimization of the process parameters such as BOD, COD, nitrogen, sulfides and chromium for an efficient treatment of tannery wastewater using lab- scale sequencing batch reactor was also performed by Andualem Mekonnen (2008).

In addition to the priority pollutants such as nitrogen, sulfates/ sulfides, carbon and chromium, tannery wastewater also harbours a number of xenobiotic compounds coming from the post tanning process. Despite the wide use post tanning chemicals, little information is available on their biodegradability and ecotoxicology. Furthermore, data on microbial communities from integrated systems involving anaerobic- aerobic SBR and constructed wetland treatment processes for the efficient treatment of tannery wastewater are lacking. Therefore, this study hypothesized that bacteria capable of degrading selected post tanning chemicals are available and it is possible to achieve biodegradation of these chemicals in a biological wastewater treatment system.

Objectives of the study

General objective

The general objective of this work is to monitor the dynamics and diversity of microbial communities controlling the biological processes of carbon and nitrogen in lab- scale reactor and field- scale integrated tannery wastewater treatment facilities and to investigate the biodegradability of selected compounds used in the retanning process by indigenous microbes from tannery wastewater.

Specific objectives

The specific objectives are:

1. To screen and identify bacteria capable of utilizing two retanning chemicals namely: Basyntan and Retanal as a sole source of carbon and nitrogen
2. To monitor microbial dynamics and identify key bacterial groups for the degradation of selected pollutants in a bench- scale level using sequencing batch reactor (SBR)
3. To study the composition, structure and diversity of microbial communities in a field- scale integrated tannery wastewater treatment system

2. Literature review

2.1. The leather

The production of leather involves the whole process of converting raw hides or skins into useful commodities such as shoes and garments from the meat industry (COTANCE, 2002). The source of hides and skins can be cows, oxen, sheep, goats, pigs, buffalo and horses, but most often the leather manufacturers use their raw materials from cattle, sheep and pigs (Heidemann, 1993). Hides and skins are processed to react with various chemical substances that prevent them from putrefaction to make them resistant to wetting and keep them supple and durable (Heidemann, 1993; Sharphouse, 1983).

The term “hide” is used for the outer covering of animals with a body surface of more than 1 square meter and a respective thickness of at least several millimeters. The term “skin” is used for the outer covering of smaller animals (Heidemann, 1993). Hides and skins contain three layers: grain, corium and flesh, containing several appendages like hair shaft and root, epidermis, sweat glands and sebaceous glands (Highberger, 1956). When the epidermis of the hide/ skin is carefully removed, a smooth layer is revealed which gives the characteristic grain surface of leather. Opposite to the grain side, the flesh side of the corium appears above the “meat”. In this layer, the fibers have a more horizontal angle of weave and fatty tissue may also be present. The corium is the most important layer because it consists of a network of collagen fibers which are intimately woven and joined together (Fig. 1). Collagen is one of the most important structural proteins that react with tanning agents during the processing (Heidemann, 1993; Highberger, 1956).

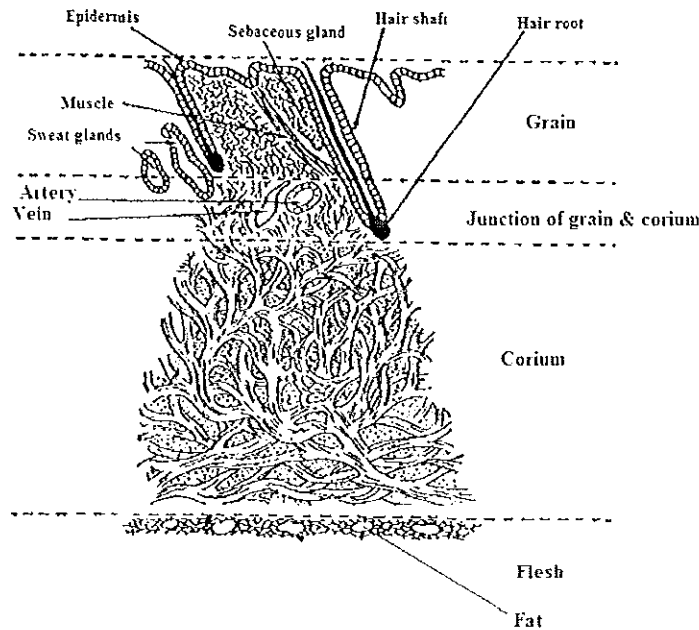


Figure 1 Cross- sectional view of a typical skin (After Sharphouse, 1983)

2.2. The industry: brief description of the processes

The process of leather production in a tannery is a chemical intensive process utilizing roughly 130 chemicals (Khan *et al.*, 1999), and the production process is generally divided into four main categories

a. The beam house operations

The first stage of beam house operation involves soaking of the hides and skins in water to restore the moisture content of the hide and skin and remove salt, dirt and manure from the hide. The process of dehairing, liming, deliming and bating subsequently follow the soaking step. The purpose of liming process is to open up the collagen structure by removing interstitial material and remove the hair and epidermis from the interior of the hide. The deliming process is used to neutralize the effects of the liming process, and to make the skin flaccid by releasing the bound water from the swollen protein fiber. The bating process removes the hair roots,

pigment and other protein substances left from the liming thus avoiding the precipitation of this matter onto the grain during subsequent tanning operations (Oke *et al.*, 2005; UNEP, 1991).

b. The tanning process

Tanning operation stabilizes the collagen fiber by using tanning agents to make it ready for further post- tanning processes. Hides can be tanned by using chrome tanning agents or vegetable tannins (Ockerman and Hansen, 1988). Wet blue is the term given to hides that have been tanned with chromium sulfate which can be stored for long periods and transported without being damaged by bacteria (IFC, 2007). Wet whites are skins and hides that are tanned using vegetable tanning materials consisting of polyphenolic compounds of two groups: the first one contains hydrolysable plant- based tannins such as chestnut and derivatives of pyrogallols such as myrobalan (Thorstensen, 1993), whereas the other group makes up the condensed tannins that are derivatives of catechol (Bienkiewicz, 1983). Leather that is tanned using vegetable tannins is much firmer than the chrome- tanned counterparts (Ockerman and Hansen, 1988).

c. The retanning process

Retanning, also called post- tanning operation, involves neutralization and fat liquoring to improve the feel and handle of leather and provide frame retarding, water and abrasion resistance properties (Jankauskaite *et al.*, 2012; Anilin and Fabrik, 2007). Furthermore, fat liquoring lubricates the leather and reestablishes the fat content. Retanning provides a characteristic nature of fullness on the finished product, thus represents a very important phase in the leather processing. Retanning can be carried out by employing various substances such as phenolic and naphthalene resins, melaminic resins, acrylic resins and polymers (Anilin and Fabrik, 2007).

Polymers include mainly polyacrylics, i.e. polymerization products from acrylic acid derivatives. These chemicals can be used with glutardialdehyde in pre- and post-tanning processes for filling and softening, as auxiliaries during fatliquoring and sometimes as replacements tannins in combination with vegetable extracts (Anthon, 1998; Zhang and Wang, 2009). They are strongly linked with peptide chain and chromium during the process (Fig. 4).

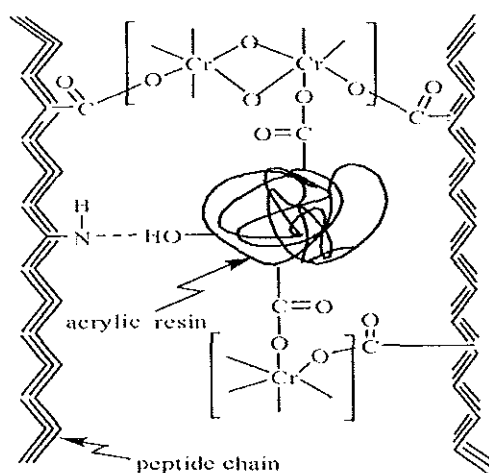


Figure 2 Interaction of resins (acrylic as example) with peptide chain and chromium
(After Zhang and Wang, 2009)

Melaminic Resins as retanning agents

Melaminic resins are condensation products from formaldehyde with amino and amido compounds like urea, melamine and cyanamid (dicyandiamide). The amine resins are polymers synthesized by condensation of urea, formaldehyde and melamine (2, 4, 6- tramino- 1, 3, 5-triazine) (Naviglio et al., 1996). The formaldehyde undergoes an addition reaction with amino group of urea or melamine with the formation of N-methylol groups as shown in Figure 2. Urea-formaldehyde resins are synthesized and chemically modified by reaction with a sulphating agent to form a

a.

urea

methyloleurea

dimethylolurea

b.

melamine

formaldehyde

trimethylolmelamine

Figure 3 Condensation of urea (a) and melamine (b) using formaldehyde (After Naviglio *et al.*, 1996)

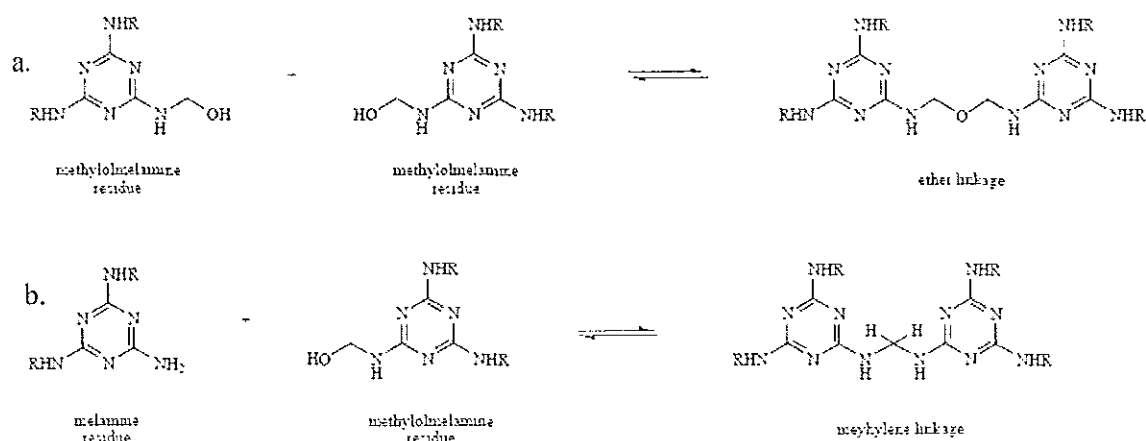


Figure 4 formation of melamine resin by the addition of two methylolmelamine residues (a) or a methylolmelamine residue with melamine residue (b) (After Naviglio *et al.*, 1996)

Phenolic and naphthalene resins as retanning agents

Phenolic and naphthalene resins are polymers synthesized using phenolic, naphthalene and their derivatives condensed with urea and formaldehyde. An example of phenolic resin also used in this study and its structure is displayed in Fig. 5. The synthesis reaction which is patented by BASF in 1913 involves reaction of the basic phenolic and/or naphthalene constituents under acidic conditions which results in attachment of the aromatic compounds to one another with the aid of formaldehyde through methylene bridges. Then they are adjusted to the optimum degree of condensation by making them binuclear or trinuclear and made watersoluble by sulfonation or sulfomethylation which are finally adapted by buffering to meet the application requirements (Anilin and Fabrik, 2007; Piotte *et al.*, 1995). Phenolic and naphthalenesulfonic retanning agents are useful on chrome tanned side leathers and splits. When used on chrome tanned leather, they specifically impart to the chrome leather a soft fullness and relaxed grain. These characteristics of mellowness and

softness are very desirable in glove, garment and soft type leathers (Danhong and Bi, 2010). Besides their use for retanning leather, phenolic and naphthalenesulfonic resins are also applied as drying agents, dye dispersants and solubilizers in the textile, paper and metal industries (Riediker et al., 2000; Piotte et al., 1995).

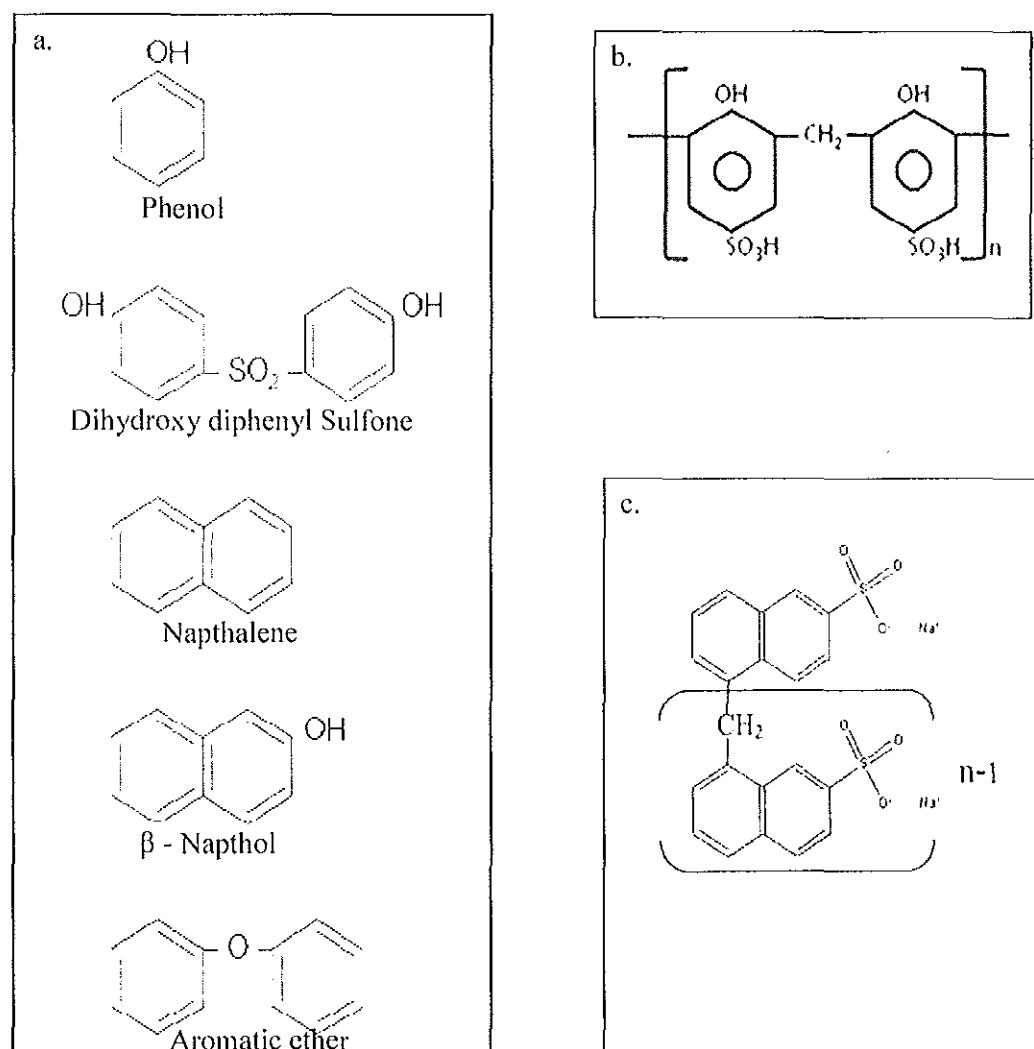


Figure 5 (a) Basic constituents of phenolic and naphthalene resins, (b) Structure of phenol formaldehyde condensate (b) Structure of naphthalene formaldehyde condensate (After Anilin and Fabrik, 2007; Sundarapandiyan *et al.*, 2010; Song *et al.*, 2003)

d. The finishing process

The final process mainly involves the application of paint to the leather surface to make the leather look good for longer while withstanding weather and wear (Anilin and Fabrik, 2007). The paint that is applied is usually a mix of water or other solvent to dilute the pigments/ dyes and resins that give color to the finish and bind to the leather surface to give toughness, stretch and flexibility to the finished product (Gerhard, 1996). Depending on the nature of the skin structure, different leathers need different amounts of finish applied to them. Some leathers are very lightly finished while others may need a lot of finishing (Anilin and Fabrik, 2007).

2.3. The importance of the leather industry and market

Africa has a considerable livestock wealth and is an important part of the economy. This leather industry sector contributes a significant portion to the national incomes of many Sub-Saharan countries including Ethiopia (UNIDO, 2001). In the sub-Saharan Africa, the livestock population is composed of 113 million bovine (7.8%), 128 million sheep (12%), and 153 million goats (24.4%) of the global population (Arunga, 1995).

Ethiopia is one of the few sub-Saharan countries where production of leather garments and other goods has developed to a reasonable standard and some Ethiopian factories are able to export these products to industrialized countries (FAO, 1998). Since 1993, Ethiopia has banned rawhides export to international market and recently has used high export levies on wet blues to encourage export of finished leathers (Mahmud, 2000). This is due to the fact that low prices for raw hides and skins combined with limited progress in processing to a semi-manufacture stage of production leads the country to loss of income. Thus value-added hide and skin manufacture is considered to be more preferable than selling raw hides and skins, as

long as the manufactured commodities are customer- tailored to avoid difficulties when trying to become internationally competitive. Thus a resource-based, value-adding, agro-industrial manufacture makes enormous economic sense for countries like Ethiopia, as they have considerable renewable livestock resources (Arunga, 1995; Mahmud, 2000).

2.4. Characteristics of wastewater of the leather industry

Though the leather-making process uses the by- product of the meat industry, it in turn generates by-products and wastes. The tanning process is almost wholly a wet process that consumes high amount of water, estimated to be 34 – 56 m³ of water per ton of hide or skin processed (Ludvick, 2000), where out of the total water consumed, 85% is discharged as a wastewater (Teodorescu and Gaidau, 2007). It is known that only 20% of the wet salted hides/skins are converted into commercial leather, while 25% becomes chromium-containing leather waste (CCLW), and the remainder becomes non-tanned waste or is lost in wastewater as fat, soluble protein and solid suspended pollutants (Alexander *et al.*, 1991). Therefore, environmental pollution is a difficult problem for the leather industry.

The characteristics of the wastewater vary considerably from tannery to tannery depending upon the size of the tannery, chemicals used for the specific process, amount of water used and type of final product produced by a tannery. The variations of effluent characteristics also occur through each working day in a tannery. According to Calheiros *et al.* (2007), average COD and pH analyzed in one day were 2010 mg /l ($\pm$ 516) and 6.98 ($\pm$ 0.05), respectively, whereas 2068 mg/l ($\pm$ 446) and 7.93 ($\pm$ 0.08) respectively, in another day. Table 1 summarizes the pollution load discharged from individual tannery processing operations.

Table 1 Characteristics of tannery wastewater from different sources

	Apayd in <i>et al.</i> , 2009	Calheiro <i>s et al.</i> , 2007	Ganesh <i>et al.</i> , 2006	Haydar <i>et al.</i> , 2007	Kongja o <i>et al.</i> , 2008	Kotesw ari and Ramani bai 2003	Lefebv re <i>et al.</i> , 2005	Seyou m Leta 2004	Orho n <i>et al.</i> , 2000	Ram <i>et al.</i> , 1999	Szpyrk owicz <i>et al.</i> , 2005	Thaniga vel, 2004
COD	3700	2250±565	4800±350	1320 - 54000	4100-6700	8000	2200	11123±563	2155	3114	2426	5650
BOD	1470	1000±88	-	840 - 18620	680-976	930	-	2983±259	-	1126	-	-
NH₄	180	-	225±18	-	144-170	-	-	122±8	166	33	335	-
Cr	-	0.027±0.075	95±55	41 – 133	41623	11	-	32±6	50.9	83	29.3	-
S²⁻	440	-	-	800 - 6480	-	228	-	630±67	35.6	55	286	-
TS	-	-	10,266±1460	-	-	-	-	-	-	18884	-	19755
SS	2690	92±36	2820±140	220 - 1610	600-955	2004	5300	-	915	1147	-	5025
VSS	1260	-	1505±90	-	-	1660	1300	-	578	-	-	-
pH	7.4	6.14±1.1	7.06 ± 0.26	-	7.0– 8.7	-	7.7	10.8-0.1	7.79	10.5	7.7	8.2- 8.5
TDS	-	-	18800-19700	-	-	15152	36800	6646±557	-	17737	-	14750

Most of the studies on pollution load of tanneries did not include chemicals that are involved in the process after the tanning step. This is partly because the pollution load of the chemicals used in the post- tanning process is included in some parameters such as COD and TDS. The other reason is the absence of fast and cheap method to detect these particular chemicals. Reemtsma *et al.* (1995) reported the presence of benzothiazoles in tannery wastewater in three forms: benzothiazol (BT), methylthiobenzothiazole (MTBT) and monobenzothiazole (MBT), with a dominance of MBT at a concentration of 3.3 - 6.9 $\mu\text{mol/L}$. These compounds have been detected in tannery wastewater samples by Fiehn *et al.* (1994) in concentrations of 655 $\mu\text{g/L}$ MBT, 10.5 $\mu\text{g/L}$ BT, and 39 $\mu\text{g/l}$ of MTBT. A recent report by UNIDO (2011) indicated that only 22% of all the chemicals used for post- tanning and finishing process is taken up and remained in/ on the leather, whereas from the remaining waste chemicals (88%), 23% belongs to fat liquors and 20% to dyestuffs.

2.5. Impact of discharging properly untreated water

The discharge of properly untreated wastewater to the aquatic environment can result in the accumulation of various pollutants in the environment, especially in water bodies. The consequences are loss of biodiversity, livelihood and of water quality which affects the whole ecosystem (Lefebvre *et al.*, 2005; Seyoum Leta *et al.*, 2003; UNEP, 1999). Therefore, treatment of wastewater is necessary to correct wastewater characteristics in such a way that the treated effluent can be reused or disposed off without causing an adverse effect on the receiving water bodies. Tannery wastewater treatment is important

not only to comply with environmental legislation but also to improve the competitiveness of the leather sector in the international trade (Mahmud, 2000).

Tannery wastewater is mainly characterized by being strongly alkaline with a high salt and chromium contents (Bajza and Vreck, 2001). Studies show that only 50 – 60% of the chromium applied is taken by the leather the rest is discharged as waste (Rajamanickam, 2000). As a non- essential mineral for microorganisms and other life forms, excess amounts of chromium exerts toxic effect on them after cellular uptake (Singanan *et al.*, 2007). Moreover, uptake of excess amounts of chromium by higher organisms like mammals has also been reported to have carcinogenic effect (Allison, 1996; Chaudhry, 1994).

The high amount of nitrogen in tannery effluent enriches plant nutrients which results in a proliferation of water weeds and algae leading to problems of eutrophication (USEPA, 2008; Seyoum Leta *et al.*, 2004). Nitrogen in the form of ammonia is toxic to certain aquatic organisms and is rapidly oxidized to nitrate, creating a high oxygen demand and depleting dissolved oxygen in surface water (Seyoum Leta *et al.*, 2004). Organic and inorganic forms of nitrogen may cause eutrophication in fresh water and coastal waters. High concentration of nitrate can also harm young children by causing methemoglobinemia (USEPA, 2008).

The sulfide content of tannery effluent results from the use of sodium sulfide, sodium hydrosulfide and the breakdown of hair in the dehairing process. When the pH of the effluent falls below 9.5, hydrogen sulfide is evolved from the effluent that creates unpleasant smell and causes toxicity for many forms of life (Buljan *et al.*, 2000).

Hydrogen sulfide gas is also fairly soluble, and when dissolved by condensation, weak acids can be formed which cause corrosion problem (USEPA, 2008). Sulfate is produced by the use of sulfuric acid, or through using products with high sodium sulfate content and is broken down by anaerobic bacteria to produce sulfide and odor. Soluble sulfate also causes a problem by increasing total salt concentration in the surface water and ground water (Bosnic *et al.*, 2000).

Many xenobiotic compounds produce a variety of biological effects, which is revealed when they are characterized mainly using bioassays. Unlike the extensive literature on the environmental impact of other xenobiotic pollutants, database available on the impact of the leather tanning processes based on retanning chemicals is scarce. The toxic and ecotoxic effects of benzothiazoles have been described by Recmetsma *et al.* (1995). The authors measured the luminescence inhibition (EC_{50}) of *Vibrio fischeri* and found at a concentration of 3 mg/L benzothiazole and the inhibition of nitrification in sediment columns at concentrations of 0.1 - 0.3 mg/L for benzothiazole and mercaptobenzothiazole. Hendriks *et al.* (1994) tested the acute toxicity of mercaptothiobenzothiazole and benzothiazole on *Daphnia magna* and found EC_{50} values of 10 mg /l for mercaptothiobenzothiazole and 50 mg /l for benzothiazole. Earlier study on the toxicity of Benzothiazole in mice had shown an LD_{50} at 900 mg /Kg, when administered orally (Sax, 1984).

With all the impacts discussed above, the discharge of properly untreated tannery effluent affects the ground water table of the surrounding locality to certain radius. Mondal *et al.* (2005) showed that a single tannery can cause pollution of ground water

about a radius of 7 and 8 Km. This will make the surrounding water source unsuitable for drinking, irrigation and for general consumption.

2.6. Treatment methods of tannery effluents

The methods that have so far been used for the treatment of tannery wastewater include physico-chemical, biological and plant- based (phytological) treatment methods. Physical system is a primary treatment which involves screening and sedimentation which is also used prior to biological treatment. The chemical system involves chemicals which are used to improve the settling abilities of suspended solids prior to a solids removal stage or adjust the properties or components of waste water prior to biological treatment (Song *et al.*, 2000; Tiglyene *et al.*, 2008).

Tertiary treatments use either physical methods such as ozonation, or chemical methods such as chlorination or other methods such as reverse osmosis to purify the water from biological agents as well as high salt concentration and make the water potable (Paschoal *et al.*, 2009; Preci *et al.*, 2009; Song *et al.*, 2004). Some of the biological treatment systems include pond systems, trickling filter, activated sludge process and sequencing batch reactors, while plant- based treatment systems are used for polishing wastewater. Some of the biological and plant- based systems used for the treatment of tannery wastewater are discussed below:

2.6.1. Activated sludge

Activated sludge process can be described as a complex enrichment culture comprising a mixture of generalist and specialist microorganisms (Blackall *et al.*, 1998). It is the most

widely used biological treatment process because the recirculation of the biomass allows microorganisms to adapt to changes in the wastewater composition by a relatively short acclimation process (Doan and Lohi, 2001). The entire process is based on the growth of microorganisms in the wastewater forming flocs, and when allowed to settle result in a relatively clear liquid free of organic material and suspended solids. Part of this growth is then wasted and the remainder is returned to the system (Dohse and Heywood, 1996).

According to Haydar *et al.* (2007), a BOD and COD removal of 90 and 80%, respectively can be achieved using activated sludge for tannery wastewater treatment. A study on biodegradability of fat liquor chemicals in activated sludge was conducted by Danhong *et al.*, (2008). Accordingly, six fat liquor chemicals were respirometrically assessed and four of the six chemicals namely: sulfated castor, neat foot oil, fish oil and lecithin were found to be biodegradable in the activated sludge while chlorinated paraffin and alkyl sulfonyl chloride remained recalcitrant.

2.6.2. Sequencing batch reactor

The sequencing batch reactor (SBR) is a fill and draw system involving activated sludge for wastewater treatment. In this system wastewater is added to a single batch reactor, treated to remove undesirable components and then discharged. Equalization aeration and clarification can be achieved using a single batch reactor (Mahvi, 2008). Sequencing batch reactors usually operate by a cycle of periods consisting of fill, react, settle, decant and idle (USEPA, 1999). The duration, oxygen concentration and mixing in these periods could be altered according to the needs of the particular treatment plant.

The difference between SBR and activated sludge system is that the SBR performs equalization, biological treatment and secondary clarification in a single tank using a timed control sequence. In activated sludge system, these unit processes would be accomplished by using separate tanks (USEPA, 1999). According to Dinesh *et al.*, (2004), a BOD and COD removal of 85 – 93% and 70 – 75%, respectively can be achieved for tannery wastewater using SBR. A work done by Andualem Mekonnen (2008) also reported a removal of 85%, 38%, 35% and 99.9% for COD, TN, NH₄ and Sulfide, respectively.

2.6.3. Constructed wetland

Historically, uncontrolled discharge of wastewater to natural wetlands led to an irreversible degradation of many wetland areas. This has led to studies on the understanding of the mechanism of natural wetland ecosystem for pollutant assimilation and the design of constructed wetlands to treat wastewater (Pries, 1994). Studies on the feasibility of constructed wetland for wastewater treatment were initiated in the early 1950's in Germany that was followed by the United States in the late 1960's, which then increased dramatically in scope during the 1970's. To date, the use of artificial wetlands for water and wastewater treatment has gained considerable popularity worldwide (Pries, 1994).

Wetlands constructed for wastewater treatment are classified as surface flow or free water surface (FWS) systems and subsurface flow (SSF) system. Free water surface (FWS) most resembles natural wetlands both in the way they look and the way they provide treatment. Both designs can be used to treat wastewater from individual and community sources, but FWS are more economical for treating large volumes of wastewater

(Sinclair, 2000). Bacteria play an important role in wastewater treatment using FWS system. Aerobic bacteria thrive in wetlands wherever oxygen is present, especially near the surface, while anaerobic bacteria thrive in the lower substrate where there is little or no oxygen and convert the wastes into methane, carbon dioxide and biomass (USEPA, 1993).

In subsurface flow (SSF) wetland, a cell is filled with a treatment media, such as rock or gravel, which is placed on top of lining on the cell bottom (Reed, 1993). The saturated media and soil, together with the emerging plants' roots provide a greater surface area. This facilitates bacterial attachment and growth on the various surfaces and enhances break down of organic materials which, together with natural chemical processes, are responsible for approximately 90% of removal of pollutants (USEPA, 1993). All in all, in subsurface constructed wetland water goes through a series of purification process, which includes biological degradation, filtration, sedimentation and adsorption resulting in the significance reduction of organic compounds, suspended solids, nitrogen compounds, and pathogen (Reed *et al.*, 1987; Reed, 1993).

In properly functioning subsurface constructed wetland (SSF) system, the wastewater flows just below the media surface and remains unexposed to the atmosphere and saturates the layer below. The SSF system is prone to clogging if not properly managed. To solve this problem, studies recommend the application of organic loadings lower than 67.25 Kg BOD₅/ha/day (Metcalf and Eddy, 1991) or in the range of 67 -- 157 Kg BOD₅/ha/day, and for TSS 45 -- 168 Kg TSS/ha/day (USEPA, 1999). Garcia *et al.* (2004) have also suggested that an organic loading of 200 Kg/ha/day is optimal for urban wastewater to obtain a BOD₅ removal reaching as high as 90%.

Different researchers have investigated the wide use of constructed wetlands for different types of wastewater, including domestic (Kaseava, 2003; Kyambadde, 2005; Berhanu Genet, 2007), industrial (Prabu and Udayasoorian, 2003; Maine *et al.*, 2006; Sohsalam *et al.*, 2007; Calheiros *et al.*, 2007), agricultural runoff and dairy (Pucci *et al.*, 2000) and polluted river water (Jing *et al.*, 2001), and have been shown significant improvements in water quality in these systems. Studies have shown the use of SSF for different types of wastewater. Calheiros *et al.* (2007) reported TSS removal of 92% and 57% at a HRT of 6.8 days and 3.4 days using *Typha latifolia* for tannery wastewater treatment.

2.7. Microbial ecology: Overview of approaches

Microorganisms are responsible for most of the biogeochemical cycles such as recycling of carbon, nitrogen, phosphorous and sulfur; that shape the environment and influence all lives on Earth. Moreover, they are potential players in the decomposition of xenobiotic compounds (Field *et al.*, 2006; Garbeva *et al.*, 2004). Microbial ecology deals with the study of microbial interactions with each other and their environment, attempting to answer questions that arose while discovering and characterizing any natural or artificial ecosystem (Amann *et al.*, 1998; Riesenfeld *et al.*, 2004). Some of the biochemical- and molecular- based approaches for studying microbial community composition over time and space in response to environmental changes are discussed below.

2.7.1. Culture- based methods

Culture techniques to characterize microbial ecology generally involve sample dilution and spread plate inoculation for isolation and characterization of microorganisms. To

maximize the cultivable fraction of microbial communities, media other than the commonly known growth media have been devised to mimic natural environments in terms of composition and concentration of nutrients, oxygen gradient and pH (Kirk *et al.* 2004).

Despite these efforts, large number of the microorganisms remain unculturable, including 85–99% of those in water (Staley and Konopka, 1985), 99–99.9% of soil bacteria (Clardy, 2007), over 90% of the bacteria in wastewater (Manz *et al.*, 1994) and 93–99.9% of the bacteria in drinking water (Hammes *et al.*, 2008). Although viable in their natural environments, these organisms do not grow under laboratory conditions and remain in a “viable but nonculturable” (VBNC) stage (Oliver 2005). Such organisms may be abundant or very active but remain untapped by standard culture methods; hence there is a need for approaches that can better reveal the complex profile of the different microbial communities in different ecosystems.

2.7.2. Culture- independent methods

Culture-independent approaches use nucleic acids, lipids or proteins as a primary source of information for studying microbial communities (Handelsman, 2004). The most common methods include analyses of genes of evolutionary significance such as 16S and 18S rRNA (ribosomal RNA) for prokaryotes and eukaryotes, respectively (Amann *et al.*, 1998). Over the last few decades, a wide variety of molecular techniques have been developed for describing and characterizing functional diversity of microorganisms using

sequences of functional significance (Schloss and Handelsman, 2004; Schloss and Handelsman, 2003).

2.7.2.1. Clone Library Method

The clone- library method is the most widely used method to analyze PCR products collectively extracted and amplified from an environmental sample (De Santis *et al.*, 2007). It involves cloning amplified gene fragments followed by sequencing, which are compared to known sequences in a database such as GenBank, Ribosomal Database Project (RDP), and Green genes (De Santis *et al.*, 2006). The sequences are then assigned to the correct taxonomic level at sequence similarity cut-off values ranging from 80 to 97% (De Santis *et al.*, 2007).

While clone libraries of 16S rRNA genes permit an initial survey of diversity and identify novel taxa (Venter *et al.*, 2004), studies have shown that environmental samples like soil may require over 40,000 clones to document 50% of the richness (Dunbar *et al.* 2002). As an example, a study by Rastogi *et al.* (2010) studied the microbial community composition of deep subsurface soils affected by previous mining activity in USA by using 230 clone sequences, and only revealed a partial view of the whole picture present in the soil.

Rarefaction analyses of the clone libraries also indicated that diversity was not exhaustively sampled due to insufficient clone sequencing, which is a common drawback for clone library- based study. Despite its being labor-intensive, time-consuming and expensive, clone library is still considered the “gold standard” for preliminary microbial

diversity surveys (De Santis *et al.* 2007). With the advent of fast, reproducible and cost effective sequencing methods, great progress is expected in this method for microbial diversity analysis.

2.7.2.2. *Fingerprinting methods*

Genetic fingerprinting generates a profile of microbial communities based on analysis of DNA length polymorphism of the PCR products amplified from environmental collective DNA (Ramette, 2009). Fingerprinting approaches have been used to compare and infer relationships between microbial communities and effect of various factors on microbial communities (Muyzer 1999; Ramette, 2009). Some of these techniques include ARDRA, T-RFLP, DGGE/TTGE, SSCP, ARISA, and RAPD. Among these, ARDRA and TRFLP will be discussed briefly.

A. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Amplified ribosomal DNA restriction analysis (ARDRA) is based on DNA sequence variations present in PCR-amplified 16S rRNA genes (Smit *et al.* 1997). The PCR product amplified from environmental DNA is generally digested with tetracutter restriction endonucleases (e.g., *AluI*, and *HaeIII*), and restricted fragments are resolved on special agarose or polyacrylamide gels. Besides rapid monitoring of microbial communities over time and comparative diversity studies in response to changing environmental conditions (Heyndrickx *et al.*, 1996; Smit *et al.* 1997), ARDRA is mainly used for estimating OTUs in environmental clone libraries based on restriction profiles of clones, which reduces massive sequencing of clones (Heyndrickx *et al.*, 1996).

B. Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) uses fluorescently labeled primer at the 5' end during the PCR reaction. The resulting PCR products are digested with restriction enzyme(s), and terminal restriction fragments (T-RFs) are separated on an automated DNA sequencer (Thies, 2007). Since only the terminally fluorescent labeled restriction fragments are detected, community diversity is estimated by analyzing the size, numbers, and peak heights of the resulting T-RFs (Schutte *et al.*, 2008). Each T-RF is assumed to represent a single OTU or ribotype.

With recent developments in bioinformatics, several Web-based T-RFLP analysis programs have been developed, which enable researchers to rapidly assign putative identities based on a database of fragments produced by known 16S rDNA sequences (Abdo *et al.*, 2006). Similar to ARDRA, a T-RFLP pattern is characteristic of the restriction enzyme(s) used, and more than two enzymes should typically be applied (Thies, 2007). One pitfall of T-RFLP method is that it underestimates community diversity because different bacterial species can share the same T-RF length (OTU overlap or OTU homoplasy). Nonetheless, the method does provide a robust index of community diversity, and T-RFLP results are generally very well correlated with the results from clone libraries (Fierer and Jackson 2006).

In order to understand the influence of abiotic parameters on microbial communities of various environments, various studies have been conducted employing T-RFLP analysis. Tiquia (2005) studied the diversity and dynamics of bacterial and fungal communities in manure composts at different stages of composting and indicated the effects of temperature and organic matter content on the diversity of microorganisms indicated by

marked shifts of T-RFLP fragment patterns. In a recent study, Stomeo *et al.* (2012) longitudinally assessed soil microbial community structure in various spots of the McMurdo ice-free Dry Valley region in Antarctica using T-RFLP and statistically determined the strong influence of potassium and calcium salts together with organic carbon on the distribution and structure of soil microbial populations in the region.

2.7.2.3. Single cell- based approaches

The study of individual microbial cells in a culture-independent manner involves established methods such as microautoradiography, fluorescence in situ hybridization (FISH), stable isotope probing of nucleic acids, flow cytometry and cell sorting (Amann *et al.*, 1998; Günther *et al.*, 2009; Kleinstüber *et al.*, 2006). The FISH method was used to track a bioaugmented strain, *Brachymonas denitrificans*, in biological reactors for the efficient removal of nitrogen in the system (Seyoum Leta *et al.*, 2005). In another study, the dynamics of bacterial populations in agricultural soils treated with s-triazine herbicides were followed using different molecular probes targeting the different subdivisions, revealing a sharp change in the abundance of γ -Proteobacteria populations in treated soil compared to control soil with no s-triazine treatment (Caracciolo *et al.*, 2010).

Flow cytometry and cell sorting

Flow cytometry is a technique which involves measurements on individual cells within the size range of 0.5 μm to 40 μm diameter using the principles of light scattering, light excitation and the emission of fluorochrome molecules (Shapiro, 2002). Light scattering occurs when a particle or a cell flowing in a fluid stream deflects incident laser light. The extent to which this occurs depends on the physical properties of a cell, namely its size

and internal complexity. The forward scatter light (FSC), which refers to light scattered at low angles provides information on cell size (Julia *et al.*, 2000), and light scattered in an orthogonal direction, also known as side scatter or (SSC) provides information about granularity and cell morphology and is collected by a different detector (Shapiro, 2002). These characteristics, together with fluorescence intensity are determined using an optical-to-electronic coupling system which records how the cell or particle scatters incident laser light and emits fluorescence.

Flow cytometry in microbial cell analysis is used for rapid cell counting in studying heterogeneous bacterial populations (Müller and Nebe-von-Caron, 2010), strain improvement in industrial microbiology (Müller *et al.*, 2010), and sorting of bacteria for further molecular analysis (Comas-Riu and Rius, 2009). When employed in conjunction with fluorescent dyes, it is possible to measure biological parameters such as nucleic acid content, metabolic activity, enzyme activity and membrane integrity (Joux and Lebaron, 2000; Walberg *et al.*, 1999).

Among a wide range of fluorescent dyes in use, 4', 6-diamidino-2'-phenylindole (DAPI), which intercalates with the AT rich sites of DNA is widely used for applications in environmental microbiology (Günther *et al.*, 2009). DAPI labeling produces high-resolution sub-community patterns. Günther *et al.* (2009) conducted a study on composition and dynamics of microbial community from a domestic wastewater treatment plant and differentiated up to 20 different sub-communities on the bases of forward scatter (FSC) versus DNA content clustering.

Although flow cytometry helps identify cells within microbial communities that share distinct physiological characteristics, the technique cannot reliably link these properties with the metabolic state of the individual cell. Cell sorting of interesting sub communities is a powerful tool for resolving natural communities. Cell sorting allows to control fluorescent-based knowledge and to yield profound information by including subsequent culture- independent analysis such as T-RFLP and sequencing technologies (Müller *et al.*, 2009).

Some of the applications of cell sorting include detection of metabolically active species within communities (Kalyuzhnaya *et al.*, 2008; Günther *et al.*, 2009). In addition, cell sorting of individual uncultivable microbial cells in combination with single-cell DNA sequencing was used to generate reference genomes of uncultured taxa from a complex microbial community ,and analyze biogeography and metabolic adaptation by comparing metagenomic data records (Stepanauskas and Sieracki, 2007; Woyke *et al.*, 2009). Thus, bacterial flow sorting is an intermediate between different experiments to increase the resolution of a flow- cytometrically disintegrated community.

2.7.3. Functional Microbial Ecology: Linking Community Structure and Function

Understanding how microbial communities function in natural environments is a central goal in microbial ecology (Wellington *et al.*, 2003). By linking ecological processes in the environment with specific microbial sub- communities, it is possible to answer questions like what factors and resources govern the enormous genetic and metabolic

diversity in an environment. Such understanding can be applied in bioremediation techniques, energy generation processes, and in various biotechnological industries (Gibson and Muse, 2004).

In earlier studies, RNA extracted from environmental samples revealed the active microbial communities (Torsvik and Ovreås, 2002). Because RNA is an indicator of functionally active microbial populations, the amount of rRNA in a cell roughly correlates with the growth activity of bacteria. In addition to mRNA of functional genes allows the detection and identification of bacteria and actually express key enzymatic activities under specific conditions (Wellington *et al.*, 2003). Using this approach, genes that are useful for ammonia oxidation, nitrogen fixation, denitrification, and sulfate reduction have been amplified from RNA isolated from microbial communities to obtain insights into key microbial processes (Hansel *et al.* 2008; Lorenz *et al.*, 2002; Rondon *et al.*, 2000).

An alternative and newly emerging approach is based on the combination of cell sorting for interesting sub-communities that are either very stable or very fluctuating with phylogenetic or proteomic approaches (Koch *et al.*, 2013). Such combination is a powerful tool to gain insight into community functioning. Kleinstuber *et al.* (2006) used combined approaches involving cell sorting and molecular phylogenetic characterization to identify key organisms involved in degradation of hydrocarbons of diesel fuel under different salinity levels. Their findings revealed *Sphingomonas spp.* as a key organism in lower salinity levels whereas *Ralstonia spp.* and members of the genera *Halomonas*, *Dietzia* and *Alcanivorax* were found as key organisms prevailing in higher salinity conditions in diesel fuel degradation schemes.

A recent study by Günther and colleagues (2012) used combined approaches involving cell sorting and molecular phylogenetic characterization to identify key organisms behind stable performance of a municipal wastewater treatment system including maximum phosphate accumulation. Monitoring complex community is less challenging and inexpensive when combining flow cytometry with appropriate downstream molecular techniques (Müller *et al.*, 2010; Günther *et al.*, 2012).

2.7.4. Challenges and prospects in methods for analyzing microbial communities

All of the approaches available for community structure and function analysis have advantages and limitations associated with them, and none provides complete access to the diversity of complex microbial communities. Both culture- dependent and independent methods have their own pitfalls and are usually associated with bias (von Wintzingerode *et al.* 1997).

Most of the culture- independent approaches are technically challenging though conceptually simple. Biases associated with DNA extraction such as incomplete or preferential lysis of certain microbial cells can distort the community composition, richness, and microbial community structure (Handelsman, 2004). Biases associated with PCR include inhibition by humic acids, which are generally coextracted with DNA from soil. Annealing efficiency and specificity of primers sometimes cause preferential amplification of certain templates, which affects the quantitative assessment of microbial diversity (Feinstein *et al.*, 2009; Handelsman, 2004). Formations of PCR artifacts such

as chimeric molecules, deletion mutants, and point mutants could also lead to misleading results (von Wintzingerode *et al.* 1997).

To minimize some of the risks, Feinstein *et al.* (2009) suggested the use of several validated DNA extraction methods including DNA purification steps and dialysis or diluting template DNA and pooled DNA extracts prior to PCR analysis., However, these processes might lead to loss of DNA and PCR efficiency.

Considerable progress has been made in the characterization of microbial communities by the application of genomic, transcriptomic and proteomic approaches. Moreover, a combination of single cell- based approaches such as flow cytometry and cell sorting with molecular techniques are suggested to interrogate and monitor the diversity, function, and ecology of microorganisms (Riesenfeld *et al.*, 2004).

It is also important to note that the art of microbial cultivation should continue to provide culturable representatives of taxa that are of interest for physiological and phylogenetic reasons (Rappe and Giovannoni, 2003; Sait *et al.*, 2002). Such use of the above mentioned and other combined approaches reveals the interactions between genes, proteins and environmental factors, providing new insights into environmental microbiology.

2.8. Microbial degradation and transformation of xenobiotic compounds

The term xenobiotics, derived from two Greek words: *xenos*- meaning foreigner and *bios*- meaning life, refers to both manufactured and natural chemicals that are foreign to

the biological system and require catabolic pathways different from the pathways used for commonly available compounds (Rieger *et al.*, 2002). Xenobiotic substances are becoming major hurdles in municipal and industrial sewage treatment systems as well as agricultural runoff; due to the growing number of newly synthesized substances some of which resistant to biodegradation (Rieger *et al.*, 2002). Prominent examples are synthetic organochlorides such as plastics and pesticides, naturally occurring organic chemicals such as polyaromatic hydrocarbons (PAHs) and some fractions of crude oil and coal (Haritash and Kaushik, 2009; Swoboda- Colberg, 1995).

It has long been known that solid and liquid waste treatment systems and natural attenuation processes rely on microorganisms for the removal of the recalcitrant organic compounds (Young and Cernigla, 1995).

High interest in using microorganisms for environmental cleanup began after the huge oil spill of Exxon Valdez in Alaska, in March 1989 (Young and Cernigla, 1995). Since then, many microorganisms are being studied for their ability to degrade hazardous chemicals such as polyaromatic hydrocarbons (PAHs), benzene, toluene, xylenes (BTXs), polychlorinated biphenyls (PCBs) and chlorinated solvents such as perchloroethane (PCE) and trichloroethane (TCE) (Rieger *et al.*, 2002). For enhanced biological removal of these recalcitrant pollutants, effort is made to optimize the natural ability of the microorganism to catabolize the compound by enhancing the bioavailability of the compound and minimizing abiotic stress to the microorganism (Portier *et al.*, 1994). The resulting benefits that could be derived from the use of microorganisms in various biotechnological processes for removal of recalcitrant pollutants have stimulated research worldwide (Young and Cernigla, 1995). As a result, several microorganisms capable of

degrading different recalcitrant compounds have been characterized both at a single isolate- level and community levels (Rieger *et al.*, 2002). The enzymes responsible for catabolism of some of the compounds have also been identified and putative metabolic pathways have been proposed. This review describes about the biodegradation of two compounds involved in the current study.

Phenol

Phenol is found naturally in decaying dead organic matters and in coal; and anthropogenically it is the basic structural unit for a variety of synthetic organic compounds including agricultural, industrial and pharmaceutical chemicals (Anselmo and Novais 1984). Degradation of phenol occurs as a result of the activity of a large number of microorganisms including bacteria, fungi and actinomycetes (Anselmo and Novais 1984; Farrell and Quilty, 1999; Valli and Gold, 1991). Some of the bacterial species include *Bacillus sp.*, *Pseudomonas sp.*, *Acinetobacter sp.*, *Achromobacter sp.* (Reviewed in Basha *et al.*, 2010). Among these, the genus *Pseudomonas* is widely studied for the biodegradation ability of phenolic compounds (Kotresha and Vidyasagar, 2008; Song *et al.*, 2009).

Studies on phenol biodegradation have resulted in bringing out the possible pathways with the enzymes involved in the process. The enzymes identified include oxygenases (monooxygenases and dioxygenases), hydroxylases, peroxidases, tyrosinases and oxidases (Mahiudddin *et al.*, 2012). Like other aromatic compounds, the critical step in the metabolism of phenol is the destruction of the resonance structure by hydroxylation and fission of the benzoid ring to an intermediate known as catechol by dioxygenase-

catalysed reactions in aerobic conditions. Subsequently, the catechol is degraded via ortho- or meta- cleavage pathway (Fig. 6).

Both the ortho- and meta- cleavage pathways of catechol result in ring opening (Evans, 1947, cited in Basha *et al.*, 2010). Studies on the phenol and benzoate biodegradation pathway of the strain *Streptomyces setonii* revealed an ortho- cleavage pathway using catechol 1,2 dioxygenase (Basha *et al.*, 2010; Mahiudddin *et al.*, 2012). A similar study on the bacterial strain, *Serratia plymuthica* showed that the bacteria was able to tolerate phenol up to a concentration of 1050 mg/L and degradation via ortho- pathway, in the presence of ring cleaving enzyme catechol 1, 2-dioxygenase (Nilotpala and Ingle, 2007).

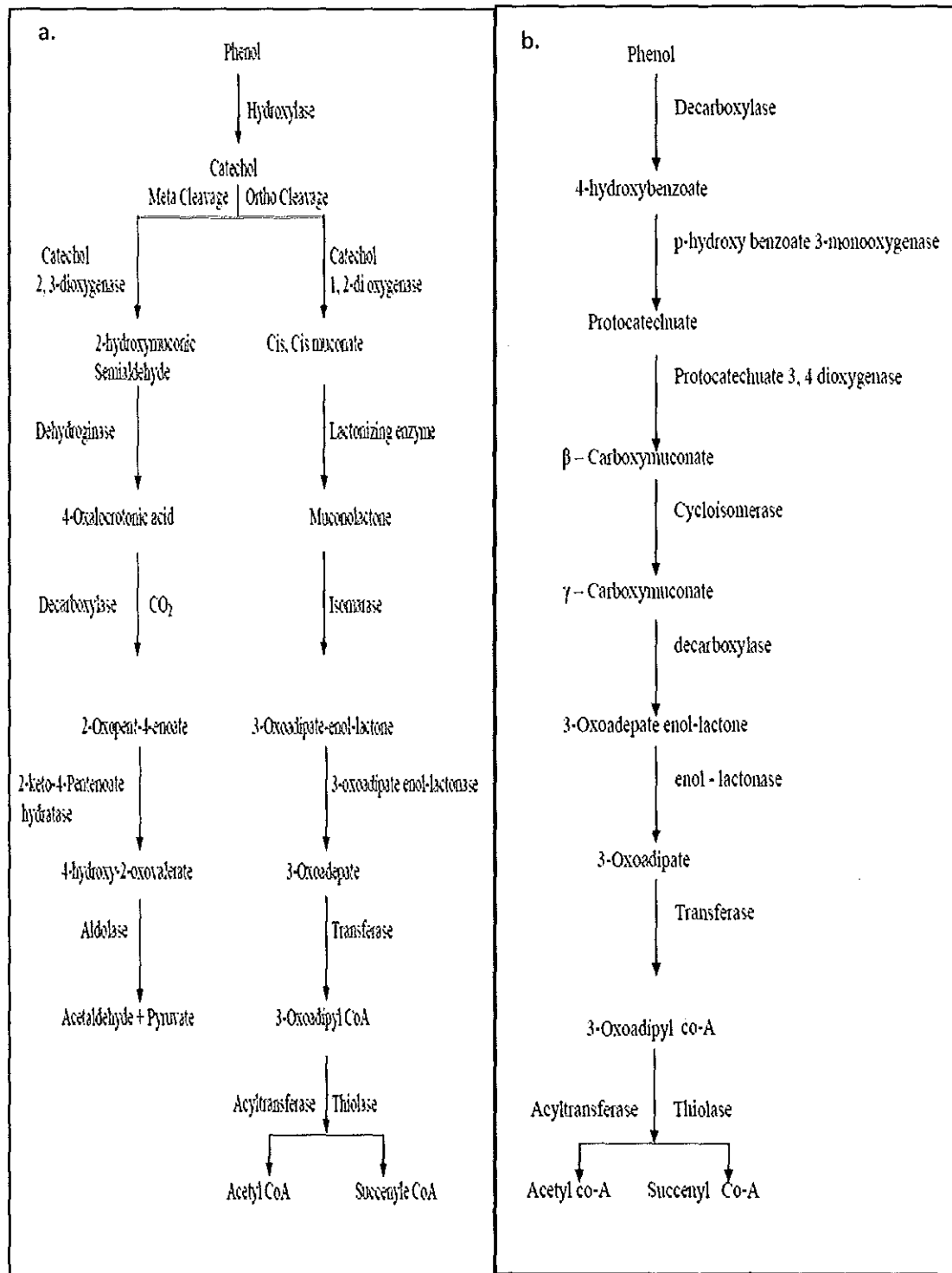


Figure 6 Proposed Phenol biodegradation pathways with the enzymes involved in aerobic conditions (a) and in anaerobic conditions (b) (After Basha *et al.*, 2010 and Mahiuddin *et al.*, 2012)

The microorganisms which utilize phenol by the aerobic pathway are *Acinetobacter calcoeticus*, *Pseudomonas* species and *Candida tropicalis* (Ahmed, 1995; Powlowski and Shingler, 1994; Bossert and Compeau, 1995). The genus *Pseudomonas* has been known for its versatility in utilizing different aromatic substrates, thus an attractive organism for use in waste water treatment applications (Mahiudddin *et al.*, 2012). Anaerobically, phenol can also be degraded by carboxylation in the para position to 4 - hydroxybenzoate by the enzyme decarboxylase (Fig. 6). The bacterial strains *Thauera aromatica* and *Desulphobacterium phenolicum* have been reported to degrade phenol under anaerobic conditions (Basha *et al.*, 2010).

The biological wastewater treatment processes used to treat phenolic wastewater include Activated sludge, fluidized, packed-bed and moving bed biofilm reactors (Basha *et al.*, 2010). Holladay *et al.* (1978) conducted comparative study of phenol degradation levels in stirred tank, packed bed and fluidized bed reactors and reported that fluidized-bed bioreactor was the most efficient in degrading phenolic wastes, followed by packed bed and stirred tank reactor. In this study, the factors affecting degradation rate were reported to be biomass development, feed concentration, liquid and air flow rates.

Melamine formaldehyde condensate

Melamine is a chemical commonly used in the manufacture of plastics, flame retardants; and is also a metabolite of the insecticide cyromazine (Miao *et al.*, 2009). In the leather industry, a condensate of melamine formaldehyde plays an important role in the final ability of water repellency and water adsorption of the leather in addition to its role in the filling and softening of the leather (Naviglio *et al.*, 1996). Melamine formaldehyde belongs to the triazine family, in which other members have been used extensively as

nitrogen fertilizers and herbicides for weed control (Allan *et al.*, 1989; Bhatti and Zuber, 2009); and their accumulation and persistence in the environment is well known (Miao *et al.*, 2009).

Melamine has attracted worldwide attention due to the marked event of tainted milk powder which resulted in a large number of renal failure in infants and young children in China in 2008 (Miao *et al.*, 2009). In December 2008, more than 294 000 cases of urinary tract stone were reported in China due to the consumption of melamine contaminated infant formula (Miao *et al.*, 2009). Earlier in March 2007, melamine tainted pet feed had caused an outbreak of renal disease and associated deaths of hundreds of cats and dogs in the United States (USFDA, 2008). The investigations revealed that melamine and one of its metabolites, cyanuric acid, are able to form an insoluble salt, may precipitate in kidneys, and cause renal functional failure (Miao *et al.*, 2009).

Various studies reported the biodegradation of melamine by soil pseudomonads and strains of the genera *Klebsiella* and *Micrococcus* (El- Sayed *et al.*, 2006; Garcia *et al.*, 2008). Biodegradation of melamine by most pseudomonads is effected via a stepwise deamination reaction producing the intermediates, ammeline, ammelide and cyanuric acid (Cook and Hutter, 1981). *Klebsiella terrigena* strain DRS-1 was able to deaminate melamine successively in a similar way to the other pseudomonads previously studied (Shelton *et al.*, 1997). In all cases, cyanuric acid is produced, a compound metabolized by several soil bacteria.

Melamine and the other members of the triazine family metabolized based on enzyme-catalyzed deamination and hydrolytic reactions (Cook, 1987; Wackett *et al.*, 2002). The most extensively studied enzymatic catalysis mechanism is of Atrazine, a chlorinated member of the s- triazine family (Radosevich *et al.*, 1995). Atrazine mineralization has been studied in *Pseudomonas* sp. strain A (Mandelbaum *et al.*, 1995). In this strain, displacement of the three substituents on the s-triazine ring is mediated by three enzymatic steps involving the enzymes AtzA, AtzB and AtzC (Garcia *et al.*, 2008). The enzyme AtzA, catalyzes the hydrolytic dechlorination of atrazine (chlorinated triazine) to yield hydroxyatrazine. AtzB subsequently catalyzes hydroxyatrazine deamidation, yielding N-isopropylammelide, and the third enzyme, AtzC, metabolizes N-isopropylammelide stoichiometrically to cyanuric acid and N-isopropylamine (Garcia *et al.*, 2008). Cyanuric acid is subsequently metabolized to CO₂ and NH₃ by AtzD, AtzE, and AtzF (Garcia *et al.*, 2008; Radosevich *et al.*, 1995).

The genes encoding atrazine catabolism in *Pseudomonas* sp. strain ADP, identified as *AtzA*, *AtzB*, *AtzC*, *AtzD*, *AtzE* and *AtzF* have been reported to be localized to plasmid pADP-1, which has been completely sequenced (Radosevich *et al.*, 1995). The above mentioned catabolic genes and their respective enzymes have also been identified in several other gram-negative bacteria such as *Ralstonia*, *Agrobacterium*, *Pseudaminobacter* and *Rhizobium* (Bouquard *et al.*, 1997; Radosevich *et al.*, 1995; Struthers *et al.*, 1998; Topp *et al.*, 2000).

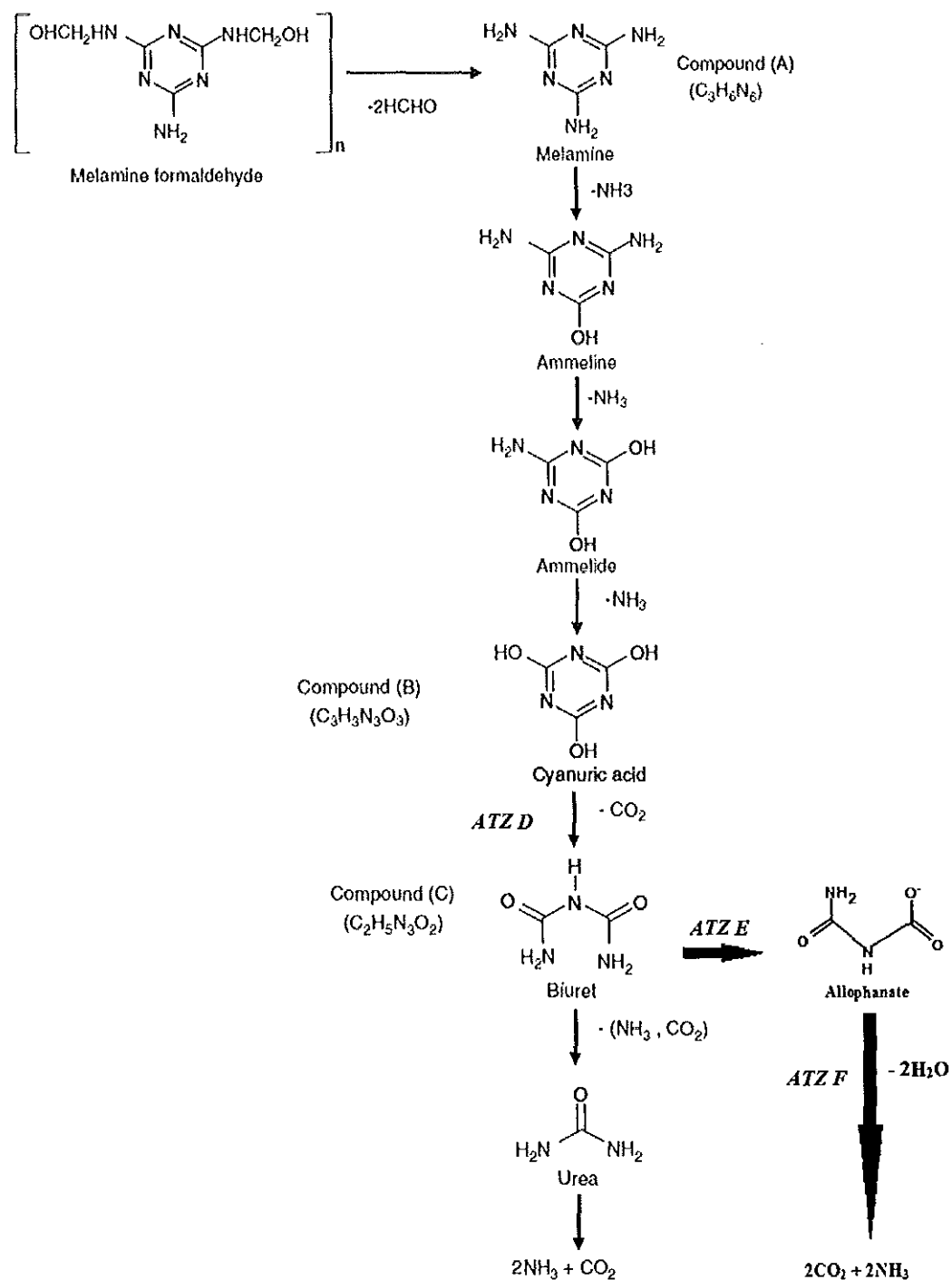


Figure 7 Proposed metabolic pathways of Melamine formaldehyde resin as studied using the strains of *Micrococcus* and *Klebsiella* (After El- Sayed *et al.*, 2006; Garcia *et al.*, 2008)

3. Materials and Methods

3.1. Description of the study site

The study was conducted at a pilot-scale wastewater treatment facility which was installed in the vicinity of Modjo tannery in Modjo town. Modjo town is located 70 km south of the capital Addis Ababa, located at 8° 35' North and 39° 10' East with an altitude of 1825 m above sea level (EMA 1988). The mean annual rainfall was within the range of 680 and 2000 mm and mean annual temperature was between 15 and 30°C (Isaias Tadesse *et al.*, 2004).

Modjo Tannery manufactures various types of leather from sheep and goat skin and cattle hide. During the study period, the unit processed an average of 3399 goat skin, 2564 sheep skin and 255 cattle hide every day (Mulatu Kebede, personal communication). According to a data provided by the factory, the tannery processes an average of 25 – 30 ton of skin to wet blue and crust leather and generates about 300 m³ effluent daily.

Previously, all wastewater streams were diverted into a channel for screening and directly discharged into the nearby Modjo River that ends up in Koka reservoir. Currently, a chemical treatment plant is installed by the company to treat its effluent through flocculation and coagulation. In 2010, a pilot anaerobic- aerobic reactor integrated with constructed wetland system had been established by the BIO-EARN project which treats about 50% of the effluent coming out from the factory. This system will be described further in section 3.4.1.

For the sake of simplicity, the studies described in this dissertation are divided into three phases: phase I dealing with isolated bacteria, phase II dealing with experiments

conducted on lab- scale reactor and phase III dealing with studies conducted on field- scale reactor integrated with constructed wetland.

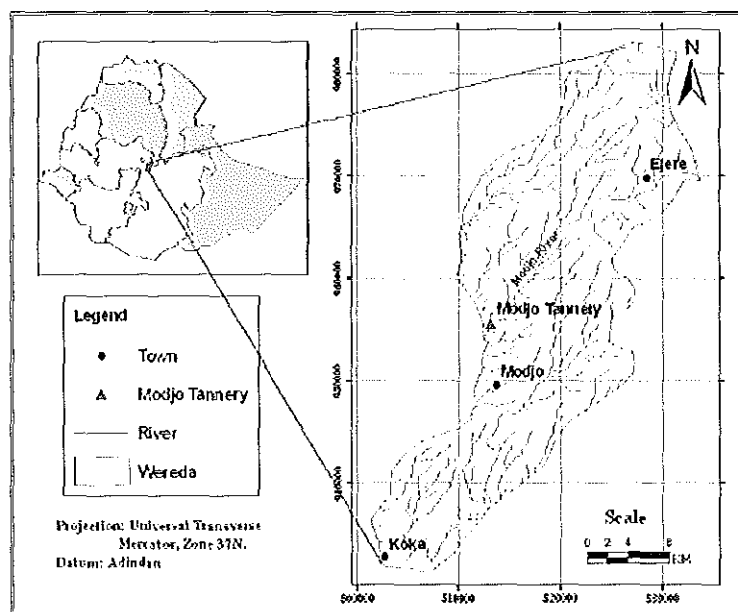


Figure 8 Map of the study area (After Asaye Ketema, 2009)

3.2. Phase I: Characterization of microorganisms capable of growing on retanning agents from tannery wastewater

3.2.1. Sample collection

Wastewater samples were taken from a collection pit of the effluent discharged from the different process units of the Modjo Tannery. The pit served as a temporary storage site of composite wastewater prior to screening and grit removal. The water samples were collected using sterile bottle and transported to laboratory in an ice box and immediately transferred to -20 °C freezer until use.

3.2.2. Isolation and purification of microorganisms

The wastewater samples were filtered using filter paper (Grade 1, Whatman, USA) to remove large particles such as hair and other residues. The filtrate was then serially diluted (10^{-2} - 10^{-5}) into 150 ml of sterile minimal medium prepared as in Veeranagouda *et al.* (2006) (Table 2) in 250 ml Erlenmeyer flasks and supplemented with equal amounts of retanning agents known as Basyntan-AN and Retanal MD-80 in the following concentrations in g/l: 0.1, 0.25, 0.5, 0.75 and 1.0. The pH of the minimal medium was 8.1, and was left unadjusted. Basyntan-AN and Retanal MD-80 were kindly supplied by the Modjo Tanning industry and served as the main sources of carbon and nitrogen. The cultures were incubated at 30°C on a rotary shaker at 100 RPM (Gallenham, England) for seven days. After a series of subculturing in the liquid medium, 100 µl of the suspension from each culture was dispensed onto solid minimal medium (Table 2) and amended with the different concentrations of Basyntan and Retanal.

Different colonies were picked from the plates and streaked onto a solid minimal medium supplemented with the retanning agents as described above and incubated at 30°C for at least 7 days. A series of repeated streaking was performed to ensure the purity of each isolate. The purified bacterial isolates were labeled using ordinal numbers prefixed by B or R based on their growth on Basyntan or Retanal. The pure colonies were kept on minimal medium agar slants at 4°C until further use and preserved at -20°C in 40% glycerol for long term storage.

Table 2 Composition of minimal medium used in this study (Veeranagouda *et al.*, 2006)

Component	Amount (g/l)
K ₂ HPO ₄	6.8
KH ₂ PO ₄	1.2
MgCl ₂	0.01
MnCl ₂	0.01
CaCl ₂ x 2H ₂ O	0.1
FeCl ₂ x 7H ₂ O	0.1
Na ₂ MoO ₇ x 2H ₂ O	0.006
Agar agar (for solid medium)	15

3.2.3. Optimization of the concentration of retanning agents in the medium

To determine the effect of various concentrations of the retanning agents Basyntan and Retanal on growth of the isolates, 20 randomly selected isolates were taken and loop-full amount from each isolate was inoculated in 20 Erlenmeyer flasks containing 150 ml liquid minimal medium with the following concentrations of the retanning agents: 0.1, 0.25, 0.5, 0.75, 1, 1.25 and 1.5 gram per liter of Basyntan- AN and Retanal- MD80 in 1:1 ratio. The cultures were incubated at 30°C on a rotary shaker at 100 RPM (Gallenham, England) for 12 days. Sample from the cells in the liquid medium were taken every 24 hours and cell numbers determined using a Neubauer counting chamber (Neubauer Improved Haemocytometer, Germany). Using a phase- contrast microscope (Zeiss, Germany), bacterial cells were visualized and counted according to the method described in Estridge and Reynolds, (2007). The concentration that yielded the highest average cell number was taken as the optimal concentration for further experiments.

3.2.4. Screening of isolates for growth on optimized amount of retanning agents

Based on the optimal concentration (0.5 g/L) of Basyntan and Retanal in which the representative isolates showed maximum growth, all the the isolates was inoculated in 250 ml Erlnmeyer flasks containing 100 ml of minimal medium containing the optimal concentration and incubated at 30°C in a shaking incubator (Gallenham, England) at 100 rpm for 15 days. Samples were taken periodically (every 36 hrs) from each flask to calculate cell numbers on a counting chamber under microscope as described previously. The isolates that showed typical bacterial growth were selected for further growth and degradation experiments.

3.2.4.1. Protein determination

Protein content of the screened isolates was performed in batch cultures in 250 ml flasks containing 100 ml of the minimal salts medium amended with 0.5 g/l Basyntan and Retanal (1:1). Isolates were inoculated by picking a single colony and transferring into the culture medium and incubated for six days at 30°C on a rotary shaker at 100 RPM (Gallenham, England). Sample from each culture was removed every 12 hours starting from the 36th hour and protein was analyzed using Bradford's test (Stoscheck, 1990). For this purpose, a stock solution of 1 mg/ml of bovine serum albumin (BSA) dissolved in water was prepared as a standard solution. It was then diluted to 100µg/ ml and pipetted into reaction tubes to make 1 to 10 µg BSA in a total volume of 100µl. In other test tubes, 5µl of the test samples was pipetted into each test tube to which distilled water was added to make a final volume of 100 µl. Then, 1.0 ml of Bradford's reagent was added to each tube and mixed thoroughly. The absorbance of each solution was determined by a

spectrophotometer (Beckman DU-40, USA) at 595nm. The absorbance was then plotted as a function of BSA concentration to calculate the best-fit straight line.

3.2.4.2. Ammonia determination

The selected isolates were further screened for the consumption of one of the retanning agents, Retanal MD80, by measuring the production of ammonia based on the study by El- Sayed *et al.* (2006). The isolates were inoculated by picking a single colony into a 250 ml Erlenmeyer flask containing 100 ml of the mineral salts amended with 0.5 g/l Retanal. Starting from the 36th hour, sample from each culture was removed every 12 hours till the sixth day and ammonia production was measured spectrophotometrically using the HACH kit (DR/2012, HACH, Loveland, USA).

3.2.5. Effect of pH and temperature

The isolates were tested for their ability to grow at temperatures of 20, 25, 30, 35 and 40 degree Celsius combined with a pH range of 6 to 10. Solid minimal medium supplemented with Basyntan and retanal was prepared as described above. The pH of the mineral mix was adjusted by titrating it with an appropriate volume of either 1.0 N NaOH or HCl prior to the addition of the Agar. 100µl of each isolate from minimal liquid culture at the actively growing stage of the cells was spread onto the plates. Appearance of colonies on the Petri- plates with the medium after incubating them for 15 days was used as an indicator of growth.

3.2.6. Chromatographic determination of residual retanning agents

3.2.6.1. Standards

The standards used in this study were the retanning chemicals, where the stock solutions of each of the standards (5 g/l) were prepared in distilled water. Working solutions of 0.1, 0.5 and 1 g/l were prepared by diluting the stock solutions. The above mentioned working concentrations were also prepared by mixing the two chemicals in 1:1 ratio.

3.2.6.2. Sample preparation for extraction

Isolates were inoculated by picking a single colony into a 250 ml Erlenmeyer flask containing 100 ml of culture medium containing the mineral salts amended with 0.5 g/l Basyntan and Retanal (1:1) and incubated for 26 days at 30°C on a rotary shaker at 100 RPM (Gallenkamp, England). Samples periodically taken from the liquid culture medium were centrifuged at 12000 RPM at 4°C for 10 min (Heraeus fresco 21, Thermo Scientific) and the supernatants were filtered with 0.2 µm syringe filters (EMD Millipore). Basyntan-AN and Retanal-MD80 were analyzed from the culture and a medium without bacteria was used as a control. For injection, 800 µl of the filtrate were loaded into the injection vials of the instrument.

3.2.6.3. Chromatographic analysis

The concentrations of residual Basyntan-AN and Retanal-MD80 from the collected samples were analyzed by an Acquity™ ultra performance liquid chromatography (UPLC) system (Waters, Germany) using photodiodearray (PDA) detector. For the mobile phase, phosphate buffer (0.1 M, pH 7) was prepared from monobasic and dibasic potassium phosphate salts dissolved in sterile water. The buffer was filtered with 0.2 µm

syringe filter (EMD Millipore), autoclaved and degassed in a sonicator bath (Elmasonic, Germany). Methanol and HPLC- grade water were used to clean the column prior to and after analysis.

Separation was achieved using Acquity™ UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm particle size; Waters, Germany) as a solid phase and phosphate buffer pH 7 as mobile phase. Before running the analysis, a standard curve was calculated using 0.1, 0.5 and 1 gram/liter working concentrations of the retanning chemicals. The chemicals were detected at 278 nm, 40°C with a flow rate of 0.4 ml/min.

3.2.7. Kinetic experiments

Growth kinetics of one of the isolates was determined using the following initial concentrations of Retanal (g/ L): 0.1, 0.25, 0.5, 0.75, 1 and 1.25 in liquid minimal medium as described before. Cultures were incubated at 30°C for 15 days and growth rate was monitored at 12 hour intervals. K_s (half saturation constant) and μ_{max} (maximum growth rate) were determined based on the appropriate model fitting the data (Copeland, 2000).

3.2.8. Characterization and identification of bacteria degrading retanning agents

3.2.8.1. Morphological and Biochemical characterization

The selected isolates were grown on Nutrient Agar medium (DIFCO) and were characterized for cell morphology under phase- contrast microscope (Zeiss, Germany) using 10 X 100 magnification. Image was recorded with a DXC-9100P camera (Sony,

Japan) and captured using image analysis with the Openlab 3.1.4 software (Improvision, USA).

The isolates were tested for their Gram reaction using the Gram staining test (Conn *et al.*, 1957, cited in Brown and Hopps, 1973). They were also grown on the selective MacConky agar and Slantez- Bartley agar (SIFIN, Germany).

3.2.8.2. Genomic DNA extraction

DNA was extracted from four isolates using microwave boiling method used by Kleinstuber and colleagues (2006). Briefly, single colony from a 24 hours incubated plate was picked and added into 100 µl of sterile distilled water and mixed well. The cell suspension was then heated in microwave oven at 2400 Watt for 20 seconds. Lysates were maintained at -20°C until used.

3.2.8.3. Amplification of 16S rRNA gene

Using the extracted genomic DNA, PCR was performed to amplify the gene for smaller subunit of ribosomal RNA with the bacteria universal primers 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT 3'). The PCR reaction mix contained 8.5 µl of PCR- grade water, 12.5 µl of Hotstart Taq Master Mix (Qiagen, Germany), 1µl of 2 pmol of the forward and reverse primers, 1 µl of DMSO and 1µl of 2.5ng DNA, with water in the negative control making a final reaction volume of 25 µl. The reaction conditions were fed to the thermal cycler (PTC-200 Thermal Cycler, MJ research) as follows: 4 min at 94°C and 30 cycles of 45 s at 94°C, 1 min at 58°C an 2 min at 72°C, followed by a 10-min extension step at 72°C. Amplification was checked by electrophoresis on a 1.5% agarose gel stained with

ethidium bromide and visualized under UV transilluminator. 1kb molecular weight standard (Invitrogen, Germany) was used to estimate the size of the amplified fragment.

3.2.8.4. 16S rRNA gene sequencing and database search

The PCR products were purified with Promega PCR cleaning kit (Wizard® SV gel and PCR- cleanup system, USA). Partial DNA sequencing of purified PCR products was performed using a BigDye RR Terminator AmpliTaq FS Kit version 3.1 (Applied Biosystems, Germany) and the sequencing primers 27F and 519R (5' GWATTACCGCGGCKGCTG 3') (Kleinstauber *et al.*, 2006). Electrophoresis and data collection were carried out on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Germany). The DNA sequences were edited and assembled using the software Sequencher version 4.10.1 (www.genecodes.com). Similarity searches against the NCBI database and the Ribosomal Database Project were carried out using the Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the Sequence Match tool was used to search for similar sequences compiled by the Ribosomal Database Project II Release 9 (<http://rdp.cme.msu.edu>). The sequences were submitted to GenBank for the assignment of accession numbers and nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers JX876856 – JX876859.

3.2.8.5. Phylogenetic analysis

In order to verify the taxonomic classification resulting from the database retrieval, a phylogenetic analysis was performed. First, the 16S rRNA gene sequences obtained from this study and similar sequences downloaded from GenBank as well as two sequences downloaded as outgroups were aligned using multiple alignments method of CLUSTAL-

X software. Evolutionary distances were calculated by Tamura-Nei model. Phylogenetic tree was constructed by Neighbor-Joining method by MEGA4 software (Tamura *et al.* 2007), and a bootstrap analysis of up to 1000 iterations was carried out for evaluating the branches of the constructed tree.

3.2.8.6. Data presentation and analysis

Data from growth and degradation experiments was graphically presented using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, (www.graphpad.com). Comparison of mean degradation efficiencies was analyzed using One Way Analysis of Variance by employing Scheffe's Post Hoc test on SPSS (version 16.0) software.

3.3. Phase II: Analysis of microbial community dynamics and identifying key sub-communities for degradation of synthetic retanning agents in lab-scale Sequencing Batch Reactor

3.3.1. Reactor set-up and operation

A bench- scale sequencing batch reactor (SBR) with a volume of 2 L was used for the experimental study discussed in this section. The reactor was made of a 2mm thick plexiglass cylinder with an internal diameter of 100mm (Biostat MD, Braun, Germany). It was fitted with a fermenter lid containing a turbine propeller stirrer, an air line with attached bubble diffuser, a dissolved oxygen probe, pH probe, a temperature sensor and heating element; and an effluent withdrawal line (Fig. 9). The reactor operated at a constant temperature maintained at 30°C throughout the running period. The pH of the reactor was left uncontrolled to be in the range of 7 to 10. Dissolved oxygen was

measured using a polarographic Oxygen sensor. Data of the bioreactor was collected using MFCS/Win 1.1 (Braun Biotech International).

The SBR was operated continuously in cycles of around 72 hours. One cycle consisted of a fill phase (working volume 1.5 L) for 1 hr, an aerated phase of 15 hr with stirrer at 150 rpm. During this phase, the reactor was flushed with sterile air to a saturation of 100%. A phase of 8 hours with no aeration and mixing followed the aerated phase. These periods were repeated three times followed by settling, decanting 1000 ml of the total volume, idle and refilling (1000 ml) phases (Fig. 10).

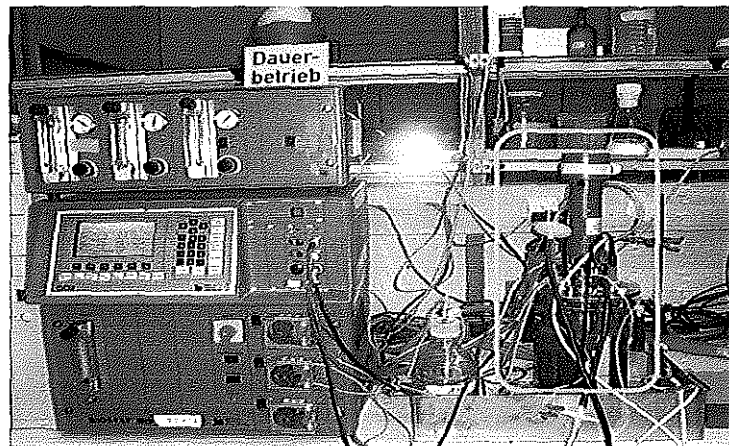


Figure 9 Experimental setup used for the work showing a fully automated 2L SBR with online monitoring of DO, pH and temperature.

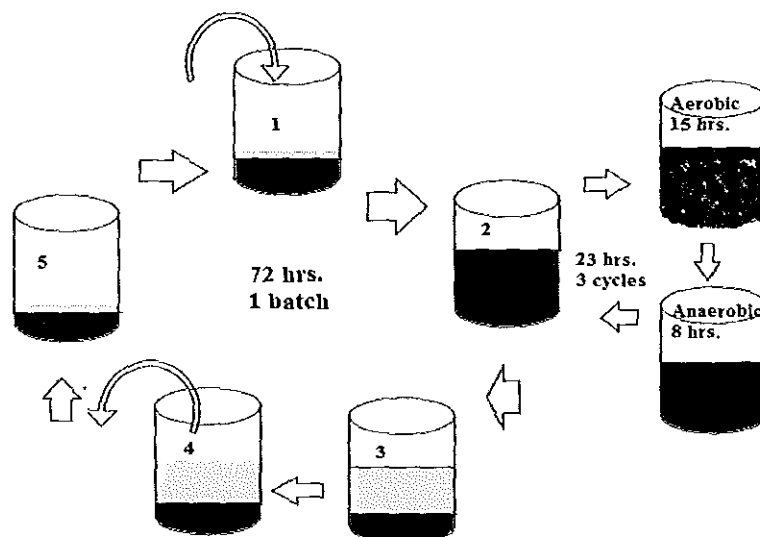


Figure 10 Schematic presentation of the different phases of the lab- scale sequencing batch reactor

3.3.2. Biomass and feed characteristics

The reactor was initially seeded with 500 ml of acclimatized tannery sludge mixed with synthetic wastewater with composition listed in Table 2 (phase I of the study) containing 0.5 g/l of Basyntan and Retanal. Synthetic wastewater and the retanning agents were prepared in stock to serve as influent for the sequencing batch reactor (SBR). The composition of the synthetic wastewater medium was as listed in Table 3. The medium was autoclaved to prevent bacterial activity in the feed vessel. Stock preparation (10 g/l each) of Basyntan and retanal was prepared and autoclaved. Both the synthetic wastewater and the retanning agents were stored at room temperature until use. The influent (feed) to the SBR was a mixture of the following components: 350 ml of

composite effluent from Modjo Tannery, 500 ml of synthetic wastewater and 150 ml of the retanning chemicals Basyntan and Retanal. The concentrations of the retanning agents being added to the feed were progressively increased at every batch to minimize any potential toxic effects at the beginning of the experiment. The following concentrations of the retanning chemicals were used: 0.1 g/l at the first batch, 0.2 g/l at the second batch and 0.5 g/l in all subsequent batches.

Table 3 Composition of synthetic wastewater (After Amare Gessese *et al.*, 2003)

Components	Amount (g/l)
Sodium Acetate	0.7
Casein	1.0
Na ₂ S ₂ O ₃	0.5
FeCl ₂	0.05
K ₂ HPO ₄	0.2
KH ₂ PO ₄	0.1
CaCl ₂	0.05
MgSO ₄ .7H ₂ O	0.02

3.3.3. Sampling

Samples were taken from the influent and effluent fluids at each batch of the reactor for analysis regarding measurements of total nitrogen (TN), Ammonium nitrogen (NH₄-N), chemical oxygen demand (COD), total organic carbon (TOC) and retanning chemicals Basyntan and Retanal. For microbial studies employing flow cytometry, sludge was sampled at the end of the reaction in each batch. For microbial DNA- based studies which employed terminal restriction fragment length polymorphism (T-RFLP) and a cross- sectional clone- library- based sequencing, sludge samples were collected at the end of the reaction in each batch. In addition, sludge samples were taken at the end of

anaerobic and aerobic cycles from the following batches: 1, 2, 12, 13 and 14 to see if there is any difference in cluster pattern and microbial composition in the presence and absence of oxygen.

3.3.4. Analytical measurements

Measurement for TOC was done using TOC analyzer (Shimadzu, Japan), COD, TN and NH₄-N were analyzed spectrophotometrically (DR/2010 HACH, Loveland, USA) according to the HACH instructions. The concentration of Basyntan and Retanal compounds were analyzed by an Acquity™ ultra performance liquid chromatography (UPLC) system (Waters, Germany) as described in section 3.2.6.

3.3.5. Flow cytometric analysis

Flow cytometric measurements were carried out using the MoFlo cell sorter (DakoCytomation, Fort Collins, CO) equipped with two water-cooled argon ion lasers (Innova 90C and Innova 70C from Coherent, Santa Clara CA). Excitation of 580mW at 488nm was used to analyze the forward scatter (FSC) and side scatter (SSC) as a trigger signal at the first observation point, using a neutral-density filter with an optical density of 2.3. DAPI (4'-6-Diamidino-2'-phenylindole) fluorescence was passed through a 450/465 band pass filter. Photomultiplier tubes (models R 928 and R 3896) were obtained from Hamamatsu photonics (Hamamatsu city, Japan). Amplification was carried out at linear or logarithmic scales, depending on the application. Data were acquired and analyzed using Summit software (DakoCytomation, Fort Collins, CO). The dot plots were gated in such a way that distinct subcommunities were included in the gates and cell counts therein were calculated. Fluorescent beads (polybead microspheres 0.483um diameter; Flow Check BB/Green Compensation Kit, blue alignment grade,

reference 23520, polyscience) were used to align the MoFlo (coefficient of variation 2%). Also, an internal DAPI-stained bacterial cell standard was introduced for tuning the device up to a coefficient of variation not higher than 6% (Müller and Nebe-von-Caron, 2010).

3.3.6. Cell sorting

Cell sorting was performed from two samples of the 14th batch (between 936 – 1008 hrs) on selected sub communities represented as clusters using the four-way-sort option at high speed (12 m/s). The single and one-drop mode was chosen for separating at least 500,000 cells at a rate of 2,500 cells per second (Koch *et al.*, 2013). The cells were sorted into nucleic-acid-free glass flasks. The cells were separated from the mixed community using DNA-DAPI fluorescence intensity and forward scatter signals with different gate settings. The sorted cells were then separated from sheath buffer by centrifugation at 20000g for 25 min. The pellets were stored at -20°C until use for DNA extraction. To serve as control samples for downstream analyses, 1 ml of unsorted cells and 1 ml of sheath buffer were stored together with the cells.

3.3.7. Genomic DNA extraction

3.3.7.1. DNA extraction from sludge samples

DNA was extracted from the sludge samples using the PowerSoil™ DNA Isolation Kit (MoBio, West Carlesbad, CA, USA) following the manufacturer's protocol. Extracted DNA was visualized using Agarose gel electrophoresis run in 1X TAE buffer and carried out at 100V for 30 min. DNA purity (A260/A280 and A260/A230) and mass

concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

3.3.7.2. DNA extraction from sorted cells

DNA extraction from sorted cells was performed using Chelex solution as described in Giraffa *et al.* (2000). 70 µl of Chelex solution was added to the frozen sorted cells and to the unsorted cells, 300 µl was used. The mixture was vortexed for 10 seconds before and after vortexing for 10 sec. The samples were then centrifuged at 7000 RPM for 5 minutes and the clear supernatant was carefully transferred into pre-chilled microfuge tubes.

3.3.8. PCR

3.3.8.1. PCR for TRFLP: DNA from sludge samples and sorted cells

Bacterial 16S rRNA gene fragments were PCR amplified with the forward primer 27F-FAM, (5'AGAGTTTGATCMTGGCTCAG 3') which was labeled at the 5' end with phosphoramidite fluorochrome 5-carboxyfluorescein (Biomers.net, Germany) and the unlabelled reverse primer 1492R (5'GGTTACCTTGTTACGACTT 3'). PCR was performed in 25 µl reaction mixtures containing 12.5 µl Taq PCR mastermix (QIAGEN, Germany), 5 pmol of each primer (supplied by MWG Biotech, Germany) and 1 to 4 µl template DNA extracted from sludge samples taken at the end of each reaction of the bioreactor as described above. For the sorted cells, the reaction mix was composed of 12.5 µl Taq PCR mastermix, 5 pmol of each primer and 10.5 µl of the extracted DNA. Amplification was performed using PTC-200 Thermal Cycler (MJ research) with the following cycle parameters: 4 min at 94°C and 30 cycles of 45 s at 94°C, 1 min at 58°C and 2 min at 72°C, followed by a 10-min extension step at 72°C. The results were

checked by electrophoresis on a 1.5% agarose gel and visualized using a gel documentation system.

The PCR products obtained from the DNA of sludge samples and sorted cells were purified using the wizard SV PCR clean-up system (promega, Germany) and quantified on the agarose gel electrophoresis and ethidium bromide staining using the GeneTools program (Syngene, United Kingdom).

3.3.8.2. PCR for clone library construction

Extracted DNA from sludge sample taken at the end of the thirteenth batch was selected for clone library construction. PCR was performed using primers and PCR reaction conditions as described previously in section 3.2.6.2. The PCR product were purified by adding 250 µl of absolute ethanol, 90 µl of water and 10 µl of 5M Sodium acetate to the tube and centrifuging in cold (4°C) for 20 minutes at 14000RPM (Heraeus fresco 21, Thermo Scientific). 300 µl of 70% Ethanol was added to the pellet and centrifuged for 15 minutes at 14000RPM, 4°C then the pellet was allowed to dry before resuspending with 10 µl TE buffer (Kleinstüber *et al.*, 2006).

3.3.9. Terminal restriction fragment length polymorphism (TRFLP) analysis

Purified PCR products were digested using the restriction endonucleases *HhaI*, *MspI* and *RSaI* (New England Biolabs, Germany). A 10 µl reaction mixture contained 20ug of DNA and 10 units of the restriction enzymes. Samples were incubated at 37°C overnight and then precipitated with sodium acetate (pH 5.5) and ethanol. The dried DNA samples were resuspended in 20 ul HiDi formamide containing 1.5% (V/V) GeneScan-500 ROX standard (Applied Biosystems). Samples were denatured at 95°C for 5 min and chilled

on ice. The fragments were separated by capillary electrophoresis on an ABI Prism 3100 genetic analyzer (Applied Biosystems), and their relative peak areas were determined by dividing the individual T-RF area by the total area of peaks within the threshold of 50 to 650 bp. Only peaks with relative fluorescence intensities of at least 20 units were included in the analysis.

3.3.10. Cloning, ARDRA screening and sequencing of 16S rRNA genes

Purified PCR products were cloned using a QIAGEN PCR cloning kit. From the cloned cells, about 200 clones were picked and screened for the appropriate insert size by PCR by using the vector-specific primers M13uni (-21) (5'-TGTAACGACGGCCAGT-3') and M13rev (-29) (5'-CAGGAAACAGCTATGACC-3'). In order to dereplicate clones with similar sequences, positive clones were screened by amplified ribosomal DNA restriction analysis (ARDRA) as follows: the PCR products were digested with HhaI (New England Biolabs, Germany) and separated electrophoretically on 2% MetaPhor agarose (Cambrex; distributed by Biozym, Germany). ARDRA patterns were analyzed using the Phoretix 1D software (Nonlinear Dynamics, United Kingdom). A hierarchical cluster analysis was performed applying the Jaccard similarity index and the complete Linkage algorithm for calculating a similarity dendrogram. Representative clones from clusters were selected and sequenced.

3.3.11. Sequence analysis

Partial DNA sequencing of cloned 16S rRNA gene amplicons was performed using a BigDye RR Terminator AmpliTaq FS kit version 3.1 (Applied Biosystems, Germany) and the sequencing primers 27F and 519R. Electrophoresis and data collection were carried out on an ABI PRISM 3100 Genetic Data Analyzer. Sequence chromatograms

were edited and trimmed using Sequencher® version 4.0 (Gene Codes Corporation, MI, USA).

3.3.12. Data analysis

Data of temperature, dissolved oxygen level, redox potential and pH was collected from the MFCS software and processed in Excel worksheet as a text delimited file. Data of the abiotic parameters and degradation data of the retanning agents in the course of the bioreactor was graphically presented using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, (www.graphpad.com). Gate template was created and the number of cells in each gate was determined using the software FlowJo (Tree star inc., Switzerland). Correlation analyses between the abiotic parameters and the gated sub communities were done by the Spearman's rank- order correlation coefficient using the program R (<http://CRAN.R-project.org>) Version 2.14.0 and the resulting correlations were presented as a heat map using the "heatmap" function in the package gplots (Gregory *et al.*, 2011).

For the TRFLP data, the signal peaks were separated from noise peaks following the statistical approach designed by Abdo *et al.* (2006). The resulting normalized data were used for the subsequent analyses. Theoretical T-RF values of the dominant phylotypes represented in the clone library were calculated using the NEB cutter (<http://tools.neb.com/NEBcutter2/index.php>). These values were confirmed experimentally by T-RFLP analysis using the corresponding clones as templates. Relative T-RF abundances of representative phylotypes were determined based on the percentage relative peak areas of the corresponding fragments and graphically presented using SPSS version 16 (www.SPSS.com).

3.4. Phase III: Bacterial community structure and diversity of pilot- scale anaerobic-aerobic reactors integrated with constructed wetland for the treatment of tannery wastewater in Modjo town, Ethiopia

3.4.1. Description of pilot- scale treatment system

The pilot-scale wastewater treatment facility was installed in the vicinity of Modjo tannery, Ethiopia, 75 km south of the capital Addis Ababa. The treatment facility has started operation since September 2010. As depicted in Fig. 11, the system consists of tanks for primary screening and grit removal, two anaerobic reactors, each of having 3.6m diameter and 3.3m height; an aerobic reactor having 4m diameter and 3.3m height, followed by a vertical subsurface-flow constructed wetland (31 m long, 16.40 m wide, 1.30 m deep and filled with medium- sized gravel) vegetated with the perennial grass *Phragmites australis* (Cav.). Wastewater was fed into the anaerobic reactors to undergo reaction for 24 hrs. Then, the water coming out from the anaerobic reactors was directed to the aerobic reactor to react with aeration and mixing for 12 hrs. The water was collected from the aerobic reactor into a collection tank, where it was continuously fed into the constructed wetland with a loading rate of about 120 Kg/BOD₅/ha/day.

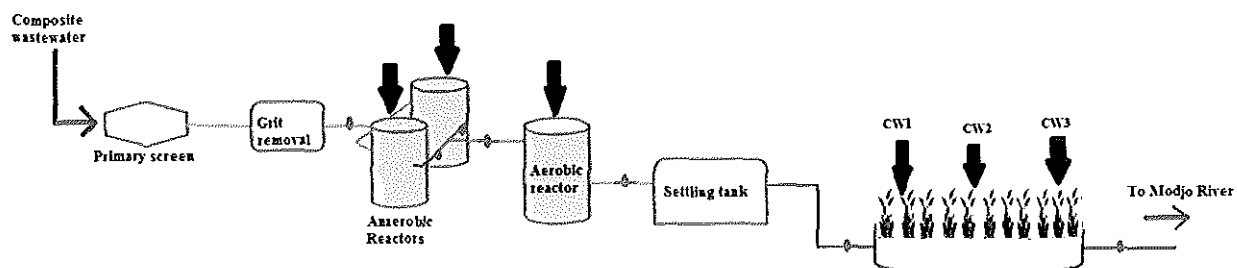


Figure 11 Schematic presentation of the pilot tannery effluent treatment site comprising anaerobic-aerobic reactors integrated with constructed wetland system. Sites where samples were taken are shown in block arrow

3.4.2. Wastewater sampling and analytical methods

To preview the performance of the integrated system, samples of raw and treated wastewater were taken three times on the 6th, 13th and 20th of January 2012. The water samples were processed and analyzed for a number of parameters to assess the biological degradability of the pollutants. COD, total N, NH₃-N, NO₃, NO₂, SO₄ and S²⁻ were measured spectrophotometrically (DR/2012, HACH, Loveland, USA) according to the company's instructions. BOD, total suspended solids and volatile suspended solids were determined following Standard methods (APHA 1998). Total chromium was determined using direct air- Acetylene flame method of atomic absorption spectroscopy (AAS) according to standard methods (APHA 1998). Total suspended solids (TSS) was analyzed using gravimetric method by evaporating the sample at 105°C and measuring the residue using balance (SCALTEC, SBA 32). Total dissolved solids (TDS) and pH were measured using conductivity meter (ELEMETRON, CC401, Spain) and pH meter (Jenway Ltd., England).

3.4.3. Sludge sampling

For analysis of microbial community residing in the treatment system, sludge samples were collected from the anaerobic and aerobic reactors. To achieve maximum recovery of microbes from the constructed wetland, rhizosphere samples were collected from around the root zone of the plants and gravels. To get a representative data of microbial populations from the constructed wetland, samples were collected from three randomly selected spots of the wetland (Fig. 8) and designated hereafter as CW1, CW2 and CW3. Sampling was performed three times on the 6th, 13th and 20th of January 2012, on the same dates of wastewater sampling. All samples were collected using sterile containers in duplicates at each sampling time.

3.4.4. Genomic DNA extraction and PCR amplification

DNA was extracted from the duplicate sludge samples using ZR soil microbe DNA kitTM (Zymo Research, CA., USA) according to the provided instruction. Extracted DNA was visualized using 0.8 % Agarose gel stained with GelRed (Biotium Inc.) and electrophoresis run in 0.5X TBE buffer carried out at 100V for 30 min. DNA purity (A260/A280 and A260/A230) and mass concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Bacterial 16S rRNA gene fragments were amplified by PCR using the primers 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3') as described by Kleinstuber *et al.*, (2006). PCR was performed in 25 μ l reaction mixtures containing 0.2 μ l Dream Taq (Fermentas, Lithuania), 2.5 μ l 10X Taq buffer, 0.5 μ l of each deoxynucleoside triphosphate (10 mM), 1 μ l (0.4 pmol) of each primer (Bioneer corporation, Korea), 18.8 μ l sterile filtered MilliQ water and 1 μ l of template

DNA (30 ng/ μ l). To minimize PCR bias, DNA extracts from the duplicate samples were pooled and served as the template DNA. Amplification was performed using GeneAmp™ PCR System 9700 (Applied Biosystems), with the cycle parameters as described previously in section 3.2.6.2. The results were checked by electrophoresis on a 1.5% agarose gel.

3.4.5. 16S rRNA gene clone library construction and sequence analysis

16S PCR products amplified from the DNA extracts were purified using QIAquick PCR purification kit (QIAGEN, Germany). For maximum coverage of the PCR products during clone library construction, the purified products collected from the five sites at three different time intervals were pooled. Cloning was performed using InsTAclone™ PCR cloning kit (Fermentas, Lithuania). A total of five clone libraries, one for each site, were constructed. From each library, 200 to 250 colonies were picked and screened for the appropriate insert size by colony PCR using vector-specific M13 primers as discussed in section 3.3.10. PCR products were purified with Ethanol and Sodium acetate purification as described before (section 3.3.10). Amplified Ribosomal DNA Restriction Analysis (ARDRA) using the restriction enzymes *HhaI* and *MspI* was performed to dereplicate and classify the clones into phlotypes based on their unique restriction pattern. Partial DNA sequencing of the M13 amplicons was performed using a BigDye® Terminator V 3.1 Cycle Sequencing Kit (Applied Biosystems) and the forward primer 27F. Electrophoresis and data collection were carried out on an ABI 3730 Genetic Analyzer (Applied Biosystems). The resulting sequence chromatograms were edited using Sequencher® version 5.0 (Gene Codes Corporation, MI, USA).

4. Results

Phase I: Characterization of microorganisms capable of consuming retanning agents from tannery wastewater

4.1. Optimization of the concentration of retanning agents for bacterial growth

A total of 141 bacterial isolates were picked, purified and characterized based on their growth on minimal medium supplemented with Basyntan AN and Retanal MD-80 as a sole source of carbon and nitrogen. Among the different concentrations of the retanning agents tested in the liquid minimal medium, the one spiked with 0.5 g/l of each agent was found to give the highest mean bacterial cell number per μl from the 48th hour to the end of the experiment (288 hr) (Fig. 12). Therefore, 0.5 g/l of each of the retanning agent was taken as the optimal concentration for cultivation- based studies involving minimal medium. Upon screening of the 141 isolates for growth on solid minimal medium spiked with 0.5 g/l of each of the retanning agents, 70 (49.8%) showed growth on the medium.

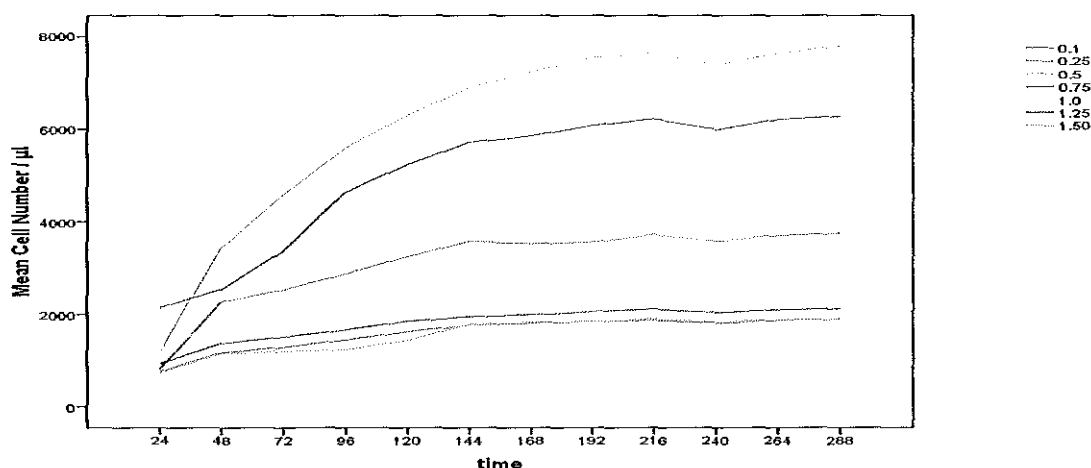


Figure 12 Comparison of different concentrations of Basyntan and Retanal (1:1) for optimal growth of randomly selected isolates (N=20) expressed as mean number of cells per µl counted every 24 hours

4.2. Production of proteins by the isolates

Ten isolates were selected based on their production of higher amount of proteins as determined by Bradford method. Out of them, the isolate designated as B45 showed increasing level of protein starting from 7 up to 46 mg/ml throughout the cultivation period, followed by R19 and B1. Highest protein concentration by most of the isolates was observed in the 132nd hour of cultivation. The concentration of protein produced by the isolates R19 and B1 at this time were 44 and 40 mg/ml. Protein production by R2 was consistently the lowest, with a maximum of 13 mg/ml in the 132nd hour of cultivation. The rest six isolates had intermediate to low production of protein, with the highest amount ranging from 24 to 36 mg/ml at the 132nd hour of cultivation (Fig.13 a & b).

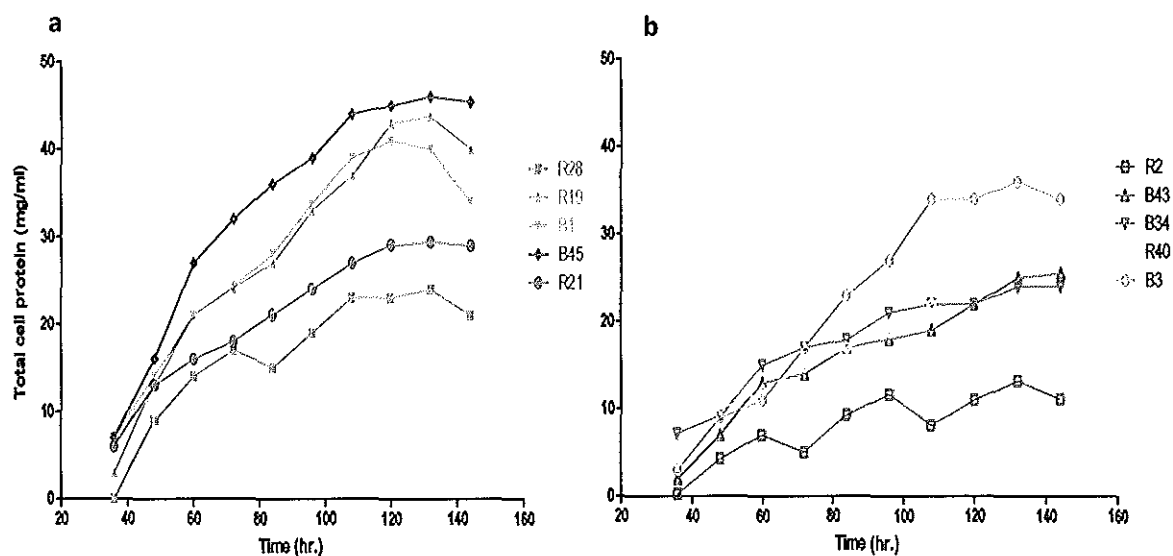


Figure 13 Total protein production of the first five (a) and the rest five (b) of the ten isolates grown in minimal medium supplemented with 0.5 g/l Basyntan and Retanal over a period of 6 days at 30°C.

4.3. Determination of ammonia during Retanal biodegradation

Preliminary analysis on the consumption of one of the retanning chemicals, Retanal MD-80 was done using the test for ammonia production. Test for ammonia was chosen based on the information of the basic constituents of Retanal as provided by the company and on the assumption that ammonia is released if biodegradation of Retanal takes place. In this analysis, the isolated B45 showed relatively the highest level of ammonia production starting from 10 mg/l in the 36th hour of cultivation, which increased up to 79 mg/l in the 96th hour. B1 was found to be the second isolate producing higher amount of ammonia reaching up to 74 mg/l in the 96th hour (Fig. 14). In this analysis, the highest production for most of the isolates was observed in the 96th hour. R19 was found to be the third isolate to produce relatively high amount of ammonia with a maximum of 73 mg/l in the

96th hour. The liquid medium devoid of bacterial cells had ammonia production reaching a maximum of 16 mg/l throughout the cultivation time (Fig. 14).

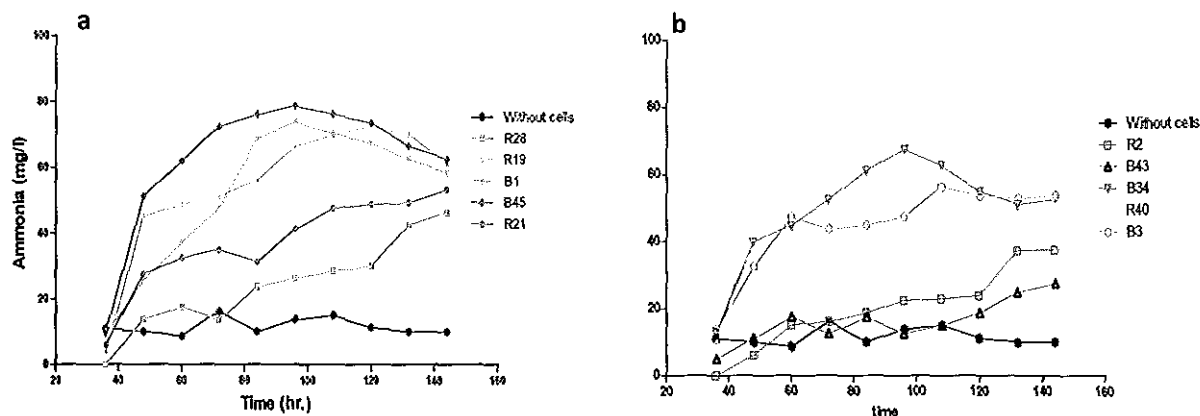


Figure 14 Utilization of Retanal MD-80 by ten isolates in (a) and (b) as determined by ammonia monitored over a time of 6 days at 30°C.

4.4. Degradation of the retanning agents by four selected isolates

4.4.1. Degradation of Basyntan

Based on Ultra Performance Liquid Chromatography (UPLC) analysis, four isolates namely B1, B45, R19 and R28 were selected due to the relatively higher degradation efficiency of the retanning agents.

For the degradation of Basyntan, the four isolates showed variations in their activities at different times (Fig. 15). At first, no degradation was observed by any of the isolates in the first 24 hours, which was changed at the 48th hour, where R19 displayed removal efficiency of 19% whereas B1 and B45 both having removal efficiency of 11% (Fig. 15). R28 showed only 6% degradation at this particular hour of incubation. An increasing trend of degradation of Basyntan was observed by B1 and B45, where the maximum

degradation for the isolate B45 took place within 336th and 480th hours of incubation and reaching up to 41% at the 384th hour. Though the isolate B1 showed increasing degradation at the 288th and 336th hours, it was not possible to follow it up due to contamination. On the other hand, degradation by the isolates R19 and R28 showed a sudden drop from 192- 288 hours of incubation, which was then increased afterwards.

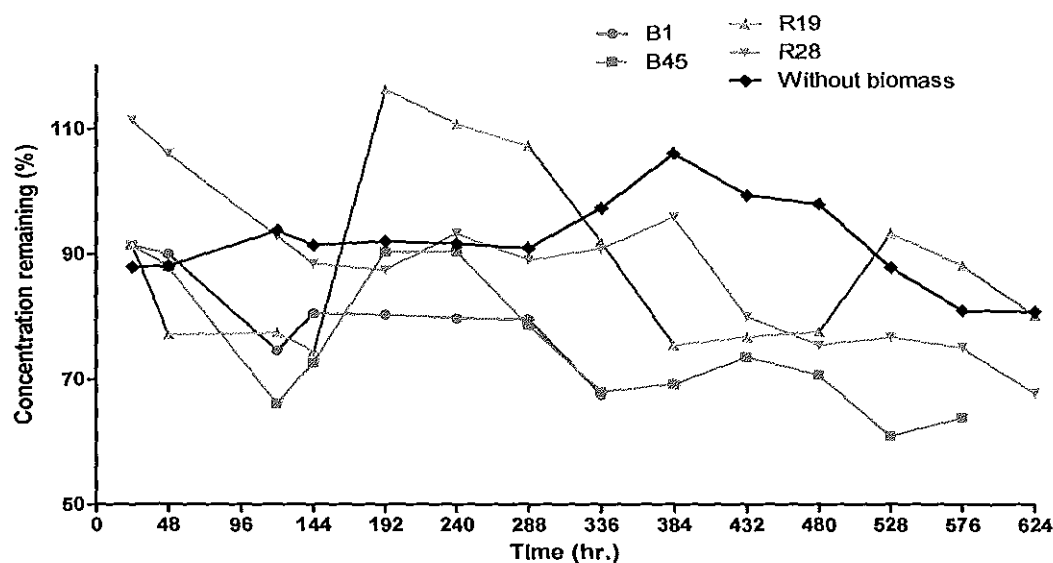


Figure 15 Degradation of Basyntan expressed in percent removal by the four isolates cultivated in liquid medium for 26 days

4.4.2. Comparison of isolates in Basyntan degradation

Upon running Basyntan as standard, three elutriation peaks were detected with retention times of 0.348, 0.405 and 0.492 minutes. For all the runs, concentrations were checked for accuracy using linear regression values (R), which showed high accuracy ($R^2 > 0.99$). For reasons of simplicity, the detected peaks of Basyntan are designated hereafter as peak 1, peak 2 and peak 3 respectively.

When ranking the three peaks of Basyntan from highest- degraded to least degraded by the isolates, peak 3 represented the portion that was most degraded by all the isolates followed by peak 2, which was the product degraded by all, except R19. Peak 1 referred to the least consumed portion of the chemicals by all the isolates except R19.

4.4.3. Degradation of Retanal

Regarding the other retanning agent Retanal, a single peak was detected at a retention time of 0.691 minutes. Consumption of Retanal was observed in the first 24 hours particularly by B1, R19 and R28 (Fig. 16). The degradation pattern of all of the four isolates showed an increasing pattern over the 624 hours of cultivation. B45 exhibited more than 50% degradation starting from the 432nd hour of incubation onwards. R28 showed increasing performance in degradation starting from the 432nd hour, with efficiencies ranging between 41 to 53%. Isolate R19 showed degradation in the range of 42 to 45% starting from the 432nd hour onwards. It was not possible to evaluate the degradation performance of B1 after the 336th hour because of contamination.

The mean degradation efficiency of B45 was 34 ± 21.5 , which was followed by R19 and R28, with 24 ± 16.9 and 28 ± 17.9 , respectively. Lastly, B1 had the mean of 23.67 ± 6.16 . In this case, the isolates did not show significant differences between their mean efficiencies for the degradation of Retanal.

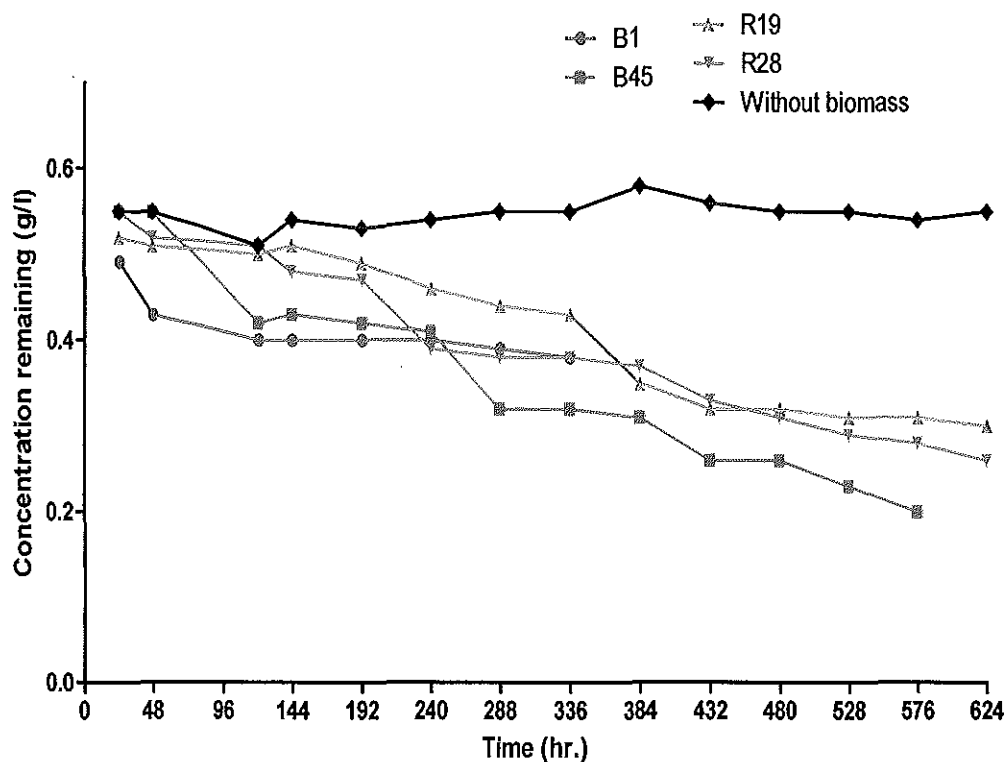


Figure 16 Degradation of Retanal expressed in percent removal by the four isolates cultivated in liquid medium for 26 days

4.5. Effect of pH and temperature on growth of four isolates on media containing retanning agents

At pH 6, B1 was observed to grow at temperatures 25, 30 and 35 degree Celsius, showing its ability to grow under acidic conditions. The other three isolates were sensitive to low pH and failed to grow in all temperatures, except B45 which grew only at 30°C. As the pH increased from 7 to 9, R28 was capable of growing at all temperatures. In the above mentioned pH range, B45 and R19 showed growth at temperature of 25, 30 and 35°C. At a temperature of 20 °C, B45 failed to grow at all pH ranges, while R19 was capable of

growing at this temperature under pH 8 and 9. On the other hand, B45 showed growth at 40 °C under pH 7 up to pH 10. In general, the four isolates were found to be mesophilic and capable of growing at neutral to alkaline pH, except B1 and B45, which also grew at acidic pH (Table 4).

Table 4 Effect of temperature and pH on the growth of the isolates on Retanning agents-amended minimal medium agar incubated for 15 days

pH	Isolate No.	20°C	25°C	30°C	35°C	40°C
6	B1	0	1	1	1	0
	B45	0	0	1	0	0
	R19	0	0	0	0	0
	R28	0	0	0	0	0
7	B1	0	1	1	1	1
	B45	0	1	1	1	1
	R19	0	1	1	1	0
	R28	1	1	1	1	1
8	B1	1	1	1	1	1
	B45	0	1	1	1	1
	R19	1	1	1	1	0
	R28	1	1	1	1	1
9	B1	0	1	1	1	1
	B45	0	1	1	1	1
	R19	1	1	1	1	0
	R28	1	1	1	1	1
10	B1	0	0	1	1	1
	B45	0	0	0	1	1
	R19	0	0	1	1	0
	R28	0	0	1	1	1

4.6. Growth kinetics of an efficient bacterium on Retanal MD-80

Based on the bacterial growth at concentrations ranging from 0.25 to 1.5 g/l Retanal in the medium, growth kinetics of B45 was determined on the log transformed values of the

cell count per μl at each time of measurement. Within the range of concentration from 0.1 to 1 g/l, the specific growth rate (V) increased with increase of Retanal concentration, with maximum growth rate reaching at around 0.5 g/l. Concentrations above this level decreased the specific growth rate (Fig. 17a). A maximum growth rate (μ_{max}) of 1450 cells $\text{ml}^{-1}\text{hr}^{-1}$ and the Monod half saturation constant (K_s) of 10.16 mg L^{-1} was calculated from the Lineweaver-Burk double reciprocal plot (Fig. 17b).

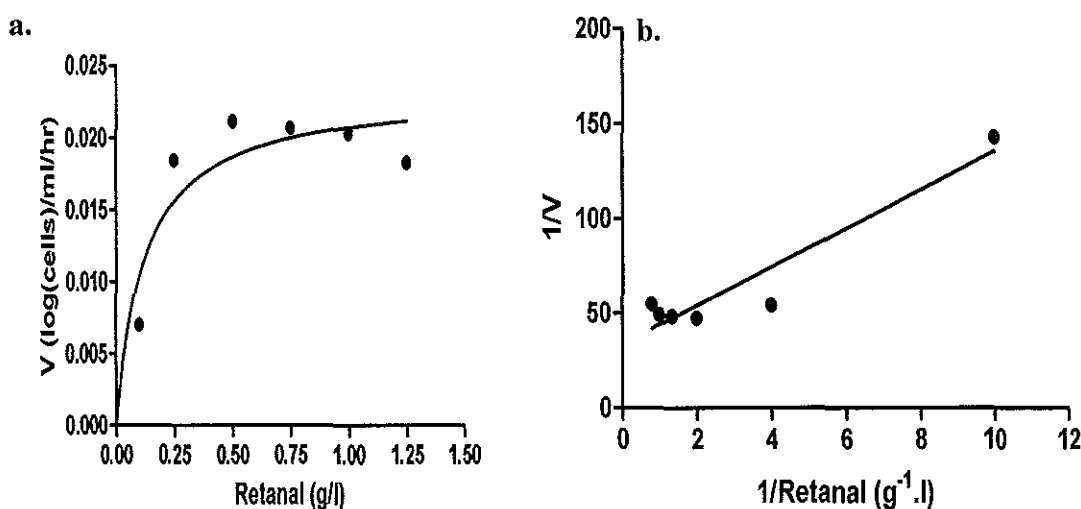


Figure 17 Relationship between growth rate of isolate B45 Retanal MD-80 concentration. (a) Kinetics fitting to the Monod model: $\mu = \mu_{\text{max}} \cdot S / (S + K_s)$. (b) The Lineweaver-Burk double reciprocal plot used to calculate the half saturation constant, K_s ($1/\mu = (K_s / \mu_{\text{max}}) \cdot 1/S + 1/\mu_{\text{max}}$)



4.7. Morphological characterization and molecular identification of the efficient isolates

4.7.1. Morphological and culture- based characterization

The microscopic structure of the isolates showed that B1, B45 and R28 were coccoid with different cellular arrangement (diplo- and monococci), while R19 was a slender rod-shaped bacterium. R19 also showed distinct colony morphology with watery texture. The rest were non- watery with different colony colors (Table 5). All of the four isolates were Gram positive. B1 and R28 showed growth on the Slantez- Bartley Enterococci selective medium.

4.7.2. Molecular identification and phylogenetic analysis

The four isolates were identified using 16S rRNA gene sequencing. The raw sequence was edited and submitted to RDP classifier for the taxonomic assignment of the query sequences. Of the four sequences, B1 and B45 were grouped under class Actinobacteridae of the phylum Actinobacteria; R19 and R28 were grouped under class Bacilli of the phylum Firmicutes. Based on the Basic Local Alignment Search tool (BLAST) of the NCBI gene bank, the sequences got their identities based on the maximum similarities with the BLAST hits listed in Table 5. The isolate B1 was identified as *Micrococcus luteus* strain B1R, the isolate B45 as *Brevibacterium ptyocampae* strain B4500R, the isolate R19 as *Lysinibacillus sphaericus* strain R1902R and the isolate R28 as *Enterococcus gallinarum* Strain R2801R.

Table 5 Morphological, cultural characterization and molecular identification of the isolates

	B1	B45	R19	R28
Gram's reaction	+	+	+	+
Cell morphology	Diplococci	Cocci, single or diplococci	Long slender rods	Cocci, single or diplococci
Colony morphology	Smooth, yellow	White, smooth	Smooth, watery	Whitish yellow, small colonies
Growth on Mackonkey agar	-	-	-	-
Slant- Bartley agar	-	+	-	+
Assigned definition and GenBank accession number	<i>Micrococcus luteus</i> strain B1R, JX876857	<i>Brevibacterium ptyocampae</i> strain B4500R, JX876856	<i>Lysinibacillus sphaericus</i> strain R1902R, JX876858	<i>Enterococcus gallinarum</i> Strain R2801R, JX876859
Maximum similarity (%)	98	98	98	97
Phylum	Actinobacteria	Actinobacteria	Firmicutes	Firmicutes

A phylogenetic tree was constructed based on the sequences of the four isolates and other similar strains obtained from the RDP and NCBI databases (Fig. 18). The phylogenetic analysis showed that all except *Lysinibacillus sphaericus* R1902R are sister species of their respective genera. *Lysinibacillus sphaericus* R1902R was found to be closely related to *Brevibacillus laterosporus* GZUB11 rather than *Lysinibacillus sphaericus* PRE16.

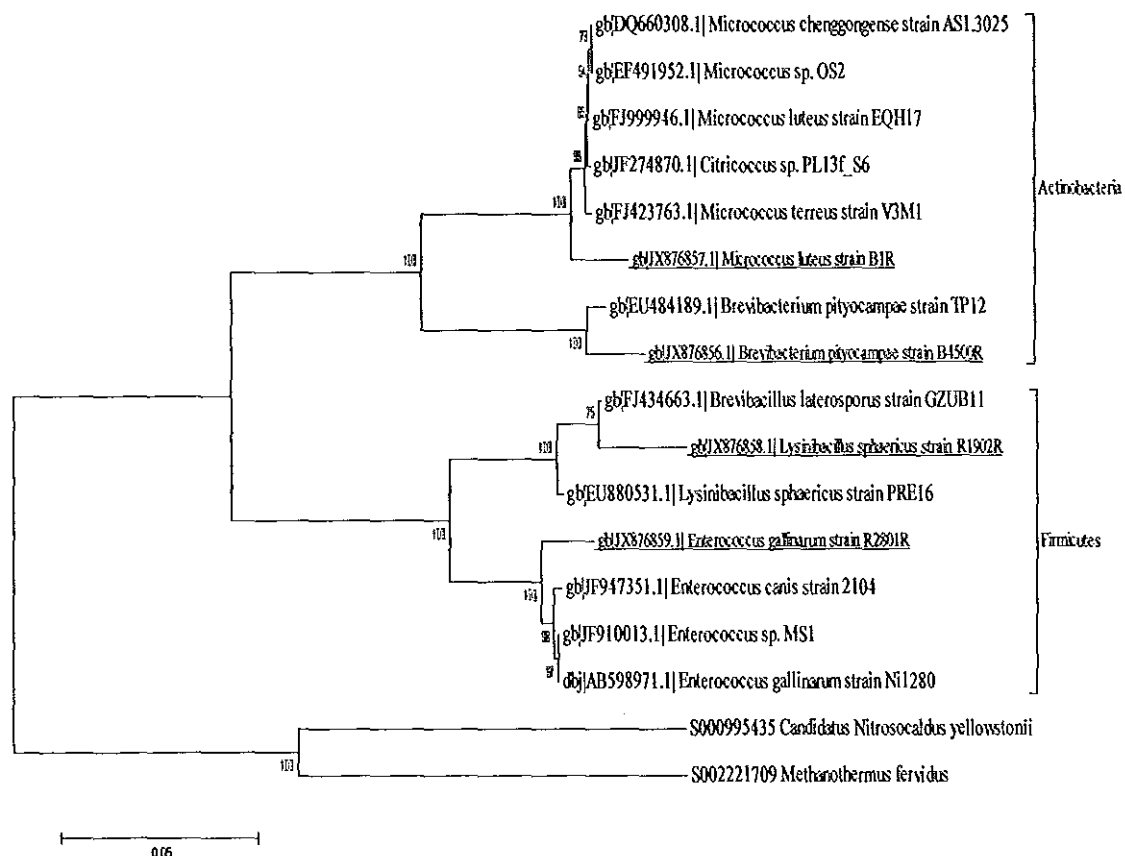


Figure 18 Molecular Phylogenetic analysis showing the relationship of the isolates (underlined) based on the 16S rRNA gene sequence analysis (1000 bootstrap for the confidence level) by Neighbor joining method. Scale bar represents 5 Substitutions per 100 nucleotides and rooting was inferred using two Archaeal sequences as outgroups.

Phase II: Microbial community dynamics and identification of key sub-communities for the degradation of synthetic retanning agents in lab-scale Sequencing Batch Reactor

4.8. Performance of the Sequencing Batch Reactor (SBR)

The influent mixed wastewater was characterized by chemical oxygen demand (COD), ranging from 793- 2650 mg/l; total nitrogen (TN) ranging from 165- 364mg/l and ammonium nitrogen (NH₄-N) ranging from 45 to 134 mg/l across the batches of the reactor. The pH at the end of each batch reaction was in the alkaline range, between 7.70 and 8.79. The reactor's performance in the removal of organic matter (COD) showed that more than 50% removal was obtained in the last four batches (Batch 11 to 14) and the 2nd batch. A maximum of 74% removal of organic matter (COD) was obtained in the 13th batch treatment of the SBR. Ammonia removal in the SBR was more than 50% from the 9th batch onwards, with a maximum of 80% in the 13th batch. The removal of total nitrogen showed decreased efficiency throughout the reaction period (Table 6).

Table 6 Removal efficiencies of COD, total nitrogen (TN) and ammonium (NH₄-N) based on the influents and effluents of the different batches of the SBR*

Batch No.	Days of batch reaction	COD			TN			NH ₄ -N			pH
		Infl.	Effl.	RE (%)	Infl.	Effl.	RE (%)	Infl.	Effl.	RE (%)	
1	0-3	793	431	46	174	98	44	47	38	19	8.03
2	3-6	1290	543	58	165	127	23	50	15	70	8.77
3	6-9	1360	948	30	247	196	21	101	22	78	8.79
4	9-12	2330	1710	27	364	293	19	116	28	76	8.63
5	12-15	2650	1420	46	358	351	2	134	53	60	8.48
6	15-18	2280	1890	17	376	352	6	109	53	51	8.54
7	18-21	2340	1210	48	299	288	4	79	31	61	8.42
8	21-24	1730	1140	34	240	225	6	45	25	44	8.27
9	24-27	2010	1240	38	268	265	1	64	20	68	7.70
10	27-30	2310	1060	54	287	282	2	72	17	77	8.32
11	30-33	2200	1100	50	302	299	1	88	27	69	8.79
12	33-36	2260	902	60	326	277	15	83	27	67	8.70
13	36-39	868	226	74	298	286	4	84	17	80	8.39
14	39-42	2300	790	66	308	264	14	79	20	74	8.38

*All parameters of influent and effluent are in mg/l, except for pH.

RE: Removal efficiency expressed in percent

The total carbon (TC) content of the influent mixed wastewater ranged from 590 and 971 mg/l of which the organic carbon (TOC) took the higher portion (461- 896 mg/ l) than inorganic carbon (IC) (86 – 146 mg/l). High efficiency in the removal of total carbon (TC) was recorded for the first two batches, which was dramatically dropped in batch 6 and then slowly increased to reach a maximum of 41% (Batch 12). The organic carbon (TOC) removal was above 50% in the first two batches of the reaction but showed a decreasing trend until batch 12; which was then improved in the last two batches.

Despite the low amount of inorganic carbon (IC) in the influent of the reactor, removal efficiency ranged between 11% (batch 1) and 60% (Batch 3) (Table 7).

Table 7 Removal efficiencies of carbon in the form of Total, organic and inorganic carbon (TC, TOC & IC) based on the influents and effluents of the different batches of the SBR*

Batch No.	Days of batch reaction	TC			TOC			IC		
		Infl.	Effl.	RE (%)	Infl.	Effl.	RE (%)	Infl.	Effl.	RE (%)
1	0-3	590	300	49	461	154	67	146	130	11
2	3-6	707	325	54	569	208	63	138	118	15
3	6-9	586	483	18	538	363	32	120	48	60
4	9-12	841	734	13	785	629	20	105	56	47
5	12-15	971	677	30	896	554	38	122	74	39
6	15-18	772	764	1	802	656	18	107	80	26
7	18-21	795	577	27	740	479	35	98	54	45
8	21-24	652	509	22	594	423	29	86	57	34
9	24-27	782	578	26	733	480	34	98	49	50
10	27-30	865	565	35	821	464	43	101	44	57
11	30-33	816	587	28	755	482	36	104	61	41
12	33-36	837	496	41	780	401	49	96	58	40
13	36-39	774	490	37	726	398	45	93	47	49
14	39-42	830	443	47	781	354	55	90	49	46

*All parameters of influent and effluent are in mg/l, except for pH.

RE: Removal efficiency expressed in percent

4.9. Fate of Basyntan in the reactor

Following start-up, 0.1g/l Basyntan and Retanal was added in the first three batches, which progressively increased to 0.5g/l on the fourth batch (reaction days 9 -12). At the end of the first batch reaction, 20 and 23 percent of peaks 1 and 2 were degraded. From batch 2 until the end of batch 6, no degradation was detected for all the three peaks. In fact, all the peaks showed unusual increment of concentration by the end of the 2nd and 3rd batches. From the 7th batch onwards, the removal of the three detected peaks showed improvement with a complete disappearance of peak 2 (the peak which appeared at the retention time of 0.405 minutes), which continued until the end of the experiment (Fig. 19). Removal of peaks 1 and 3 reached 41% and 38% respectively, by the end of the 14th batch.

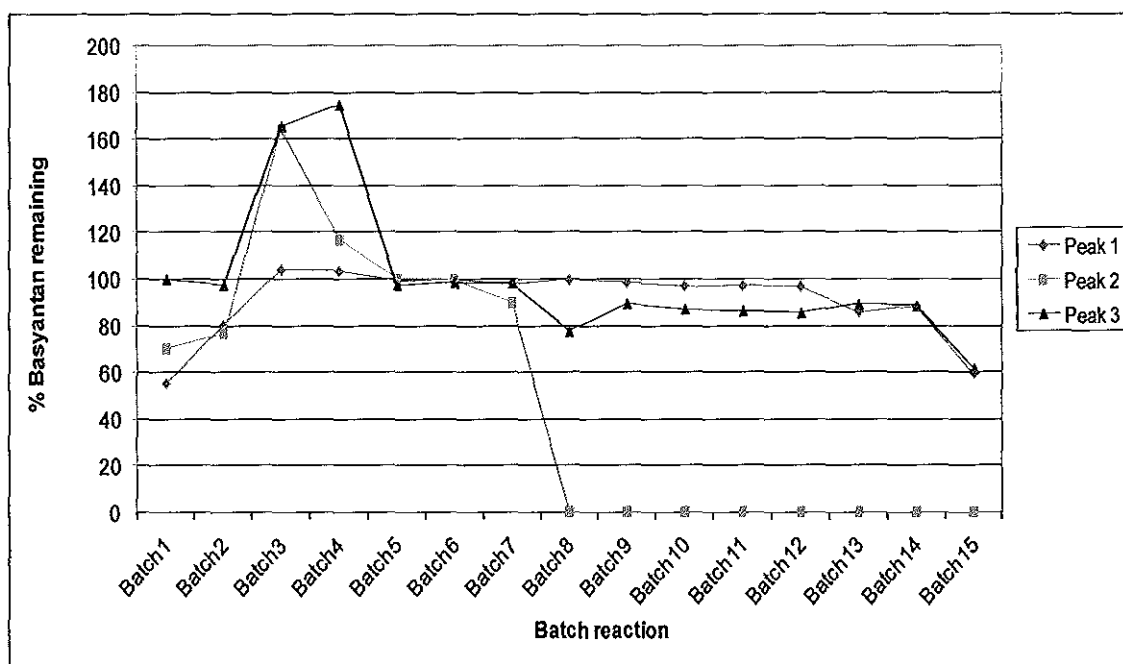


Figure 19 Removal of the three detected peaks of Basyntan calculated as the percentage disappearance of each of the peaks throughout the 42 days operation of the SBR.

4.10. Fate of Retanal in the reactor

The study showed that Retanal degradation was detected in all but batches 2, 3, 8, 12 and 13 (Fig. 20). Maximum degradation of Retanal was detected with removal efficiencies of 17%, 20%, 25% and 37% at batches 1, 6, 7 and 14.

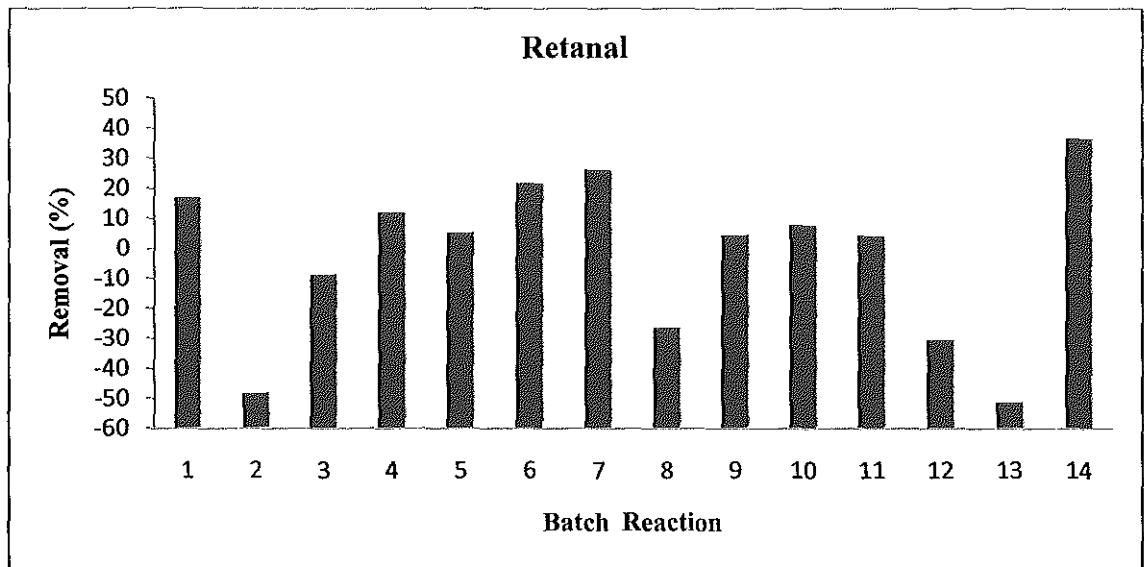


Figure 20 Removal efficiency of retanal at the end of each batch of the SBR

4.11. Dynamics of the bacterial community in the SBR

Flow cytometric characterization of the bacterial community in the sludge of the SBR revealed the dynamics, succession and shift of the microbial sub- communities during the course of reaction, with typical patterns in each batch (Fig. 21). The first dot plot in the beginning refers to the wastewater community initially seeded in the minimal medium spiked with Basyntan and Retanal for acclimatization. This plot shows the presence of

actively dividing bacterial populations expressed by the appearance of small- sized; high DNA content clusters.

Starting from the first batch of the operation of the sequencing batch reactor (SBR), changes expressed as shift of clusters in the x- and y-axes were observed in each batch of reaction of the SBR, indicating increase in cell size and proliferation activity of the microbial communities over the whole running period of the SBR.

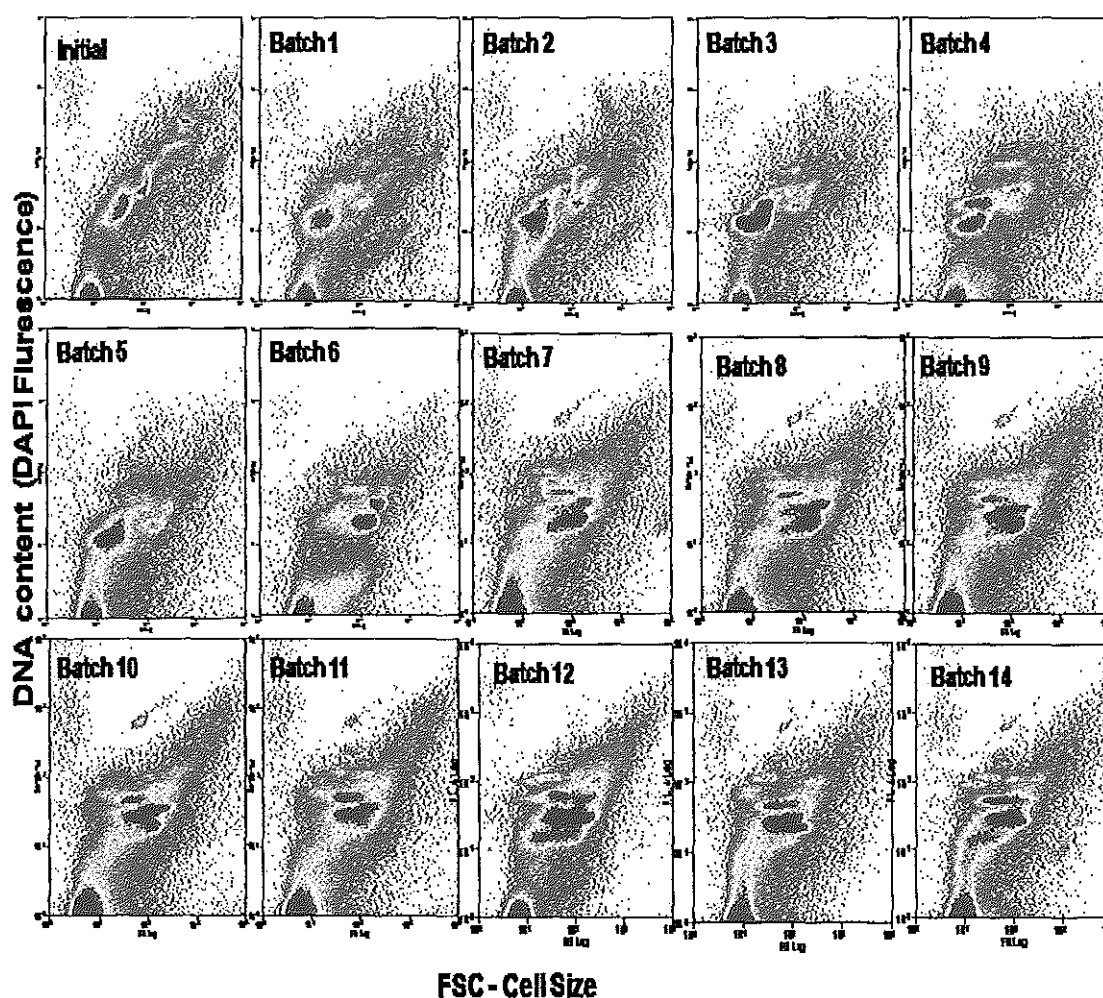


Figure 21 Bacterial community dynamics of tannery wastewater during growth on retanning agents running in sequencing batch reactor (SBR) for 45 days. The first dot plot (initial) refers to the bacterial community in the acclimatized sample used to seed the reactor at the beginning of the SBR.

At the fourth batch when the concentration of Basyntan and Retanal in the reactor was increased to 0.5 g/l, some of the cluster distributions did not exhibit any sign of depression indicating the absence of toxic effect of the chemicals on the microorganisms. A conspicuous shift of the clusters along the x- axis was observed in the sixth batch, which was then followed by intense proliferation expressed by the increase in the DNA content (y- axis) in the seventh batch. From the seventh to the 14th batch, expansion of the clusters representing different bacterial sub- communities both in cell size and emergence of new clusters in different coordinates of the dot plot was observed (Fig. 21).

4.12. Community composition in the SBR

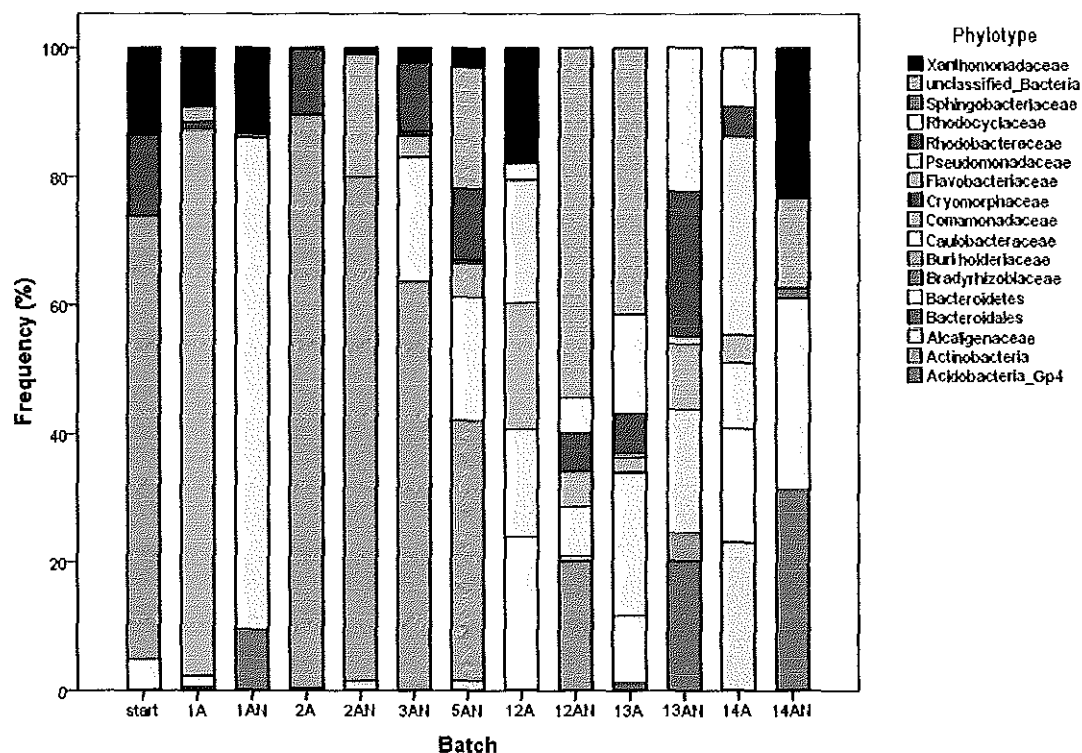
Composition of the microbial communities in the different batches was analyzed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) for the 16S rRNA gene fragment, using the restriction enzymes Hha I, Msp I and Rsa I. By affiliating the dominant T- RFs to the identified clone library sequences with their theoretical T- RF calculations, it was possible to identify peaks affiliated to 17 bacterial groups with different relative abundances (Fig. 22).

At the start-up of the SBR, the acclimatized sludge was dominated by the family Burkholderaceae with nearly 69% of the total T- RF based- phylotypes. The remaining phylotypes constituted the families Xanthomonadaceae (14%), Rhodobacteriaceae (13%) and Bacteroidetes (5%) (Fig. 22).

During the aerobic and anaerobic stages of the first batch reactions of the SBR, the families Flavobacteraceae (82%) and Caulobacteraceae (73%) were found to dominate

the microbial communities (Fig. 22). Following these groups, the family Xanthomonadaceae constituted 9% and 13% of the community in the aerobic and anaerobic stages, respectively. During this batch, the dominant bacterial family in the aerobic phase, Flavobacteriaceae was replaced by Caulobacteraceae, indicating a succession of dominant bacterial groups upon change in the oxygen content of the reactor.

In the second batch, the family Burkholderaceae was found to dominate the aerobic stage (89%) and the anaerobic stage (78%). In the aerobic stage the second most abundant group was Rhodobacteraceae (10%) whereas 19% of the community that belonged to unclassified bacteria were the most abundant group in the anaerobic stage (Fig. 22).



Start = Acclimatized sludge; A= Aerobic stage; AN= Anaerobic Stage

Figure 22 Terminal restriction fragment (TRF) abundances showing dynamics of the predominant phylotypes of the SBR at different running batches.

The anaerobic stages of the third and fifth batches were dominated by members of the family Burkholderiaceae, contributing to 40 and 65% of the community. Generally, there was a decreasing trend in the occurrence of the family Burkholderiaceae from the second to the fifth batch (Fig. 22).

In the aerobic stage of the 12th batch, members of Bacteroidetes were abundant comprising 24% of the community, followed by Flavobacteriaceae (20%), Pseudomonadaceae (19%), Xanthomonadaceae (18%), Caulobacteraceae (17%) and a least abundant (3%) group belonging to Rhodocyclaceae.

Regarding the anaerobic stage of the 12th batch and the aerobic stage of the 13th batch, it was not possible to identify the dominant bacterial groups and thus were grouped under unclassified bacteria. The families Rhodocyclaceae (18%), Rhodobacteraceae (18%), Pseudomonadaceae (25%) and Bradyrhizobiaceae (20%) were dominant in the anaerobic stage of the 13th batch; and aerobic and anaerobic stages of the last batch, respectively. In the 13th and 14th batches, additional bacterial groups such as Alcaliginaceae, Comamonadaceae, Sphingobacteraceae, Acidobacteria Gp4 and Actinobacteraceae were observed indicating ecological succession of communities in the SBR.

4.13. Community diversity in the SBR

The start- up acclimatized sludge had the least diverse bacterial population of all the samples from the SBR indicated by the Shannon's diversity index of 1.67 (Table 8). In this start stage, species richness was the lowest of all the batches ($S=1.44$) indicating few number of species present at the start. It was also characterized by unequal proportions of the different species expressed by lower equitability index (0.76) and evenness value of 0.59. Likewise, the Shannon-Wiener diversity index (H) calculated on different batches of the reactor showed an increasing trend of diversity from the start to the end of the SBR, with relatively the highest diversity observed in the 13th batch ($H= 2.52$) (Table 8).

Table 8 Diversity and richness indices of the bacterial community calculated from T-RFLP profiles of 16S rRNA genes from sludge in each batch of the SBR

Batch	No. of peaks	Shannon Diversity index (H')	richness	Equitability index	Evenness
Initial sludge	15	1.67	1.44	0.76	0.59
Batch 1	25	2.37	2.47	0.88	0.72
Batch 2	17	1.96	1.9	0.79	0.59
Batch 3	25	1.92	1.41	0.87	0.55
Batch 5	24	2.32	2.45	0.86	0.68
Batch 12	21	2.29	2.42	0.87	0.7
Batch 13	28	2.52	3.09	0.87	0.69
Batch 14	25	2.36	2.56	0.87	0.7

4.14. Functional correlation analysis

Based on visual evaluation of the histograms of the dotplots, a gate template was created representing 30 clusters during the 14 batches of the reactor run (Appendix III). From each gate, cell abundance over the entire reaction period were evaluated. Correlation analysis of bacterial cell abundance in each gate with the thirteen measured abiotic parameters revealed a number of positive and highly positive correlations between some of the abiotic factors and biotic cell abundances (Fig. 23).

Positive ($p < 0.05$) correlations were found between removal of organic matter in the form of COD and TOC (chemical oxygen demand and total organic carbon), TC (total carbon), TN (total nitrogen) and bacterial cells clustered in gates G3, G6, G11 and G12. Highly positive correlations ($p < 0.01$) were detected between removal of the retanning agents and

bacterial groups in gates G13, G14, G15 and G16. Positive correlations ($p < 0.05$) were detected between removal of the retanning agents and bacterial groups in gates G6, G12 and G20.

Considering the different peaks of Basyntan, highly positive correlation was found specifically between peak 1 ($\Delta B1$) and peak 3 ($\Delta B3$) of Basyntan and the cells in G21 and G23. The correlations between the rest of the retanning agents ($\Delta B2$ and ΔR) with the cells in G21 and G23 were still found to be positive ($p < 0.05$), suggesting the possible role of the cells in G21 and G23 for the biodegradation of Retanal and all the components of Basyntan.

Cells in G23 were found to correlate negatively with removal of ammonium, all forms of carbon (TC, TOC and IC), TN and COD suggesting the absence of any direct role in degradation of other pollutants than the retanning agents. Such a differential role in biodegradation of selected pollutants was also observed in G22 cells that are correlated negatively with removal of any of the retanning agents ($\Delta B1$, $\Delta B2$, $\Delta B3$ and ΔR), indicating the absence of direct role in the degradation of these pollutants. On the other hand, the cells in G22 were positively correlated with ammonia (NH_4), TN, TC, TOC and COD.

Negative correlations were found between the removal of the retanning agents, NH_4 , TN, TC, TOC, COD and redox potential with bacterial cells in the following gates: G1, G4, G8, G26, G27, G28, G29 and G30, which might be related to the absence of their direct role in the degradation of the mentioned pollutants. Negative correlation was also found between Oxygen level (pO_2) and pH with the bacterial cells in all gates except G1, G4,

G28, and G29, indicating the sensitivity of the bacterial cells in the 26 gates to increased levels of oxygen and alkalinity.

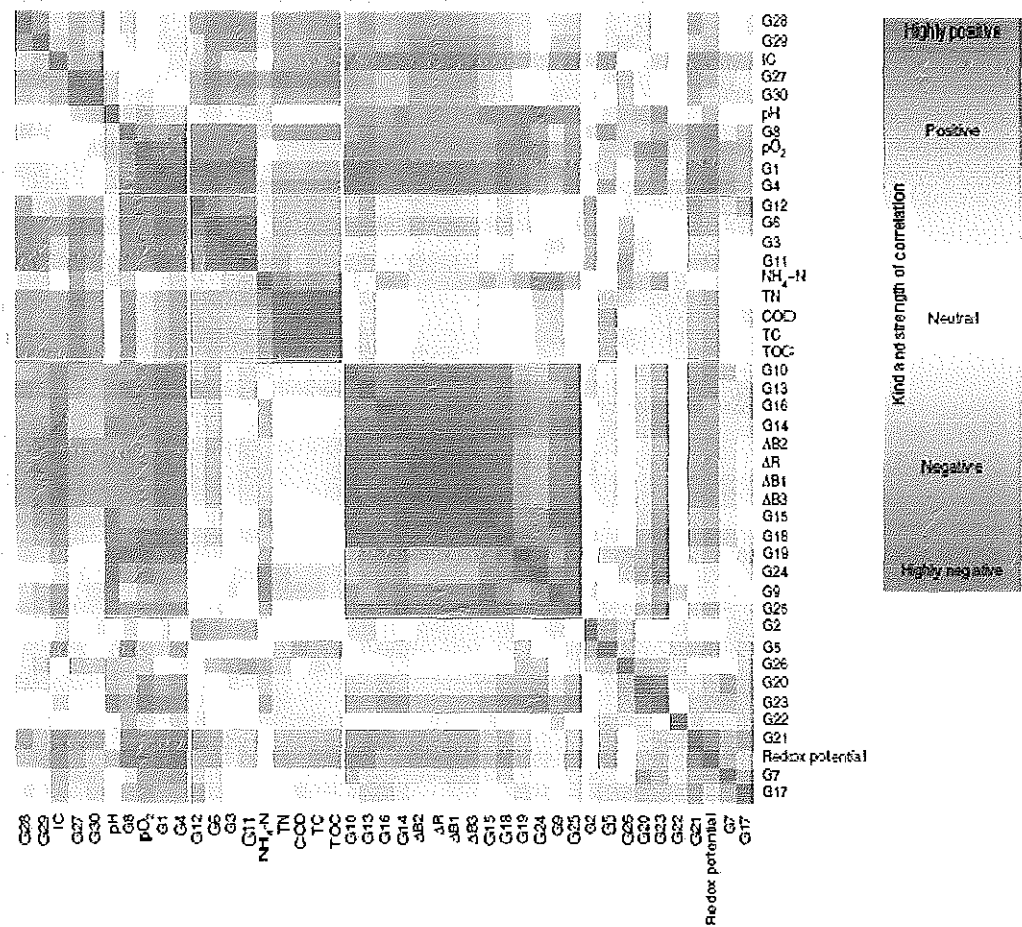


Figure 23 Correlation of 13 abiotic parameters with cell abundances in the 30 gates during the running period of the reactor.

4.15. Identification of key sub communities for the degradation of the retanning agents

In order to have a closer look at the clusters and identify members of the clusters that were consistently existed in the flow cytometric pattern from each batch of the SBR, eight of the 30 gated sub- communities namely: G1, G2, G6, G12, G14, G16, G20 and G21 were sorted to analyze the composition and abundance of bacteria in each sorted gate. From all the sorted gates, eight Bacterial families and classes belonging to the phyla Proteobacteria, Bacteroidales and Bacteroidetes were identified using terminal restriction fragments (T-RFs).

Out of the eight gated clusters, gate 14 (G14) contained the smallest portion of the sorted bacterial community with predominant members belonging to Proteobacteria (6%) and shows strong positive correlation ($P < 0.01$) with the degradation of Basyntan and Retanal (Fig. 24). The gates 16, 20 and 21 (G16, G20 and G21) which showed positive correlations with retanning chemicals degradation were dominated by members of the phylum Bacteroidetes constituting 13%, 23% and 66% respectively. Rhodocyclaceae (11%), Brucellaceae (10%) and unclassified Proteobacteria (8%) were the second, third and fourth abundant groups identified in gate 20 (G20).

Due to the biggest gate size and dense bacterial populations, gate 6 (G6) was sorted into two separate tubes of each having at least 500,000 cells. In both tubes, the most abundant cells belonged to Rhodocyclaceae (48% and 22%). The second most abundant groups in this gate belonged to unclassified Proteobacteria (16% and 8%), followed by the family Brucelleceae (8%). The families Caulobacteriaceae, Xanthomonadaceae and the phylum Bacteroidetes constitute a small proportion (15%) of the total community in

the gate. The role of the identified bacterial groups in the degradation of the retanning agents is reflected by the positive correlation ($P < 0.05$) detected between cell abundances and removal of the retanning agents (Fig. 24)

Gate 1 (G1) which was shown to have strong negative correlation with retanning agent degradation, composed mainly of Caulobacteraceae (13%), followed by a small portion of Xanthomonadaceae (2%) and Sphingomonadaceae (1%). Likewise, gate 2 (G2) was also dominated by Caulobacteraceae (20%) followed by Brucellaceae (8%) and Rhodocyclaceae (8%).

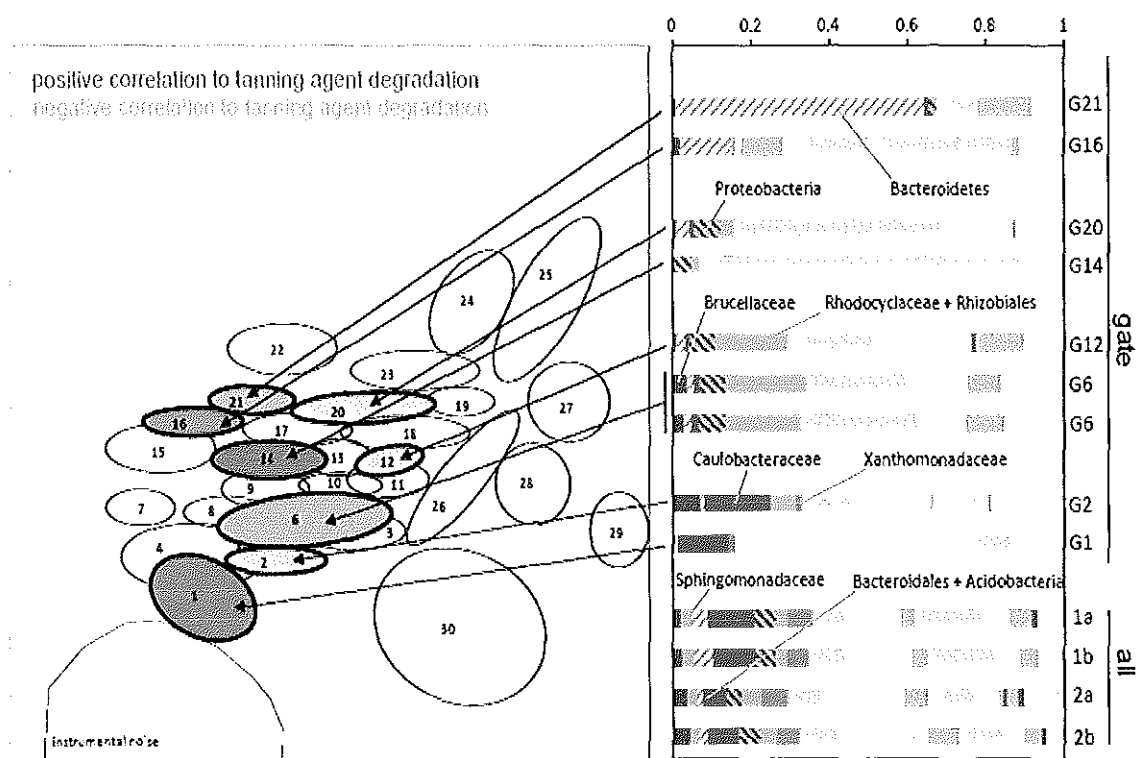


Figure 24 Taxonomic composition of the sorted gates associated with their role in the degradation of the retanning agents

Phase III: Field- scale analysis of microbial communities in anaerobic-aerobic reactors integrated with constructed wetland for the treatment of tannery wastewater

4.16. Physico- chemical characterization of influent and effluent water

Physico- chemical data showed that the untreated wastewater effluent was characterized by its high concentration of sulphate, Ammonia- nitrogen, total suspended and dissolved solids as well as high Biological and Chemical Oxygen Demands (BOD & COD). The high pH also indicated the alkalinity of the wastewater (Table 9). The overall performance of the treatment system in removal of the majority of pollutants ranged between 70 – 99%. The effluent parameters obtained for the COD, Sulfate (SO_4^{2-}), Sulfide (S^{2-}), Nitrate (NO_3) and Ammonia –Nitrogen ($\text{NH}_3\text{-N}$) were in line with the provisional emission limit values set for tannery effluents in Ethiopia (EPA 2005). The removal efficiency for total Nitrogen is low (74%) and its concentration in the effluent ($62.75 \pm 14 \text{ mg l}^{-1}$) was slightly above the discharge limit which is 60 mg/ l (Appendix IX). Likewise, the Biological Oxygen Demand (BOD) was higher than the standard limit of discharge though the removal efficiency was 94 % (Table 9).

Table 9 Average characteristics of the influent and effluent wastewaters of the integrated treatment system at the time of sludge sampling (concentrations are in mg/ l, except for pH).

Parameter	Influent	Effluent	% Removal
TN	245.25 ± 76	62.75 ± 14	74
SO ₄	800 ± 505	35 ± 61	96
TP	15.33 ± 1	4.23 ± 2	72
S ²⁻	55.50 ± 6	4.91 ± 3	91
NO ₃	310 ± 203	40.25 ± 28	87
NO ₂	2.08 ± 3	0.03	99
NH ₃	287.70 ± 178	44.28 ± 26	85
COD	12547.50 ± 3910	395 ± 139	97
BOD	4886.26 ± 266	308.91 ± 24	94
TDS	9470.50 ± 1335	2593.69 ± 344	73
TSS	1155 ± 203	92 ± 11	92
VSS	27482.75 ± 197	2272.75 ± 724	92
Total Cr	27.25 ± 3	0.95	97
pH	10.40 ± 0.3	7.66 ± 0.1	

TN: total Nitrogen, TP: total Phosphorous, TDS: total dissolved solids, TSS: total suspended solids, VSS: volatile suspended solids,
Total Cr: Total chromium

Upon looking at the relative treatment efficiency of the individual components of the biological treatment system, the constructed wetland component showed the highest removal efficiencies of total Nitrogen, total Phosphorus, Nitrite, Ammonia Nitrogen, COD, BOD, TDS, TSS and VSS (ranging from 70 – 93% removal). The anaerobic system performed well in the removal of Sulfate, Nitrate, Nitrite and Chromium, ranging from 60 – 91%. The aerobic system contributed most for the removal of reduced sulphur (S²⁻), accounting for 76%, although its contribution to the removal of total Nitrogen was less than 1% (Fig. 25).

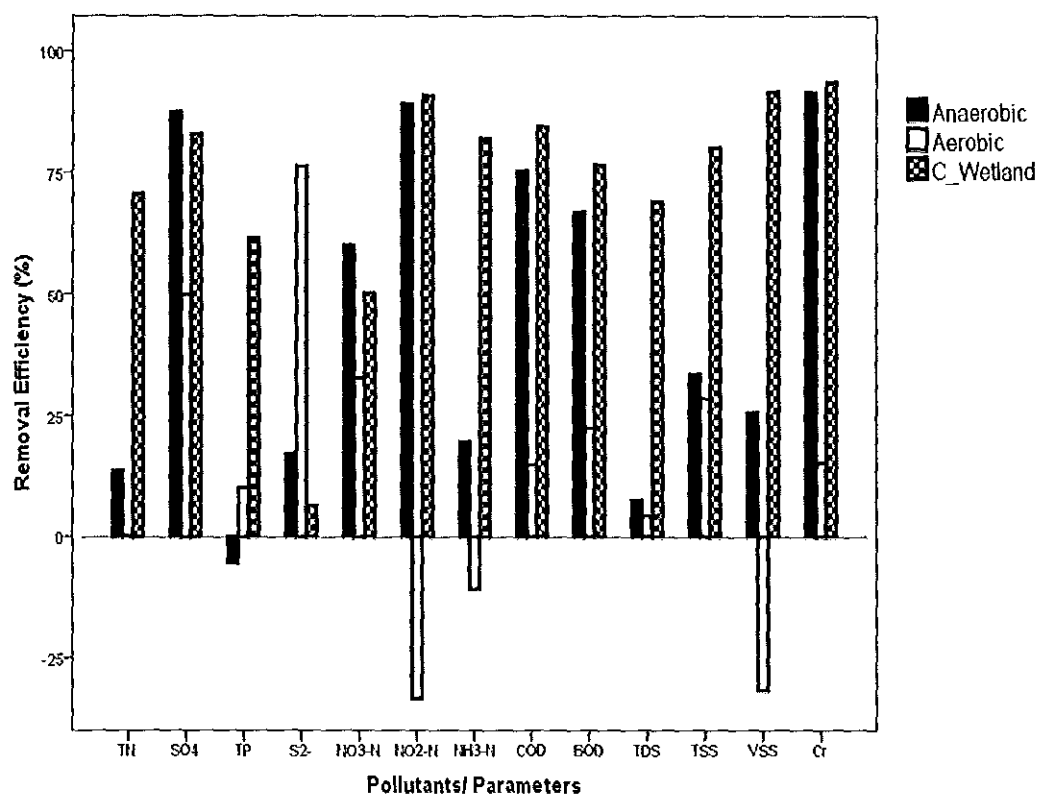


Figure 25 Relative performances of the anaerobic and aerobic reactors; and constructed wetland treatment system at each stage of the treatment of influent water.

4.17. Microbial composition in the sludge and rhizosphere of the integrated treatment system

A total of 1091 clones were screened by sequencing partial 16S rDNA inserts and 801 sequences belong to 31 phyla. 761 sequences were unique and were clustered within 255 operational taxonomic units (OTUs) on the basis of at least 97% sequence similarity. Close to 27% of the original clone sequences were removed based on the criteria for screening sequences in MOTHUR. No phylotypes were inferred from the ARDRA analysis using either of the restriction enzymes *HhaI* or *MspI*, as almost the entire clones exhibited unique band pattern. The Firmicutes were commonly found in all of the

systems, represented by 40% of all the sequences, followed by Proteobacteria and Bacteroidia, represented by 30% and 15% of the sequences, whereas the phylum Cyanobacteria accounted for 4.2%, respectively. The phyla Synergistetes and Acidobacteria and the unclassified bacterial group accounted for 2.8%, 1% and 3.6% of the total sequences, respectively (Fig. 26).

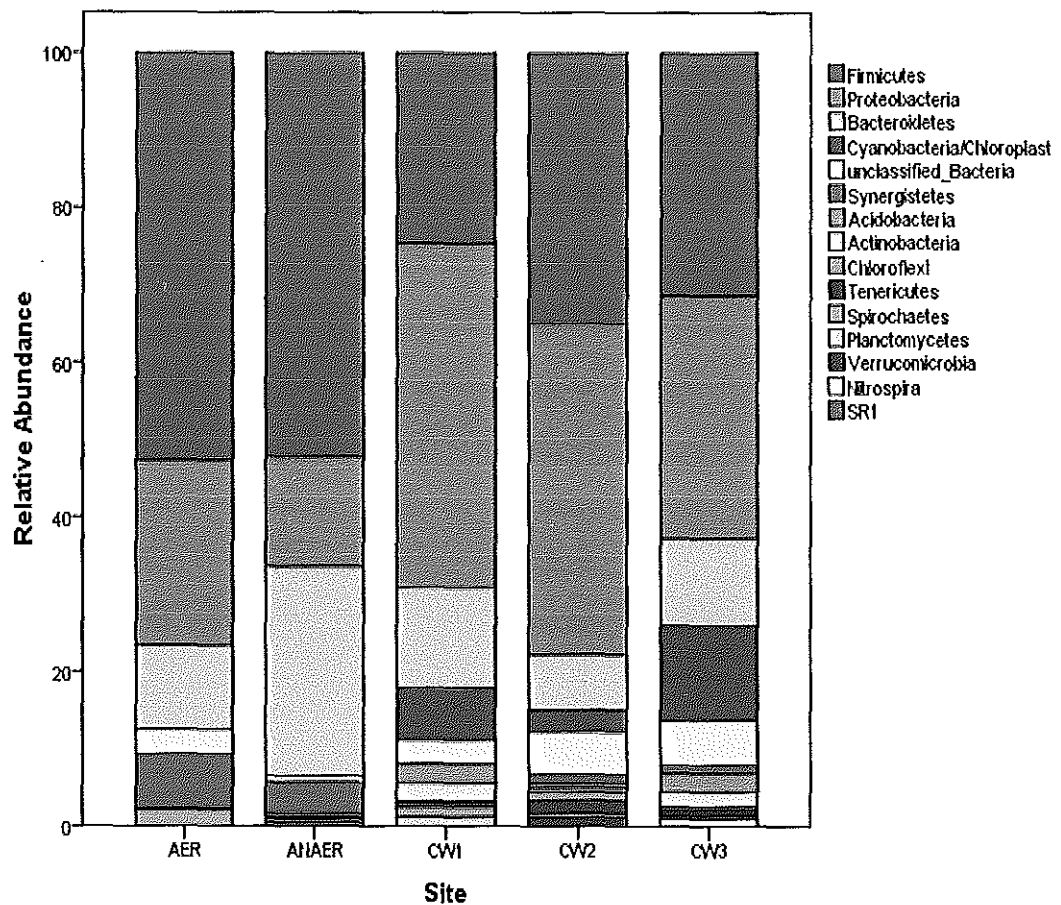


Figure 26 Composition of observed 16S bacterial phyla for the clone libraries from anaerobic, aerobic reactors and three constructed wetland sites

The phyla Actinobacteria, Chloroflexi, Tenericutes, Spirochaetes, Planctomycetes, Verrucomicrobia, Nitrospira and the uncultured candidate division SR1 comprised

a total of 3.5% of the total sequences, each of which represented by less than 1% of the sequences. In general, the phylum Firmicutes was represented by 53% of the sequences in the aerobic, 52% in the anaerobic, 44%, 43% and 31% in the three constructive wetland sites CW1, CW2 and CW3, respectively. Likewise, Proteobacteria was represented by 24% of the sequences in the aerobic reactor, 14% in the anaerobic reactor, 44%, 43% and 31% in CW1, CW2 and CW3 sites, respectively. The third largest phylum, Bacteroidetes was represented by 11% of the sequences in the aerobic reactor, 27% in the anaerobic reactor, 13%, 7% and 11% in CW1, CW2 and CW3 sites, respectively (Fig. 26). Sequences of the phylum Cyanobacteria were retrieved only from the constructed wetland sites and accounted for 7% in CW1, 3% in CW2 and 12% in CW3. Phylum Synergistetes was represented by 7% of the sequences in the aerobic reactor, 4% in the anaerobic reactor and 1% each in the CW2 and CW3 sites of the constructed wetland.

Among the Firmicutes, the class Clostridia was the most abundant class representing about 40% of the sequences in the aerobic and anaerobic reactors and 22% 32% and 29% in the three sites of the constructed wetland (CW1, CW2 and CW3 respectively). The second most abundant class, Bacilli accounted for 8% of the aerobic, 2% of the anaerobic, 3% of CW1 and 2% of both CW2 and CW3 sites. Class Negativicutes was retrieved only from the anaerobic site representing only 1% of the sequences. On the other hand, the class Erysipelotrichia was retrieved only from the aerobic site, representing 1% of the total sequence in the site. Sequences that could not be further classified under the phylum Firmicutes comprised 5% of the aerobic, 9% of the anaerobic and 1% of the CW2 site (Fig. 27).

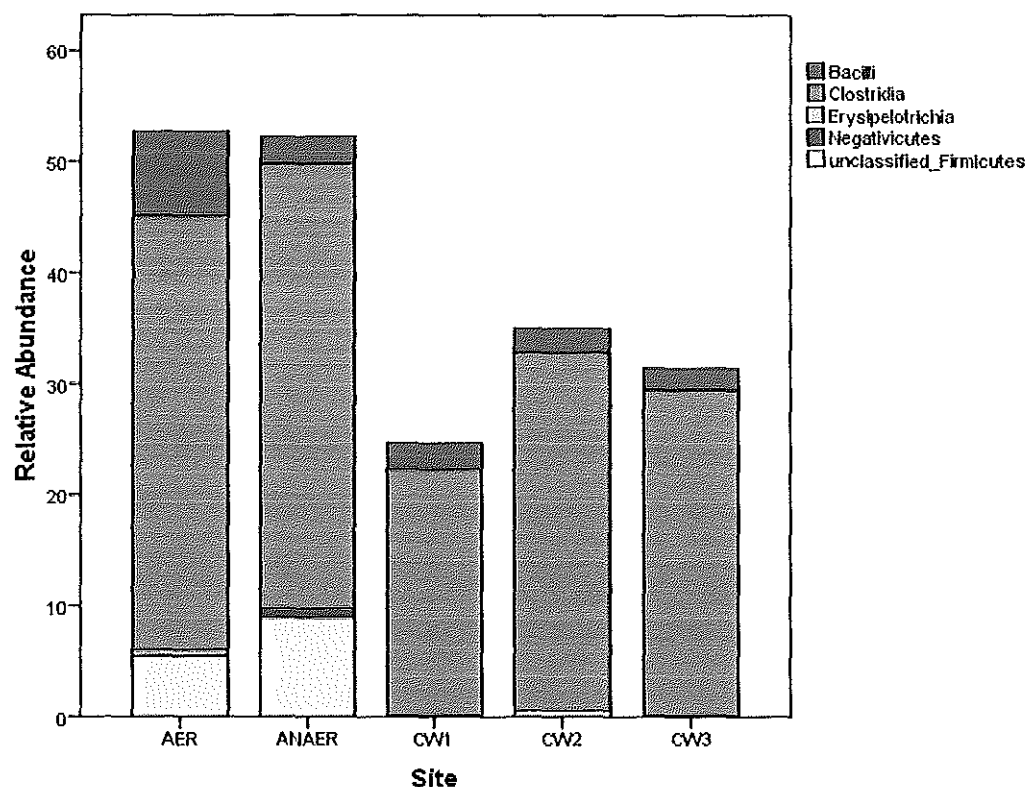


Figure 27 Class- level distribution of sequences affiliated to the phylum Firmicutes for the clone libraries from anaerobic- aerobic reactors and three constructed wetland sites

The most abundant sequences belonging to Clostridia in the aerobic reactor were closely related to the families Clostridiales Incertae Sedis XI (21%), Clostridiaceae 1 (16%), Ruminococcaceae (11%), Carnobacteraceae (9%), Peptostreptococcaceae (9%), Veillonellaceae (9%) and Clostridiales Incertae Sedis XIII (8%) (Fig. 28). In the anaerobic reactor, the dominant families were Clostridiaceae 1 (18%), Veillonellaceae (18%), Clostridiales Incertae Sedis XI (17%), Clostridiales Incertae Sedis XIII (12%), Peptostreptococcaceae (11%) and Ruminococcaceae (11%). Regarding the constructed wetland sites, the family Clostridiaceae 1 comprised 54%, 22% and 49% of the CW1,

CW2 and CW3 sites, respectively. Clostridiales Incertae Sedis XIII comprised 17%, 19% and 12% of the CW1, CW2 and CW3 sites, respectively (Fig. 28).

The family Ruminococcaceae accounted for 13% of the sequences in CW1, 9% in CW2 and 15% in CW3 of the constructed wetland site. Clostridiales Incertae Sedis XI was not retrieved from CW1 site but comprised 9% of CW2 and only 1% of CW3. Families with trace abundance in the sample sites belonged to Clostridiales Incertae Sedis XII and XI, Eubacteriaceae, Erysipelotrichaceae, Lachnospiraceae, Peptococcaceae 1, Syntrophomonadaceae and Thermodesulfobiaceae (Fig. 28).

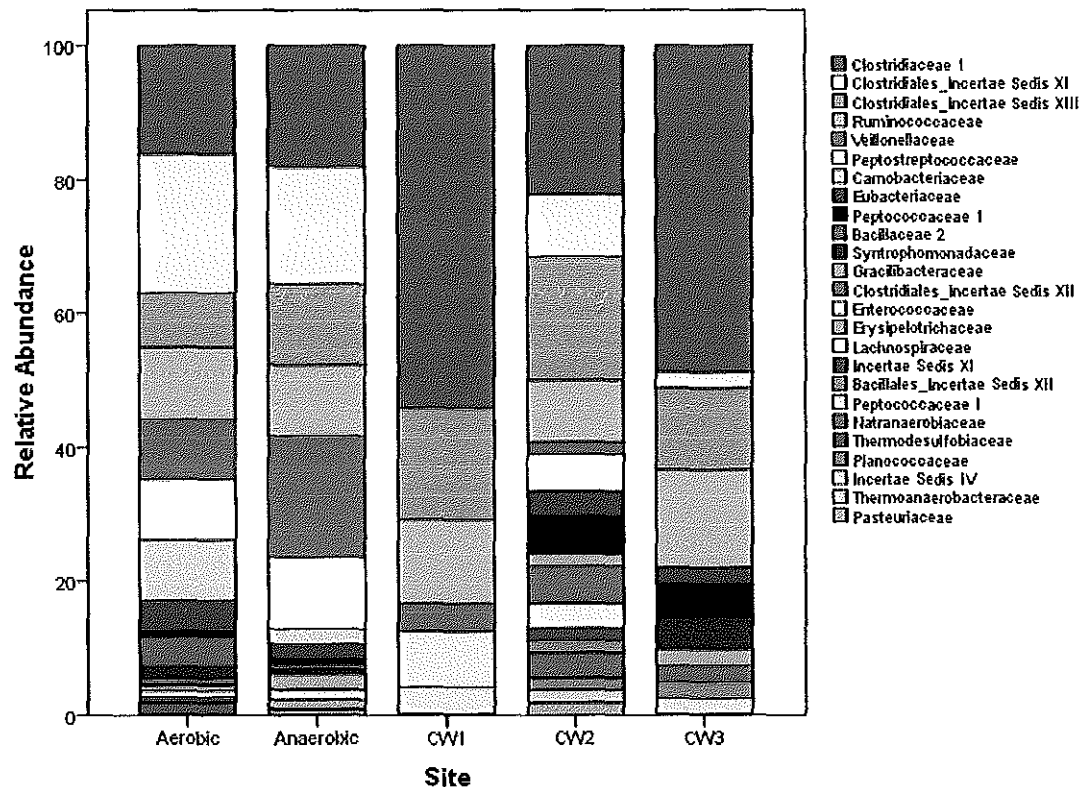


Figure 28 Family- level distribution of sequences affiliated to the class Clostridia for the clone libraries from anaerobic- aerobic reactors and three constructed wetland sites

A closer look into the Genus- level of the dominant clostridial families include the genus *Proteiniclasticum* of the family Clostridiaceae 1, accounting for 16% of aerobic, 18% of anaerobic and 50%, 22% and 46% of the three sites of the Constructed wetland; CW1, CW2 and CW3 respectively (Appendix VII). The Genus *Tissierella* of the family Clostridiales Incertae Sedis XI was dominant in the aerobic reactor (20%) and the second dominant member in the anaerobic reactor (14%) and of low abundance in the sites of the constructed wetland, with 0, 7% and 2% in the CW1, CW2 and CW3, respectively. Forty six genera including *Eubacterium* and *Acidaminobacter* were found to have less than 10% abundance. Sequences belonging to the genera *Proteocatella* and *Tissierella* were limited in the anaerobic and aerobic reactors and were not detected in the constructed wetland, while sequences similar to the genus *Acidaminobacter* were localized only to the root zone of the constructed wetland.

Among the four classes of the phylum Proteobacteria, members belonging to the class Betaproteobacteria were the most dominant sequences in the aerobic reactor (13% of the total sequences) and in the constructed wetland sites (15%, 13% and 12% of the sequences in CW1, CW2 and CW3, respectively), while 1.6% abundance was found in the anaerobic reactor (Fig. 29). The second abundant class in the anaerobic reactor was Deltaproteobacteria, accounting for 7% of the total sequences. Gammaproteobacteria was the second abundant class in CW1 and CW2 rhizosphere sites, accounting for 12% and 11%, respectively. Deltaproteobacteria was the third abundant class in all the three rhizosphere sites of the constructed wetland accounting for 9%, 16% and 11% in CW1, CW2 and CW3, respectively. Alphaproteobacteria and Epsilonproteobacteria were the ones which were represented by the lowest percentage of the population in all the sites.

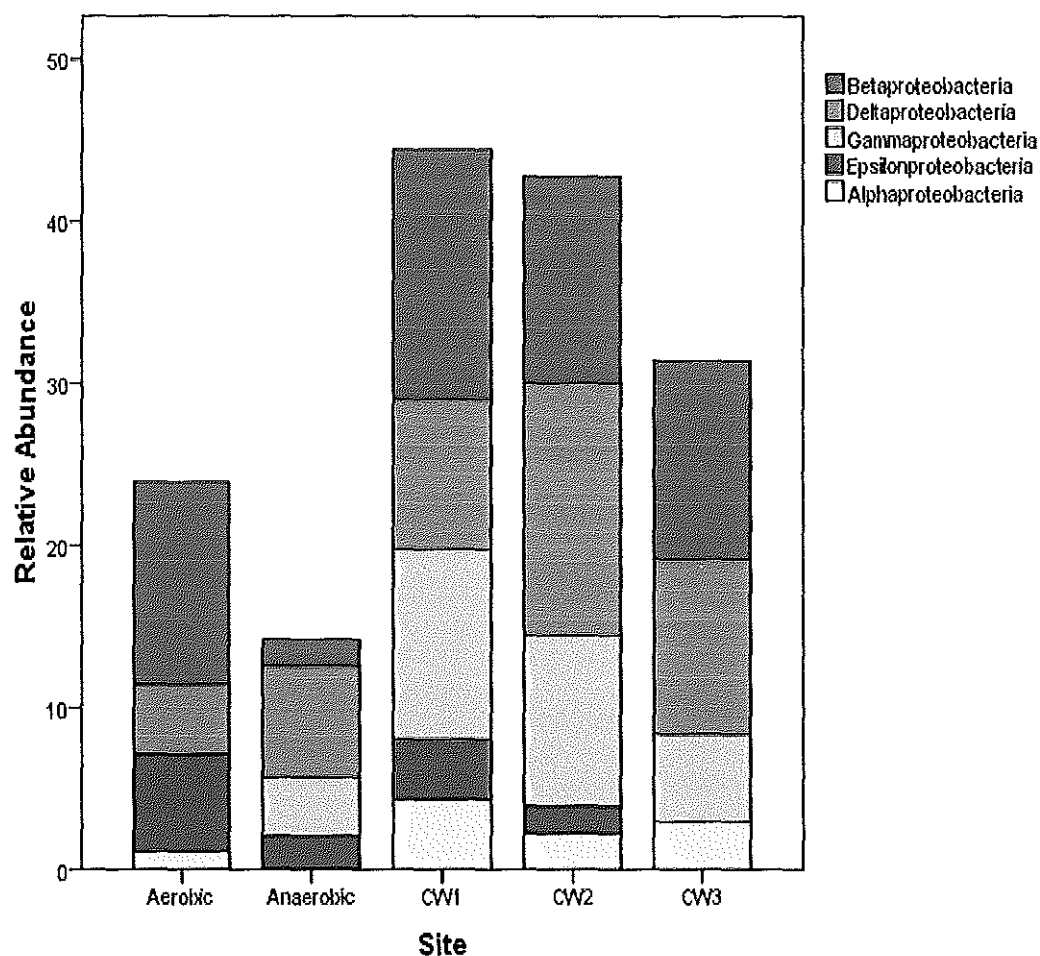


Figure 29 Class- level distribution of sequences affiliated to the phylum Proteobacteria for the clone libraries from anaerobic- aerobic reactors and three constructed wetland sites

Considering the class- level abundance of the Phylum Bacteroidetes in each reactor and rhizosphere site, members of the class Bacteroidia were the most abundant in the anaerobic reactor, accounting for 23% of the sequences (Fig. 30). In the aerobic site, the class accounted for only 4% of the total sequences. In all three sites of the constructed wetland, the class accounted for 7% (in CW1), 5% (CW2) and 6% (CW3). The classes

Sphingobacteria, Bacteroidetes incertae sedis and the unclassified Bacteroidetes accounted for less than or equal to 5% of the total sequences in each sample site.

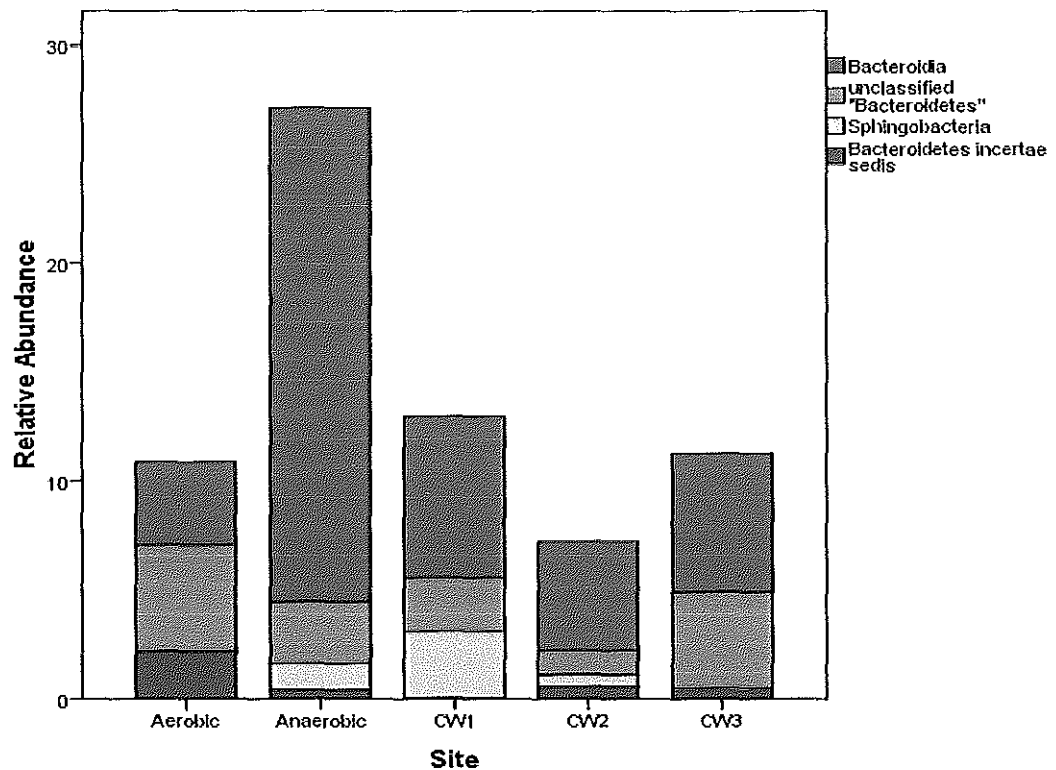


Figure 30 Class- level distribution of sequences affiliated to the phylum Bacteroidetes for the clone libraries from anaerobic- aerobic reactors and three constructed wetland sites

4.18. Evaluation of diversity and richness

The Shannon-Wiener (H) and Simpson's (D) diversity indices indicated that the constructed wetland samples showed the highest diversity, followed by the anaerobic reactor. The aerobic reactor was found to have the least diverse bacterial communities in this study. Likewise, richness estimator values S_{ACE} and Chao I reflected the high

diversity of OTUs in all sites of the constructed wetland (CW1, CW2 and CW3) than the aerobic and anaerobic reactors (Table 10).

Table 10 Estimated sample coverage, community richness and diversity estimators of the 16S rRNA gene clone libraries of Modjo Tannery effluent treatment plant samples

Library (Site)	Richness estimators ^a					Diversity indices	
	NS (N)	S	ESC	S _{ACE}	Chao I	Shan non (H)	Simpson's (D)
Anaerobic	223 (247)	83	0.78	212 (168-278)	170 (123-270)	4.2	0.034
Aerobic	151 (184)	58	0.78	141 (109-193)	124 (84-224)	3.9	0.032
CW1	125 (162)	84	0.48	524 (397-702)	257 (168-440)	5.0	0.011
CW2	131 (180)	97	0.42	325 (217-529)	301 (201-496)	5.3	0.006
CW3	131 (204)	86	0.50	425 (313-594)	265 (173-452)	4.9	0.014

Abbreviations: N, Number of clones in each library; NS, Number of unique sequences for each library; S, richness expressed by number of observed OTUs; ESC, estimated sample coverage.

^a Values in Parenthesis are 95% confidence intervals

4.19. Coverage of the populations

Rarefaction curves of the aerobic reactor and CW1 site showed curvilinear plots that leveled- off, indicating what is actually in these sites is well represented by the sequences generated from these two sites. The rarefraction curves for the anaerobic, CW2 and CW3 sites failed to level- off indicating the number of clones sequenced from these sites was not enough to cover the full bacterial diversity in the sites (Fig. 31).

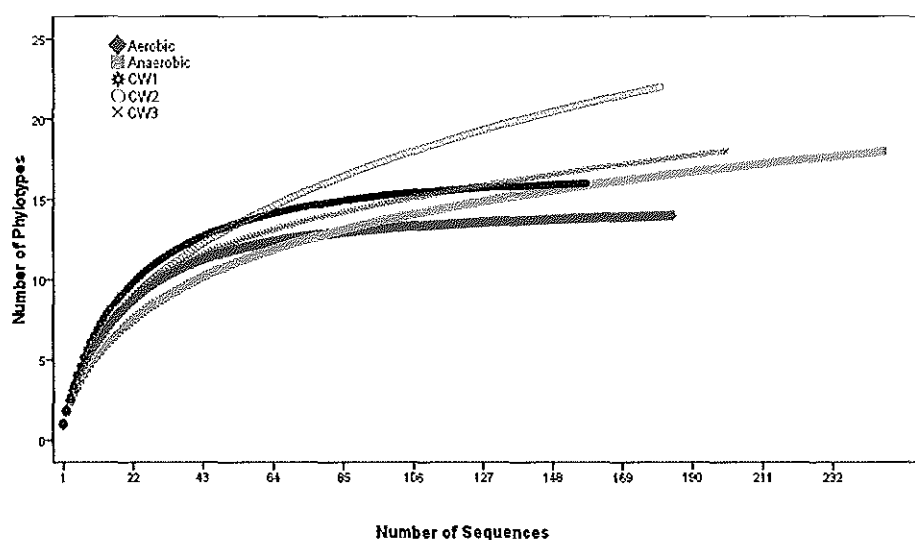


Figure 31 Rarefaction curves of observed 16S bacterial phylotypes for the clone libraries from anaerobic and aerobic reactors and the constructed wetland site

4.20. Distribution of populations across the different sites

Unifrac analysis confirmed the significant differences in composition of bacterial communities among aerobic, anaerobic and CW1 of the constructed wetland sites whereas CW2 and CW3 did not show any significant differences in their community composition ($p > 0.01$) (Table 11). Likewise, analysis using non-metric Multi Dimensional Scaling (nMDS) elucidated the rank and position of each sampling site with

respect to the bacterial phylotype and frequency, indicating the similarity sequences from two (CW2 and CW3) of the three sites of constructed wetland samples (see Appendix VIII).

Table 11 Statistical significance (P- values) of differences among the bacterial communities of sites of the biological treatment calculated based on UniFrac analysis of partial sequences of 16S rRNA gene

	Aerobic	Anaerobic	CW1	CW2	CW3
Aerobic	-	< 0.001	< 0.001	< 0.001	< 0.001
Anaerobic		-	< 0.001	< 0.001	< 0.001
CW1			-	0.003	0.001
CW2				-	0.041*
CW3					-

P-values of Weighted UniFrac test are calculated based on 1000 permutations (based on pairwise differences).
 * indicate populations not significantly different from each other (P> 0.01).

4.20. Identification of shared populations across the study sites

To determine the proportions of the bacterial populations that are shared among the aerobic, anaerobic and constructed wetland sites, comparison of the sequences clustered into OTUs from each library was performed. Three OTUs represented by 80 sequences were shared among all the sites (Fig. 32). Taxonomic identification based on BLAST and RDP analysis with a cut- off value of 97% similarity revealed that out of the 80 shared sequences, 26 sequences were members of the Family Clostridiales Incertae Sedis

XIII, 36 sequences were members of the phylum Bacteroidetes and 18 sequences belonged to unclassified bacterial group. Based on the finding (Table 11) that clone libraries of CW2 and CW3 of the constructed wetland sites did not show significant variations in their microbial composition, sequences of CW2 and CW3 were pooled and normalized (n=131) to be represented as a single site in the venn- diagram.

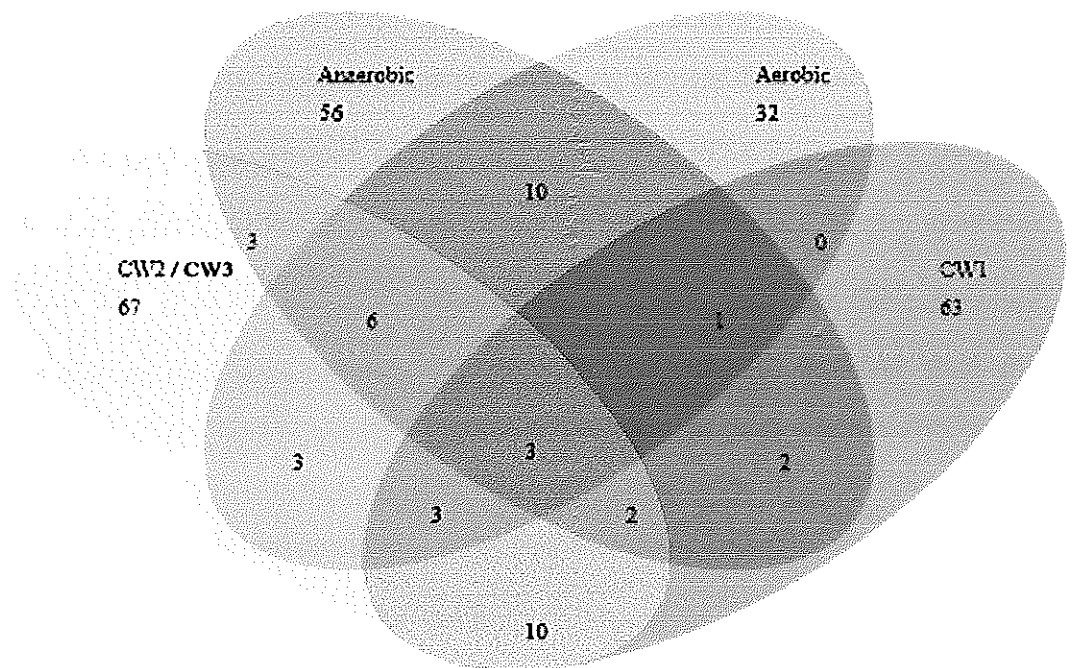


Figure 32 Venn diagram showing OTUs shared between the sample sites of the integrated biological treatment facility as determined by sequence diversity analysis.

The four bacterial strains B1, B45, R19 and R28 showed moderate to high degradation of both retanning compounds indicating the ability of the strains to metabolize the structurally different chemicals with different extents of degradation or transformation (Figs. 15 & 17). *Brevibacterium pityocampae* strain B4500R (B45) was characterized by having relatively the highest degradation ability of Basyntan and more than 50% degradation for Retanal. The Retanal degradation ability of this strain was also compounded by the highest production of ammonia (Fig. 14). Studies on different strains of the Genus *Rhodococcus* which is closely related with *Brevibacterium* had been reported to exhibit upto 100% degradation ability for para- Nitrophenol (Leilei *et al.*, 2012). A study on the biodegradation of phenol by *Rhodococcus sp.* also showed complete removal (100%) in both in batch scale and bioreactor scale at a temperature of 30°C and pH adjusted to 7 (Paisio *et al.*, 2012). Another strain of the same genera, *Rhodococcus erythropolis* SKO-1, showed 99.6% degradation of phenol in batch scale which increased to 100% upon addition of yeast extract into the minimal salt medium (Soudi and Kolahchi, 2011).

The strain identified as *Micrococcus luteus* (B1) showed the second highest degradation on peaks 1 and 2 of Basyntan (13 and 15% respectively) (Appendix II). Several studies have shown the catabolic versatility of strains of *Micrococcus luteus*, having the ability to utilize a wide range of hydrocarbons such as Napthalene (Zhuang *et al.*, 2003), carbon-based pyridine (Sims *et al.*, 1986) and involve in the bioaccumulation of metals (Lo *et al.*, 2001). Moreover, potentially toxic substrates such as malathion and chlorpyrifos (Guha *et al.*, 1997) have also been found to be consumed as a source of carbon by strains of *Micrococcus luteus*.

Enterococcus gallinarum strain R2801R (R28) showed moderate performance on degradation of Basyntan and Retanal, with more than 50% degradation of the latter. Other studies on *Enterococcus gallinarum* strains showed their ability to exhibit 100% degradation of 100 mg/l benzidine- based azo dye (Bafana *et al.*, 2009), which has structural similarities with that of Basyntan. Likewise, a strain of *Enterococcus gallinarum* isolated from Buffalo's gastrointestinal tract was found to show the degradation of tannic acid (Wahyudi *et al.*, 2010), a polyphenolic compound with similarities to Basyntan.

With the strain *Lysinibacillus sphaericus* R1902R (R19), the degradation was less than 50% both for Basyntan and Retanal hence low performance on degradation of the aromatic Basyntan (Figs. 15 & 17). Other studies showed the degradative abilities of various members of the genus *Lysinibacillus* on the phenolic compound *m*-Cresol and polycyclic aromatic compounds (Ma *et al.*, 2010; Yao *et al.*, 2011).

Analysis of the kinetic properties of the strain *Brevibacterium ptyocampae* B45 showed high maximum growth rate ($\mu_{\max}$ = 1450 cells/ml/hr) and a half saturation constant which is much lower (K_s = 10.16 mg/l) than the amount needed for optimal growth (500 mg/l). The kinetic properties of this strain was much greater than the *Micrococcus sp* which belongs to the same phylum as *Brevibacterium* and was capable of degrading melamine formaldehyde resin similar to the constituent of Retanal with a maximum growth rate ($\mu_{\max}$) of 0.83 cells /ml/ hr and half saturation constant (K_s) of 7.18 mg /l (El- Sayed *et al.*, 2006). The kinetic parameters obtained from *Brevibacterium ptyocampae* B4500R (B45) indicates the potential of the strain for use in bioaugmentation schemes in wastewater treatment plants for optimal degradation of Retanal.

To assess the combined influence of pH and temperature changes on the growth of the four characterized isolates, qualitative assessment was used in a range of pH from 6 to 10 in a temperature range of 20- 40°C. The isolate which best tolerated the tested ranges of pH and temperature was *Micrococcus luteus* B1R (B1), followed by *Enterococcus gallinarum* R2801R (R28). The above strains also grew better in the alkaline pH than the strains *Brevibacterium ptyocampae* B4500R (B45) and *Lysinibacillus sphaericus* R1902R (R19), which stood at the third and fourth levels, respectively.

Upon comparison of pH range for the growth of *Micrococcus luteus* B1R (B1) with other isolates cultivated in temperature ranges of 25 – 35°C, a strain of *Micrococcus flavus* isolated from activated sludge of a sequencing batch reactor treating mixed wastewater of nitroaromatic compounds and aniline showed growth on LB agar over a temperature range of 26- 34 °C and pH range of 5- 9 (Liu *et al.*, 2007). On the other hand, a novel *Micrococcus sp.* BRM7 strain isolated from a phosphate mine showed tolerance more than *M. luteus* B1R (B1) with a wider range of pH ranging from 5 to 12 and temperature as high as 45°C (Salem *et al.*, 2011).

Similarly, *Enterococcus gallinarum* R2801R (R28) was found to grow at pH ranging from 7 up to 9 at all the temperatures tested. Different strains of *Enterococcus sp.* have been reported to grow over a broad range of pH from 3 to 8 (Pelinescu *et al.*, 2009) and even as high as pH of 10 and a maximum temperature of 65°C (Fisher and Philips, 2009), showing better pH tolerance and thermophilic nature than that of the present study.

Brevibacterium ptyocampae strain B4500R (B45) showed pH tolerance of 7 to 9 at the temperatures ranging from 25°C up to 40°C. A similar study on *B. casei* upon growth in

minimal medium supplemented with hexavalent Chromium and pentachlorophenol (PCP) showed growth and degradation in a temperature range of 30°C up to 40°C and a pH range of 6.5 up to 10 (Verma and Singh, 2013).

Lysinibacillus sphaericus R1902R (R19) grew in the narrowest range of pH of 8 and 9 within temperature range of 20°C to 35°C which is much lower than a strain of *Lysinibacillus parviboronicapiens* grown over a wider range of temperature and pH ranging between 10°C to 37°C and 6 to 8 respectively (Miwa *et al.*, 2009).

Phase II: Analysis of microbial community dynamics and identifying key sub-communities for degradation of synthetic retanning agents in lab-scale Sequencing Batch Reactor

The performance of the lab- scale sequencing batch reactor (SBR) was evaluated on the basis of different pollution parameters and retanning agents tested on selected microorganisms (Phase I). Increasing efficiency in the reduction of COD and TOC was observed from batch 9 onwards, when the bacterial proliferation (Fig. 22) and diversity (Table 8) was getting higher, indicating the role of the bacterial communities in the removal of organic matter. Despite the fact that removal of ammonium nitrogen in the SBR was as high as 80%, poor performance with regard to nitrogen removal measured as total nitrogen (TN) was observed throughout the operational time of the SBR (Table 6). Andualetm Mckonnen and Seyoum Leta (2011) also found poor performance of TN with 38% removal in a lab- scale SBR optimized for the treatment tannery wastewater, suggesting the need for rigorous optimization of the system with regard to nitrogen removal. Another factor for the observed low efficiency in denitrification could be the

case described by Obaja *et al* (2005) on lab- scale SBR for the removal of pollutants from piggery wastewater, which indicated the need for easily biodegradable organic matter serving as a carbon source for efficient denitrification to take place in a system. In the present study the main carbon sources are limited to the retanning agents and the tannery wastewater both of which cannot be grouped under easily biodegradable carbon sources and thus may have hampered the denitrification process in the SBR.

Retanal biodegradation in the SBR did not follow a steadily decreasing pattern across the different batches of the reaction unlike the pattern observed when using single bacterial strains (Phase I of the study). In addition, sporadic increase in the concentration of Retanal towards the end of the reaction period of the SBR was observed. In these events a marked increase was observed in the absorption unit of the detected chromatogram peaks, which is an indicator of emergence of polar groups, conjugated double bonds and/or carbonyl (C=O) and imine (C=N) groups (Darnoko *et al.*, 2000). Based on the proposed metabolic pathway of melamine formaldehyde, splitting of melamine moieties and removal of formaldehyde precede melamine degradation, which involves successive deamination followed by decarboxylation and finally hydrolysis to produce urea and ammonia (El- Sayed *et al.*, 2006; Shelton *et al.*, 1997). At this level of the study, it is not possible to explain about how the pre- degradation events contribute to the increased reactive groups resulting in increased absorption units of the detected peak. In the presence of such complex bacterial communities biotransformation of the splits leading to emergence of intermediates cannot be ruled out.

The observed disappearance of one of the peaks of Basyntan from the 7th batch till the end of the experiment indicates the complete degradation of the phenolic compound

accumulation and subcommunities belonging to the family Rhodocyclaceae, Sphingobacteriales and Chloroflexi, suggesting its power to reveal index subcommunities for certain functions such as phosphate accumulation. Koch *et al.* (2013) recently developed a tool named cytometric bar coding (CyBar) to stratify the cell number variation over time in accordance to changing abiotic conditions such as substrate overload and sulfide accumulation. This additional tool helps to give a refined conclusion of the functional correlation data on which positive correlation does really imply causation.

Phase III: Field- scale analysis of microbial communities in anaerobic-aerobic reactors integrated with constructed wetland for the treatment of tannery wastewater

In this component of the study, the wastewater entering into the treatment system contained high amounts of organic and inorganic pollutants shown by high amounts of Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD). The high amounts of COD and BOD are in line with a study previously performed on Modjo Tannery effluent by Seyoum Leta *et al.* (2003), which reported a COD of 11123 ± 563.9 mg/l and a BOD of 2982 ± 259 mg/l. The concentrations of total Nitrogen, total Cr and sulfide recorded in this study were much lower than the study by Seyoum Leta *et al.*, (2003) in which 1330.0 ± 182.1 mg/l total N, 32.2 ± 5.7 mg/l total Cr and 630.4 ± 67.0 mg/l sulfide was reported. Total Dissolved Solids (TDS), ammonia and sulfate in this study were higher than the study conducted by Seyoum Leta *et al.*, (2003) in which 6646.0 ± 556.5 TDS, 122.2 ± 8.3 mg/l ammonia and 502.0 ± 82.9 mg/l sulfate were reported.

Highest reduction was achieved by the constructed wetland system with regard to Ammonia Nitrogen, COD, BOD, TSS, VSS and total Chromium, indicating the efficiency of the wetland as a secondary treatment system. This is in agreement with a study by Calheiros *et al.* (2010), which indicated the efficient capacity of constructed wetlands in polishing hypersaline water coming out from a conventional tannery wastewater treatment plant.

Based on the sequence analysis performed in this study, the phyla Firmicutes, Proteobacteria and Bacteroidetes represented around 85% of the sequences generated, with the phylum Firmicutes comprising 40% of the sequences. In a series of culture-

based studies, Calheiros *et al.* (2009; 2010) reported the microbial community dynamics of constructed wetland vegetated with different plants for treating tannery wastewater dominated by members of bacteria affiliated to Firmicutes followed by gamma Proteobacteria. Generally, members of the phylum Firmicutes are spore-forming bacteria that ensure survival in stressful environmental conditions such as hypersalinity, pH and high oxygen demand, which are typical characteristics of a tannery effluent (Calheiros *et al.*, 2010; Lefebvre *et al.*, 2006; Seyoum Leta *et al.*, 2003).

A closer look into the dominant phylum Firmicutes indicated that the class Clostridia was the most dominant bacterial class across all the sample sites, comprising 33% of the total community. Members of this class are anaerobic bacteria which were isolated from anaerobic sewage sludge and an Up-flow Anaerobic Sludge Blanket (UASB) reactor (Castello *et al.*, 2011) and constructed wetlands treating tannery effluents (Calheiros *et al.* 2010). In another study that involved tannery wastewater treatment using submerged anaerobic membrane bioreactor, it was found that the class Clostridia was dominant in the system (Vyrides and Stuckey, 2011). Other reports indicated the presence of these groups in constructed wetlands treating dairy washwater, which has less pollution load than tannery wastewater (Ibekwe *et al.*, 2003). Some of the identified Clostridia in this study such as the genera *Proteiniclasticum*, *Tissierella*, *Eubacterium* and *Acidaminobacter* have been implicated in the degradation of aromatic hydrocarbons like tetrachloroethylene and sulphate reduction (Dong *et al.*, 2011; Meijer, 1999; Webster, 2009).

Betaproteobacteria, the second most abundant bacterial class in the aerobic reactor and wetland sites (10%), comprised the genera *Azospira* and *Thauera* belonging to the family

Rhodocyclaceae and *Hydrogenophaga sp.* of the Family Comamonadaceae (Appendix VII). Although members of these genera have not been reported specifically in tannery wastewater, they were identified from oil refineries and wastewater containing aminobenzosulfate and found to be involved in reduction of perchlorate, oxidation of alkane and 4- aminobenzosulfate degradation (Contzen *et al.*, 2000; Dubbles *et al.*, 2009; Kathrynne *et al.*, 2012). In phase II of the current study, the families Rhodocyclaceae and Comamonadaceae were detected at the different batches of the reactor operation but without inference with any pollutant removal in the system. Taking their abundance into account, these members of the class Betaproteobacteria observed in this study may play a role in the functional stability of the community. Koch *et al.* (2013) also observed such “bystander” sub- communities while analyzing community dynamics in a small- scale biogas reactor, suggesting their stabilizing role in the community.

Bacteroidetes, the third abundant phylum in all the sample sites has been well implicated with the degradation of complex carbon (Burns *et al.*, 2012). The second phase study of this dissertation on a bench- scale sequencing batch reactor (Phase II) using seed sludge from Modjo Tannery wastewater revealed the key role played by Bacteroidetes in the degradation of retanning chemicals. Therefore, the presence of members of this phylum in the current study might strongly be implicated in the degradation of aromatic compounds that are used in the post- tanning process.

Deltaproteobacteria, the class comprising 9% of the reactors and wetland community, is represented by members of the genera *Desulfococcus*, *Desulfomicrobium*, *Desulfovibrio* and other genera which are exclusively sulfate and sulfur- reducing bacteria (Appendix VII). Members of these genera may be implicated to involve in oxidation of the excessive

sulfides generating from both the production process and microbial reduction of sulfur, thus contributing to the high removal efficiency (91%) of sulfides in the treatment system.

In the present study, the class Alphaproteobacteria was found to be less abundant, comprising 2% of the total sequences. This is in contrary to previous studies where they were found to be one of the dominant classes in Chromium and Arsenic contaminated soil samples taken from the vicinity of tanning industries as well as a collective tannery effluents treatment facility (Lefebvre *et al.*, 2006; Sheik *et al.*, 2012). Studies have shown that salinity is one of the environmental factors that can exert selective pressures on the microbial community that can change the composition of the dominant bacterial groups (Lozupone and Knight, 2007; Sivaprakasam *et al.*, 2008). The salinity of the wastewater in the present study (TDS: 9-12 g/l) was much higher than that of the study by Lefebvre *et al.* (2006) which might have accounted for the rareness of members of the class Alphaproteobacteria.

The higher diversity of microbial community in the constructed wetland sites than those of the aerobic and anaerobic sites might be due to the presence of the wetland plant *Phragmites australis* (Cav.), which provides more surface area for the bacteria and renders the wetland system to be less confined than the anaerobic and aerobic reactors. A comparative study by Collins *et al.*, (2004) conducted on different constructed mesocosm wetland systems for the remediation of acidic and metal contaminated water from coal pile indicated that the presence or absence of plants affect the bacterial assemblages in a wetland system that eventually affect water quality. Within the constructed wetland, the three sample sites did not show wide variability in terms of

species diversity and composition, suggesting that bacteria colonized the root zones in a similar fashion despite the observed variation in the vegetation density across the three sites.

In the current study, the overall high bacterial diversity in the anaerobic and aerobic reactors as well as the wetland sites might contribute to the performance of the system, which was expressed in the removal efficiencies of the integrated system with regard to the majority of the pollutants studied. However, in order to come up with a solid conclusion about the bacterial populations in relation to roles in removal of these pollutants, it is important to perform a longitudinal investigation of microbes in each component of the treatment system as part of a routine measurement of abiotic parameters.

Conclusion and recommendation

All in all, this study provided an investigation of biodegradability of two of the several types of retanning chemicals used in the tanning industry. It showed that individual bacteria belonging to the phyla Firmicutes and Actinobacteria have the ability to consume the retanning chemicals as their sole source of carbon and Nitrogen, which was revealed by cell growth, protein production and ammonia release. The isolate B45, identified as *Brevibacterium pityocampae* exhibited maximum degradation of 41% and 63% for Basyntan and Retanal, respectively. Despite its relatively poor degradative performance, the isolate R28, identified as *Enterococcus gallinarum*, had shown excellent growth on the retanning agents in wide range of temperatures (20°C – 40°C) at neutral to alkaline pH (pH 7 to 10) ranges.

Complete degradation (100%) of one of the compounds of Basyntan was observed in a lab- scale sequencing batch reactor (SBR) indicating better biodegradation by complex microbial communities than single isolates. The attempt to understand the microbial community structure and dynamics over the fourteen batches of the SBR using a combination of different culture- independent tools revealed the phylogenetic diversity of the community and helped interpret the putative functional roles the identified communities could play in the SBR treatment system. It was also possible to single out bacterial groups such as Bacteroidetes and proteobacteria, which have strong correlation with the complete degradation of some of the compounds in retanning chemicals.

The microbial community study at a field- scale biological treatment system provided a snapshot of the bacterial community of different biological reactors working in concert for efficient treatment of a tannery wastewater. The study revealed a highly diverse

community harboring the five sample sites with the highest diversity in the constructed wetland. The identified representative genera of the dominant bacterial classes obtained from the different sample sites, together with the key microbial groups identified from a bench- scale SBR for the removal of pollutants of synthetic origins, have been implicated in the degradation of various carbon- containing pollutants of natural and synthetic origins.

Several questions remain unanswered and for this reason, the following points are recommended:

- Detailed study should be conducted to identify the exact chemical structures present in the retanning agents to track their exact fate in the biological degradation.
- Further studies on the strains identified in this study should be conducted to evaluate the role of the bacterial strains for effective bioaugmentation in the Anaerobic- aerobic reactor for efficient degradation of synthetic compounds.
- The effect of additional carbon and Nitrogen sources on the degradation efficiency by the strains identified so far and other isolates should be studied in order to attain better biodegradation of the retanning chemicals.
- The potential inhibitory effects of these chemicals on nitrification and denitrification processes should be clearly studied on lab- scale/ field- scale treatment systems.
- Functional analysis of the microbial communities in the field- scale integrated wastewater treatment system should be conducted in order to clearly understand the contribution of each member to the performance of the biological wastewater

treatment system. This will provide a useful clue on replicating the microbial community structure in sludge of other wastewater treatment plants for maximum performance of the systems.

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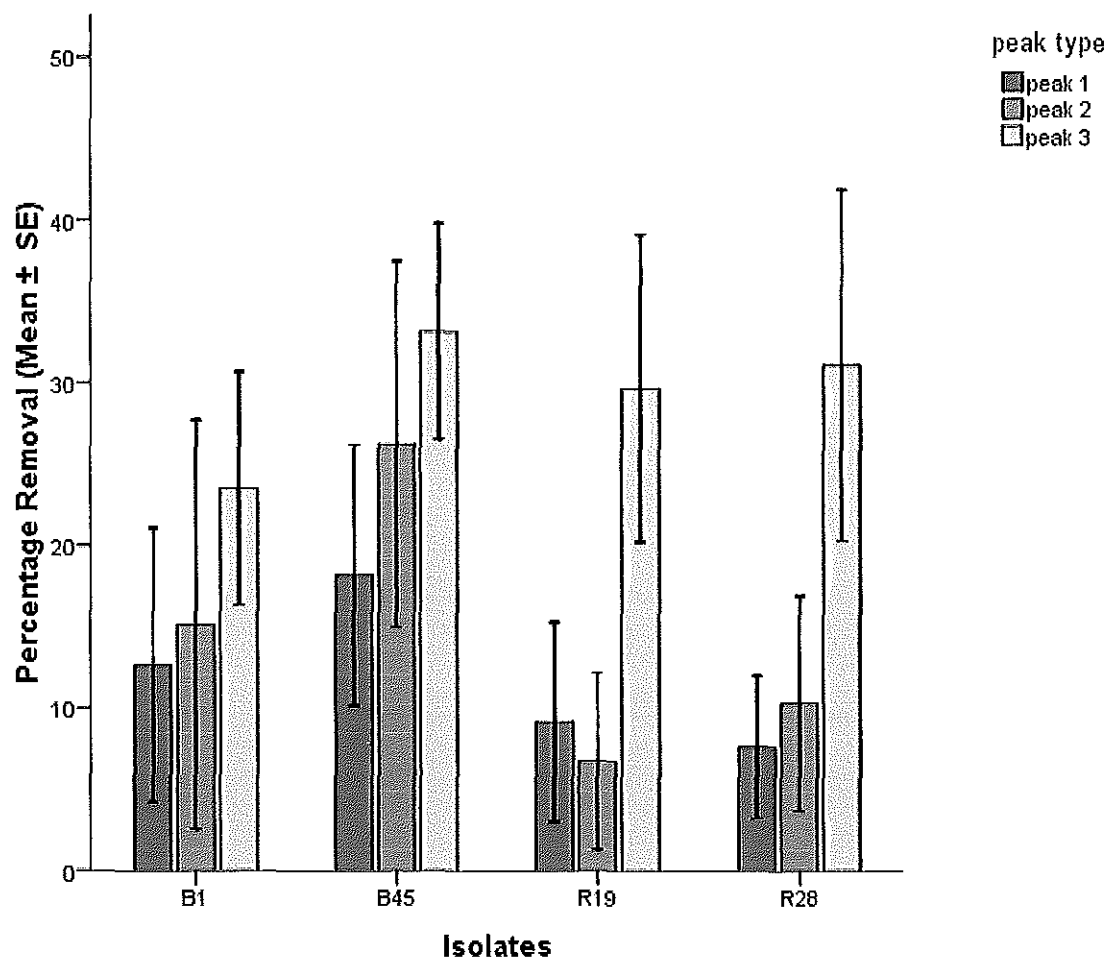
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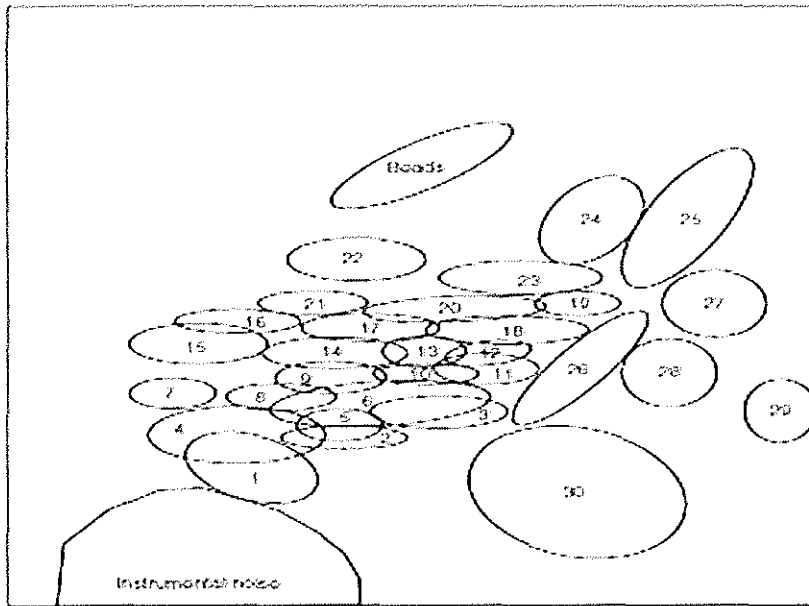
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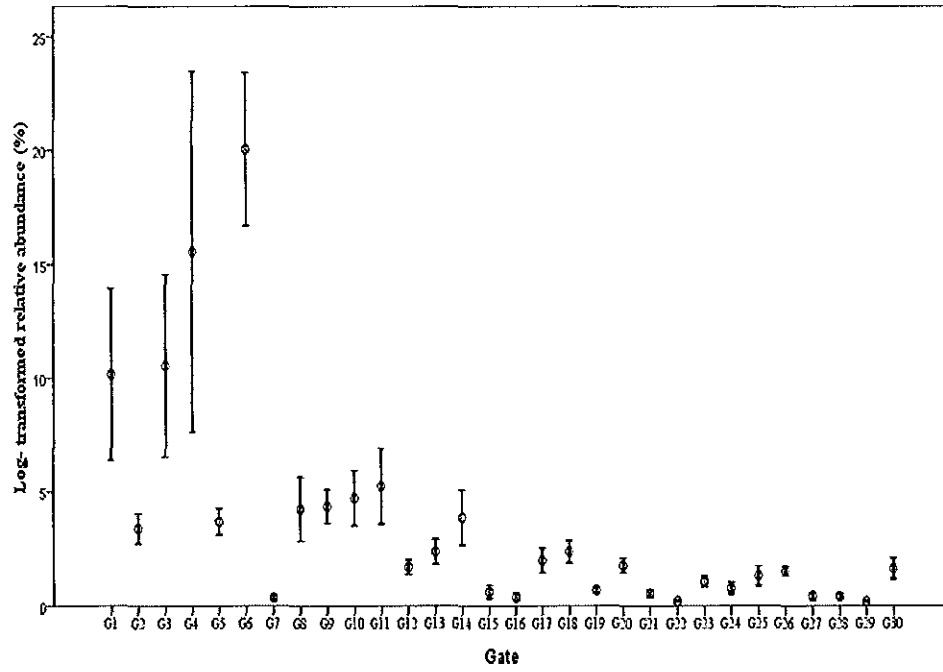
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Appendix II Mean percentage degradation of the three peaks of Basyntan by four isolates (X- axis) upon cultivation for 26 days



Appendix III Gate template created based on the pattern of each of the sub communities over the 14 batches of the SBR



Appendix IV Mean relative cell number of each gated sub community in the SBR over all the 15 batches of the SBR running period

Appendix V Representative hits of TRFLP results based on clone- library based sequences

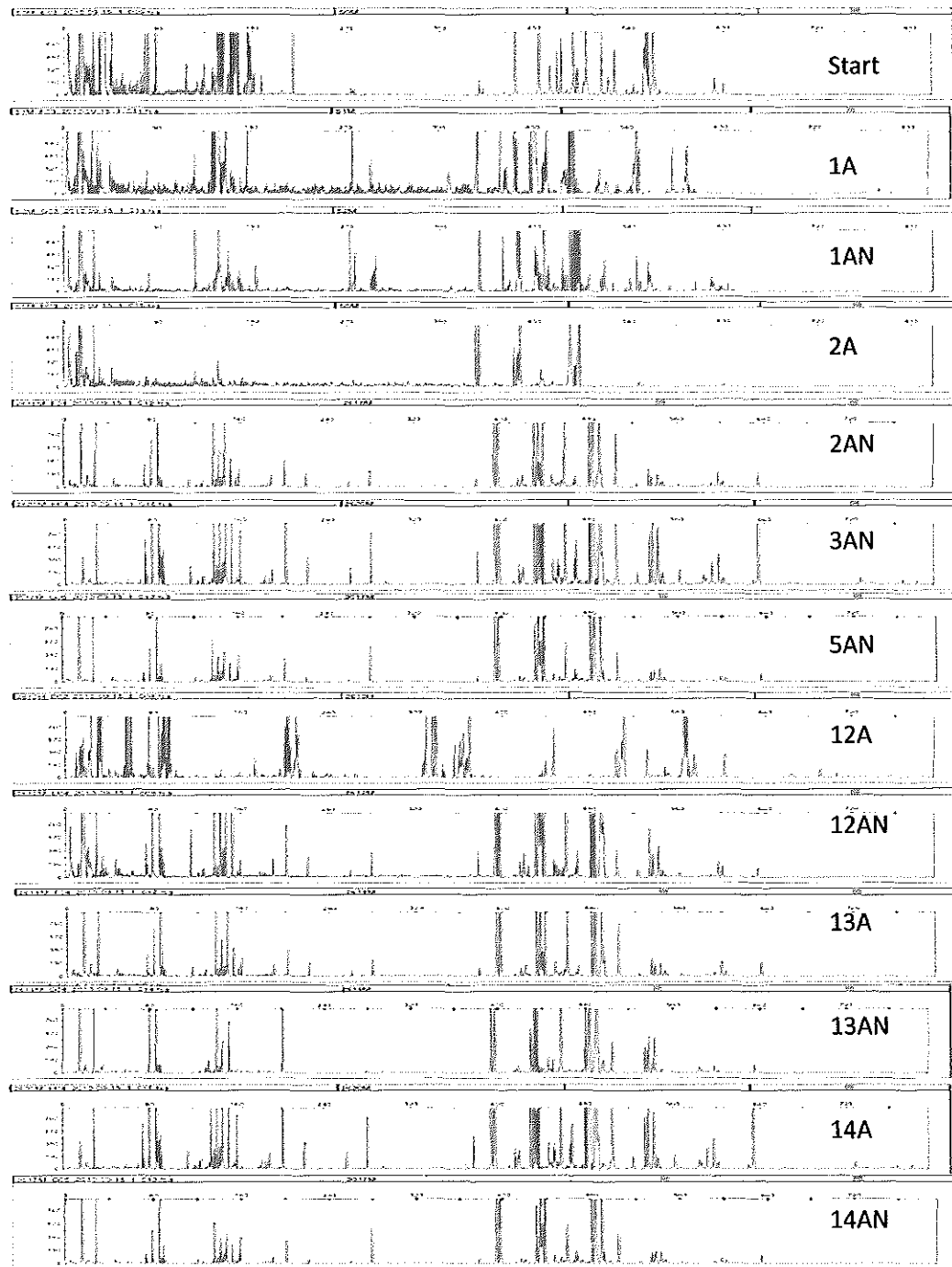
Sequence length	Highest BLAST hit	Query Coverage	Max. Identity	Taxonomic affiliation according to RDP	Restriction Fragment length	
					Rsa I	Hha I
453 bp	> gb EF486314.1 <i>Brevundimonas</i> sp. R2A 10-7 16S ribosomal RNA gene, partial sequence Length=1427	99%	98%	Alphaproteobacteria Caulobacteriales Caulobacteraceae <i>Brevundimonas</i>	422 bp	333 bp
528 bp	>gb DQ153917.1 <i>Ralstonia</i> sp. 74 16S ribosomal RNA gene, partial sequence Length=568	99%	99%	Betaproteobacteria Burkholderiales Burkholderiaceae <i>Ralstonia</i>	478 bp	n.c.s
463 bp	>emb BX640447.1 <i>Bordetella bronchiseptica</i> strain RB50, complete genome; segment 11/16 Length=349442	99%	99%	Betaproteobacteria Burkholderiales Alcaligenaceae <i>Bordetella</i>	n.c.s.	28 bp
472 bp	>gb DQ440826.1 <i>Bosea</i> sp. CRIB-10 16S ribosomal RNA gene, partial sequence	99%	99%	"Proteobacteria" Alphaproteobacteria Rhizobiales Bradyrhizobiaceae <i>Bosea</i>	422 bp	339 bp
456 bp	>emb FN433038.1 <i>Brevundimonas diminuta</i> partial 16S rRNA gene, isolate CCM29B Length=1479	100%	99%	Alphaproteobacteria Caulobacteriales Caulobacteraceae <i>Brevundimonas</i>	423 bp	331 bp

523 bp	>gb CP002290.1 <i>Pseudomonas putida</i> BIRD-1, complete genome Length=5731541	99%	99%	Gammaproteobacteria Pseudomonadales Pseudomonadaceae Pseudomonas	n.c.s	205 bp
472 bp	>emb AM989010.1 <i>Caulobacter sp.</i> AKB-2008-JO76 partial 16S rRNA gene, strain AKB-2008-JO76 Length=840	99%	99%	Alphaproteobacteria Caulobacteriales Caulobacteraceae	422 bp	332 bp
522 bp	>dbj AB246800.1 <i>Pigmentiphaga sp.</i> R-4 gene for 16S ribosomal RNA, partial sequence Length=1480	99%	98%	Betaproteobacteria Burkholderiales Alcaligenaceae	472 bp	444 bp
470 bp	> gb HQ658355.1 <i>Nitratireductor sp.</i> IITR-21 16S ribosomal RNA gene, partial sequence Length=1449	99%	99%	Bacteria "Proteobacteria"	n.c.s	61 bp
517 bp	> gb AY842900.1 <i>Flavobacterium columnare</i> strain LV339-01 16S ribosomal RNA gene, partial sequence Length=1457	99%	94%	Flavobacteria "Flavobacteriales" Flavobacteriaceae	306 bp	90 bp
471 bp	> gb HQ111159.1 Uncultured <i>Shinella sp.</i> clone cuticle_11 16S ribosomal RNA gene, partial sequence Length=1448	99%	97%	Betaproteobacteria Rhodocyclales Rhodocyclaceae Shinella	108	341

344 bp	> gb HQ693549.1 <i>Ochrobactrum anthropi</i> 16S ribosomal RNA gene, partial sequence Length=1315	100%	100%	Alphaproteobacteria Rhizobiales Brucellaceae Ochrobactrum	106	61
680 bp	> gb GU645041.1 <i>Sedimentibacter</i> sp. enrichment culture clone B19 16S ribosomal RNA gene, partial sequence Length=1041	99%	89%	"Firmicutes" "Clostridia" Clostridiales Incertae Sedis XI Sedimentibacter	94 bp	104 bp
525 bp	> gb AF493694.2 <i>Fluviicola taffensis</i> strain RW262 16S ribosomal RNA gene, partial sequence Length=1484	99%	97%	"Bacteroidetes" Flavobacteria "Flavobacteriales" Cryomorphaceae Fluviicola	314 bp	100 bp
529 bp	> gb AY635897.1 <i>Pseudoxanthomonas</i> sp. AMS6 16S ribosomal RNA gene, partial sequence Length=1025	99%	99%	"Proteobacteria" Gammaproteobacteria Xanthomonadales Xanthomonadaceae Pseudoxanthomonas	479 bp	373 bp
472 bp	> gb DQ985067.1 <i>Paracoccus</i> sp. JL1148 16S ribosomal RNA gene, partial sequence Length=1421	99%	98%	Alphaproteobacteria Rhodobacterales Rhodobacteraceae	422 bp	n.c.s
529 bp	> gb FJ529027.1 <i>Alcaligenes</i> sp. ES-JQ-2 16S ribosomal RNA gene, partial sequence Length=1502	99%	99%	"Proteobacteria" Betaproteobacteria Burkholderiales Alcaligenaceae	479 bp	67 bp

Phascolarctobacterium						
Acetobacterium	0	1	0	0	0	Eubacteriaceae
Butyricicoccus	0	1	0	0	0	Ruminococcaceae
Caldicoprobacter	0	1	0	0	0	Incertae Sedis IV
Desulfonispota	0	1	0	0	0	Peptococcaceae 1
Hydrogenoanaerobacterium	0	1				
			0	0	0	Ruminococcaceae
Lutispora	0	1	0	0	0	Gracilibacteraceae
Soehngenia	0	1	0	0	0	Clostridiales_Incertae Sedis XI
Ethanoligenens	0	0	4	4	2	Ruminococcaceae
Desulfitispora	0	0	4	0	2	Peptococcaceae 1
Clostridium sensu stricto	0	0	4	0	0	Clostridiaceae 1
						Clostridiales_Incertae Sedis XII
Acidaminobacter	0	0	0	4	2	
Dethiobacter	0	0	0	4	0	Natranaerobiaceae
						Bacillales_Incertae Sedis XII
Exiguobacterium	0	0	0	2	2	
Clostridium XI	0	0	0	2	0	Peptostreptococcaceae
Clostridium XLVa	0	0	0	2	0	Lachnospiraceae
Dehalobacter	0	0	0	2	0	Peptococcaceae 1
Dethiosulfatibacter	0	0	0	2	0	Incertae Sedis XI
						Clostridiales_Incertae Sedis XI
Finegoldia	0	0	0	2	0	
						Thermoanaerobacteraceae
Thermacetogenium	0	0	0	2	0	
Anaerofustis	0	0	0	0	2	Eubacteriaceae
Pseudoflavonifractor	0	0	0	0	2	Ruminococcaceae
Syntrophothermus	0	0	0	0	2	Syntrophomonadaceae
Thermobrachium	0	0	0	0	2	Clostridiaceae 1

Appendix X TRFLP profiles of the aerobic and anaerobic phases of the SBR



Declaration

I, the undersigned, declare that this PhD Dissertation is my own original work and has not been presented for a degree in any other university, and all sources of materials used for the Dissertation have been duly acknowledged.

Student: Adey Feleke Desta

Signature: _____

Addis Ababa, Ethiopia

This Dissertation has been submitted for examination with my approval as the research supervisor of the candidate.

Supervisor: Fassil Assefa (Ph.D)

Signature: _____

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