REMOVAL OF CHEMICAL OXYGEN DEMAND AND SULFATE IN BATCH REACTOR BY USING ANAEROBIC HALOALKAPHILIC BACTERIA

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Master Thesis

Department: Environmental Engineering

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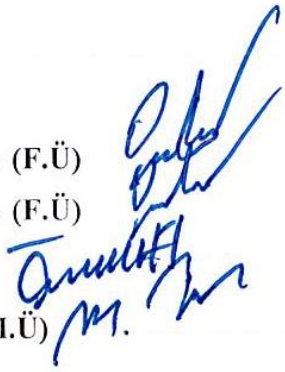

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SUMMARY

Haloalkaliphiles was considered as an ideal choice in biological treatments for alkaline wastewaters that contain toxic residues from industrial processes. They normally activated in a medium with high salinity rate of cations (Na^+, K^+), anions (Cl^-, CO_3^{-2}, HCO_3^-, SO_4^{-2}), and high pH ranging from 9-10. These microorganisms are normally implemented in anaerobic treatments on wastewater after determination and isolation of alkaliphilic bacterial under several operating conditions. In this thesis, the reductions of sulfate and organic matter have been studied under high salinity and alkalinity medium, using anaerobic Haloalkaphilic microorganisms, isolated from "Van Lake" Turkey. Several types of parameters have been examined including pH, COD, TOC, and identifying microbial species. The of the medium broth for haloalkaliphilic bacteria was at 9.74. In addition to, in the different salt concentration and alkalinity (5 g/L NaCl, 15 g/L $NaHCO_3$, and 10 g/L Na2CO3 and in the different carbon sources (5 g/L acetate, and 2.5 ml/L ethanol) at 30^oC were considered the optimum condition. As a result, it was determined that haloalkaliphilic bacteria could be used for COD and sulfate removal in biotechnological studies and anaerobic systems.

Keywords: Haloalkaliphilic bacteria; Anaerobic process; COD and sulfate removal

ÖZET

Kesikli Reaktörlerde Kimyasal Oksijen İhtiyacı (KOI) Ve Sülfat Gideriminde Anaerobik Haloalkalifilik Bakterilerin Kullanılması

Haloalkalifiller, endüstriyel işlemlerdeki toksik kalıntı içeren alkali atık sular için ideal bir biyolojik işlem seçeneği olarak kabul edilmiştir. Normal şartlarda, yüksek tuzluluk oranlı katyonlar (Na^{+}, K^{+}), anyonlar (Cl^{-}, CO_3^{-2}, HCO_3^{-}, SO_{4-2}) ve 9-10 arasında değişen yüksek pH'lı ortamlarda aktive olan bu mikroorganizmalar, çeşitli koşullar altında alkalifilik bakterinin belirlenmesinden ve izolasyonundan sonra atık suların anaerobik arıtımda kullanılır. Bu tezde, "Van Gölü" Türkiye'den izole edilen anaerobik Haloalkalifilik bakteriler kullanılarak yüksek tuzluluk ve alkali ortamlarda sülfat ve organik madde giderimi çalışılmıştır. Ayricabaslangic pH , KOI, TOC ve mikrobiyal türlerin tanımlanması gibi çeşitli parametreler de incelenmiştir. Haloalkalifilik bakteriler için besiyerinin pH değeri 9.74'tür. Ilave olarak, farklı tuz konsantrasyonlarında,ve alkali sartlarda (5 g/L NaCl, 15 g/L $NaHCO_3$) ve farklı karbon kaynaklarinda (10 g/L Na_2CO_3, 5 g-/L asetat- ve 2.5 ml/L etanol) 30 °C sıcaklıkta optimum koşul olarak kabul edildi. Sonuc olarak haloalkalifilik bakterilerin biyoteknolojik calismalarda ve anaerobik sistemlerde KOI ve sulfat giderimi icin kullanilabilecegi tespit edilmistir.

Anahtar Kelimeler: Haloalkalifilik bakteri; Anaerobik sistem; KOI ve sülfat giderimi

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ABBREVIATIONS LIST

COD	: Chemical Oxygen Demand
DGGE	: Denature Gradient Gel Electrophoresis
DNA	: Deoxyribonucleic acid
Fig	: Figure
g	: gram
h	: hour
H.B.	: Haloalkaliphilic bacteria
L	: Litter
mg	: milligram
mL	: milliliter
PCR	: Polymerase Chain Reaction
pH	: potential of Hydronium ions
RNA	: Ribonucleic acid
rpm	: round per mint
rRNA	: Ribosomal Ribonucleic acid
SRB	: Sulfate Removal Efficiency
TN	: Total Nitrogen
TOC	: Total Organic Carbon
UV	: Ultra Violet
v	: volume
w	: Wight

SYMBOLS LIST

^{o}C	**:** Celsius degree
μ	: Specifik Growth Rate, h^{-1}
μ_m	**:** Makximum Specifik Growth Rate, h^{-1}
β- carotene	**:** Beta - carotene
X_o	**:** Mikcroorganism Concentration, mL
V_R	**:** Reactor Volume, mL

1. INTRODUCTION

Since the last century to date, most of the industrial wastewaters become a complex mixture of organic and inorganic toxins with a high rate of alkalinity and salinity products Since microbial life (bacteria and archaea) is widely found on the earth, the biological technique with extreme conditions considered the most suitable way to treat and reduce the organics from wastewater. A hypersaline environment is a typical example of environments with extreme circumstances such as temperatures, light intensity, salinity, pH, nutrient conditions and oxygen. Therefore, the biological alternatives with their high alkalinity and salinity considered the most suitable approach in industrial wastewater treatment in terms of cost, pH adjustment for treatment processes, neural issues of physiochemical and in the configuration of membrane filtration sequence. Haloalkaliphiles are one of the biological sources for alternative energy that derivative from a sub-gathering of extremophile microorganisms that optimally grow in pH between 9 to10, or near-natural pH. They represent the basis for several biotechnological efforts through their enzymes and compounds that provide high growth for industrial demand in many applications. Haloalkaphilic microorganisms normally present in all three faces of life archaea, bacteria, and eukaryote. They are able to adapt under extreme habitat conditions [1-4].

Moreover, these microorganisms can also interfere in the production of different types of enzymes such as amylases cellulose, proteases, lipases, and so forth. These enzymes are extremely important for various biotechnological and industrial processes, although it is commercially available; but their import requires a lot of costs [5- 7]. Usually, they have seen in soda lakes, soda soil, and Thalasso line lakes. They normally characterized by having a high salinity rate ranging (5-30%), with presence of cations (Na^{+}, K^{+}), anions (Cl^{-}, CO_3^{-2}, HCO_3^{-}, and SO_4^{-2}) with low amount of Mg^{+} and Ca^{2+} resulting from carbonate precipitation [8, 4].

Since these habitats consist in extreme conditions in term of high salinity, alkalinity, and temperature, haloalkaliphilic microorganisms have developed two adaptive physiological mechanisms in order to prevent osmotic stress and low water activity. The first and most common method is cytoplasm storing of the organic compound in presence of osmotic pressure by using bacteria and amino acid with sugar solution as an energy source.

While the other method known as the sodium-potassium pump by intake potassium ions K^+ into the cytoplasm and pumping sodium ions Na^+ out of the microorganism's system. Although this method requires less energy than the first one, it is not quite common as it has been used by one single archaea and one order of bacteria. Recent industrial and wastewater contamination caused from heavy metals and volatile fatty acid toxicity at pH 7. They can be reduced by changing pH to alkaline condition which allows the bioreactors operate in high organic loads, also their ability to catalytic at a low water activity of non-aqueous solvent or surfactant environments [9-12]. Some recent studies have shown that, the electron acceptors and donors of Haloalkyphile-microbial are known as nitrate-reducing glucose, ammonium oxidizing, and sulfate and methane production. Though, very limited work has been done on their utilization in biotechnology and studies that applied in reactor working on an irregular premise.

As a result, haloalkaliphilic bacteria should be used in a biological treatment for waste and hazardous residues that comes from halo-alkaline industrial fields such as bioremediation, biodegradation, biofuel and renewable source for biogas formation of methane. In addition, hypersaline and alkaline environments can be substitute solution for human industrial processes like textile preparation, mineral ore, refining, pulp and paper, detergent manufacture and food industry. As these microbial interfere into several treatment fields, our work was carried out by the adaptation of the bacterial community isolated from Haloalkaphilic media for continuously using as bioreactors. Exclusively, anaerobic Haloalkaphilic microorganisms have been explored in the fermentation of lingo-calluses raw materials, producing methane and sulfate removal studies. Despite all the advantages of haloalkaliphiles compared with extremophiles, the exploitable physiology, metabolism and genetics of these bacteria to date are still limited [4, 13-15].

The aim of this thesis; is to investigate the removal of COD and sulphate in anaerobic batch system using anaerobic haloalkaliphilic bacteria isolated from the bottom sludge at different points of Van Lake.

2. HALOALKALIPHILE MICROORGANISMS

Halophile microorganisms are those microorganisms that survive and grow and reproduce under extreme conditions. They normally live in a habitat with high salinity and high alkalinity such as soda lakes and soda soil. Halophilies mostly consist of prokaryotic and some of the eukaryotes as well. In order to survive in harsh environments, they have the ability to maintain the equilibrium between low water activity and high osmotic pressure outside the cell, compared with intracellular of the organism [16]. In addition to, the concept of haloalkaliphilic is generally given to organisms that live in saline and alkaline habitats with pH optimally around (9 – 10). The most prominent characteristics of these habitats are their containment of a large amount of sodium chloride (NaCl) and sodium carbonate (Na_2CO_3) which are formed by evaporative concentration with natural high salt and high pH environment present as a part of the global saline system where mostly distributed in arid areas and high evaporated climate [13, 17- 18]. According to Ulukanli 2002 and Grant 2006 researches have shown how soda lake has some characteristics differ from other habitats in the way of consuming high rate of Mg^{2+} and availability of carbonate which provide buffering capacity to the lake waters under temperature around 30-45 ^{o}C and salinity ranging between (5% to 30% w/v) of total salt. some conditions are able to control on the ratio of salt and pH in any alkaline habitats including; geological combination in which favors the formation of alkaline drainage waters, topographical with a restricts surface that outflow from the drainage basin, and also the climate change which was conducive to evaporative concentration .Due to these conditions, the optimum growth rate of those microorganisms have been examined and widely distributed at high pH level (above 7).

Soda lake on the average of aquatic habitats has been considered as the grossest primary productive in the world that naturally occurring in environments. These lakes exhibit several colors from green, orange, purple or pink which result from the high blooming of microorganisms like algae, blue bacteria, cyanobacteria, and eubacteria or archaebacteria. In addition to the conditions related to water chemistry, and the amount microbial population. Other alkaliphilic populations that considered as the most main evolutionary and trophic groups of

bacteria and archaea includes; gram positive and gram negative, *Spirochetes*, *proteobacteria*, and methanogenic archaea, with contaminated active carbon, nitrogen, and sulfur cycling under anaerobic conditions.

The microbial diversity found in soda lake has yet to be wholly revealed, in spite of phylogenetic groups that will eventually prove to have soda lake member. Halo microbial are normally characterized by their high growth in lakes via the presence of Na_2CO_3, $NaHCO_3$, glucose or other minerals as a source of fixed nitrogen. They prevent the invasion and growth of other contaminant organisms.

There are only two alkaline environments that have been reported. The first one was about alkaliphilic bacteria that isolated from alkaline soil and water samples. The second one was based on the isolation of anaerobic phototrophic bacteria from Lake Ak-Behir. Moreover, the study of alkaline that tends to be specific and permitting the recovery of simple filtration in environments of Turkey, remain limited [7, 19]. Lake Van considered as an ideal example of naturally occurring in high alkaline environments of Turkey.

2.1. Classifications of haloalkaliphilic bacteria

One of the most important characteristics in the Halophiles is their diversity and classifications which mostly are prokaryotes and some eukaryotes as well. They can be found in all three domains of life among bacteria, archaea and eukaryotic microorganisms. As shown in Figure 2.1, it explains the major group of halophilic microorganisms. One of these groups belongs to some of algal species that blooms in the water of salterns, oceans, crystal ponds and microbial beds in hypersaline environments. They include *Chlamydomonas nivalis* and *Cyanidium caldarium*, and the most well-known halophilic algal species is the green algal *Dunaniella salina*, which dominates the algal population in saline environments. The most studied and characterized group in Halophiles is Halophilic bacteria. They exist in several types of colonies that ranging from pigmented to non-pigmented depending on salt concentration in the media. Halophilic bacteria have been recommended in which the media must be added with salt and the incubation time for subsequent growth is then assessed. In Prasongsuk 2016 study, the historical taxonomy of these microorganisms was based on a few of phenotypic or morphological characters and less attention was paid to the phylogenetics or biochemistry of

organisms. Halophilic bacteria are generally represented by archaeal bacteria, which comprised of the slightly and moderately halophilic bacteria, but most of them are eubacteria.

In hypersaline habitats such as Dead Sea, moderately and extremely halophilic bacterial are the most important groups that have received more attention in recent researches. However, slightly halophilic bacteria cannot be found in these harsh environments. Since the last decade, a few fungi have been included in halophilic microorganisms. They have been isolated from substrates with a low water activity which considered as a xerophilic phenotype. Fungi were first isolated from solar saltern, followed by in many hypersaline environmental around the world, although not restricted to any specific geographical location. Most of the reported halophilic fungi from solar salterns have either been identified as a new species or species from previous natural niches which have been unrecognized, or as those only known before as foodborne species or food contaminants. The total number of fungal order is 106, 10 of them have been reported. However, in the orders *Capnodiales, Eurotiales, Dothideales*, the halophilic character is expresses in many groups of the same order. Unlike halophilic bacteria, halophilic fungi cannot be clustered in separate phylogenetic groups. Nutrients such as nitrogen and phosphorus locations and time of sampling and dissolved oxygen levels were found to be important factors in the growth of halophilic fungi. Halophilic fungi naturally inhabit hypersaline habitats which show a halotolerant behavior. Most of these microorganisms are not required high salt ratio for their survival; they can grow in a salt concentration of any range [16].

Aharon Oren 1993 has classified *Halobacteriaceae* into six genera that sharing the same requirement of high salt concentration. These genera include; Halobacterium, Haloarcula, *Haloferax*, *Halococcus*, *Natronobacterium*, and *Natronococcus*. Two years later, a new order of halophiles was discovered and named as *Haloanaerobials* where *Halobacterium* genera belong to this order. Normally, Halobacteriales contain one family named as *Halobacteriaceae* which introduced with genera *Halanaerobacter*, *Halobacteroide*. In addition, this family was shown in accession numbers for 16S rRNA gene sequences [20-21]. The ICRC or known as (International Committee on Systematics of Prokaryotes 2001) has also discovered 19 more generals. They considered that Halobacteria as a monophyletic group, with the most distantly related species showing a 16 S rRNA gene sequence similarity of 83.2 %. Unlike Methano gene, that has shown less than 80% of 16S rRNA sequence similarity.

On the other hands, the complete list of required and recommended criteria for the determination and recognition of haloarchaeal species was proposed with three genomes which are *Halobacterium salinarum*, *Haloarcula marismortui*, and *Natronomonas pharaonis* [22-28].

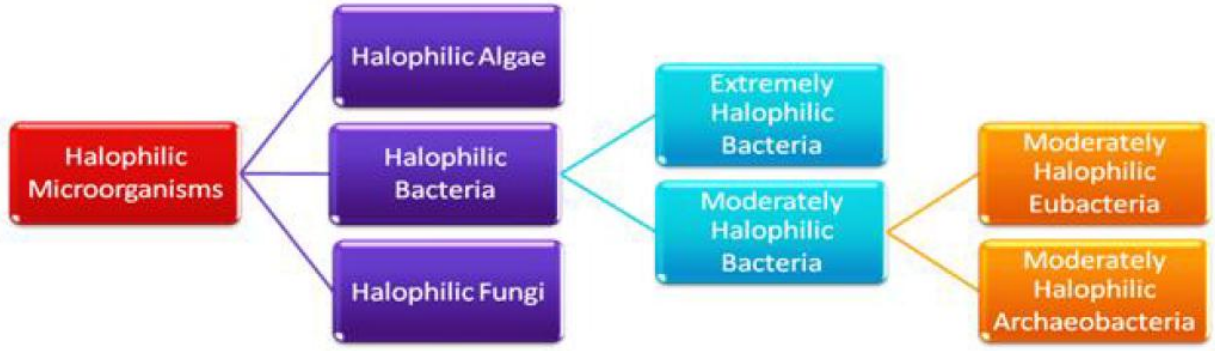


Figure 2.1. Classification of halophiles into major groups of microorganisms [16].

2.2. Habitat of haloalkaliphiles

Typically, most of the Halophiles species with their complex composition live in habitats in which salt concentration should be high enough for their growth. Extreme environments are those habitats that have a wide diversity of Halophiles characterized by high resistance to survive. However, recent technology and development in science still limited to exploration and discovery [29-32]. Some environments include; soda lakes, Dead Sea, carbonate spring, saline soil, saline lakes, and many other habitats are able to contain large numbers of halophiles. Recently these kinds of environments have been increased due to natural changes and manmade global as well. The soda and saline lake considered as the most productive habitat for alkaliphiles where the pH is more than 9, yet they haven't been very knowable due to their unavailability. Even this disadvantage, these biotas are still receiving attention from microbiologists. Moreover, Dead Sea ponds usually depend on climate conditions such as temperature, season variation, time, water retention, and nutrients that allow the diversity of halophiles to grow. The extensive growth of pigmented microbes is often imparting a red color in crystallizer alkaliphile ponds.

Comparing to other pond Dead Sea ponds support the halotolerant growth more than halophiles, and that explains how they thriving in an extreme environment of salinity and

alkalinity. As a result, Haloalkaliphiles represents several biotechnological types among industrial and environmental applications. Due to their steady increase in isolation, they have been potentially involved in biotransformation, bioremediation, and biofuel [4, 33-39].

2.3. Haloalkaliphiles growth

Through several types of research and studies, it has shown that the growth kinetics and product formation of the pure cultures with various combination of electron acceptors and donors by using the minerals of their growth medium as for enrichment. The previous works also have described the isolation procedure with the supplement salt medium according to the type of bacterial culture and media. To provide a suitable growth media for extreme halophiles and haloalkaliphiles eubacteria, they need specific nutrient diverse vitamins depending on the type of microorganisms, so that should be particular for choosing the best culture medium [40-41]. For the best growth of halophilic microbial, pH must be in alkaline level (at least two points above natural). It can be adjusted by using Na_2CO_3 $Na_2CO_3.10H_2O$, and Borax/NaOH, Na_2HPO_4/NaOH buffers systems in order to maintain pH value around 9-12. In addition to, the large scale cultures grown in bioreactors are usually controlled by a NaOH.

Most of alkali samples are cultured in saline nutrient broth. After culturing, the Haloalkaphilic organisms must be incubated under optimum temperature around 20-40 ^{0}C in 3 or 7 days for best growth. These microorganisms can be maintained in the laboratory using a number of methods.

In several previous studies, routine sub-culturing at weekly or monthly intervals has proven to be a simple method. For the maintenance of small collections, routine sub-culturing seems to be practical but it involves in contamination risk, time consuming and low recovery of cultures. As well as, changing some phenotypic properties may produce genetic instability.

Another method of preservation is cryopreservation under liquid nitrogen. In the method of bacteria that preserved in liquid nitrogen has been showing a high survival rates and good strain stability with long-term preservation after the primary isolation.

There is a chance to store this sample in sealed plates at 4^{o}C. Samples are usually taken in duration between early wet season October and November and dry season April and May by taking the sample from different sites that represent the saline environment.

The outlet of some chemical industries show a high alkalinity cannot be tolerated by non alkaliphilic microorganisms. To date, the isolation of these organisms is still obtained in different locations of habitat. An example of isolated alkaliphilic species is; Halomonas was isolated from sewage and two anaerobic alkaliphiles, namely *Clostridium paraxum* and *Clostridium thermoalcaliphilum*, were recovered from sewage digesters.

Most of our concepts about microbial life in both naturally and artificially generated alkaline environments are based on studies in which the pH of the environment and of the growth media used in laboratory studies is constant [42-43, 7].

2.4. Kinetic Growth of Haloalkaliphiles

The dynamics of microbial growth considered as the main subject in many researches of modern microbiology and biotechnology. Kinetic studies in microbiology cover all the dynamic manifestations of microbial life like; growth, survival and mortality ratio, product formation, adaptations, mutations, cell cycles, environmental effects, and biological interactions. This study provides a theoretical framework for optimal design in biotechnologies based on fermentation and enzyme catalysis, as well as on employment of outdoor activity of natural microbial populations. The main reason for using this model is to show the dynamic reaction of microbial cell growth and their enzymes activity. Furthermore, it formalizes postulated mechanisms, so that the comparison of observations and the model's predictions allows one to discard incorrect hypotheses. The biotechnologists or researchers are usually want to know many data like the time progress of product formation associated with nutrients uptake, cell growth, respiration, in order to specify the growth dynamic. Such a preliminary simulation is very useful for planning and optimizing real life experiments. Moreover, the verified dynamic mathematical models provide efficient tools to optimize the medium of target product, minimize undesirable generation of waste products, etc. Another important implication of growth dynamics is that recorded growth curves carry important hidden information about microbial cells, growth regulation, and interactions.

Depending on growth dynamic pattern, it can be distinguish the effects of products or substrate inhabitation, identify growth limiting at various. As shown in Figure 2.2 the description of microbial growth phases through exponential time [3, 44].

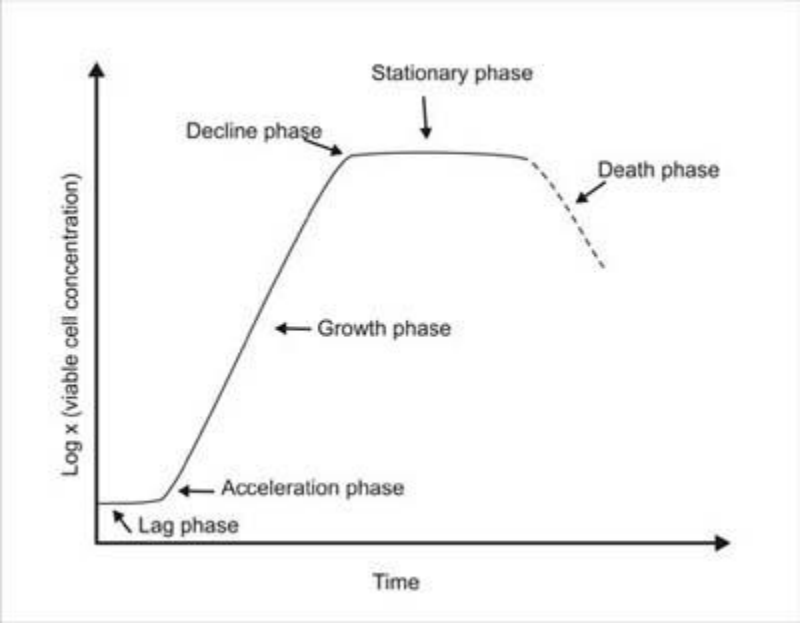


Figure 2.2. Microbial growth phases through exponential time [45].

Region 1: Lag phase: microbes are adjusting to the new substrate
Region 2: Exponential growth phase: microbes have acclimated to the conditions
Region 3: Stationary phase: limiting substrate or oxygen limits the growth rate
Region 4: Death phase: substrate supply is exhausted

Growth of the microorganism is determined by changes in the weight of biomass, and changes in substrate concentration (S) are determined using a specific, sensitive assay for the substrate. Through the equation below (2.1), it represent the specific growth rate (μ) of H.B. and (X) represent the cell population with time that described by first-order [46].

$$\mu = \frac{1}{x}\frac{dx}{dt} \tag{2.1}$$

If the cell concentration at t_o is X_o, and after an interval (t) has increased to a new concentration X_t, equation (1) can be integrated between the limits t and t_o for yield the equation of a first order reaction:

$$X_t = X_o\, e^{\mu t} \tag{2.2}$$

The specific growth rate of a given microorganism growing on a given medium is calculated graphically from results to batch growth experiments which determine the increase in biomass with time. Taking logs of equation (2) shows that in equation (3) a plot of lnX against time will be linear over the exponential period with a gradient of μ:

$$\ln X = \ln X_o + \mu t \quad (2.3)$$

2.5. Monod model

One of the most commonly used equations in biotechnology for relates the microbial growth is Monod model [47]. The relationship between the rate-limiting substrate concentration and specific growth rate in the absence of inhibitory substance often assumes the form of saturation kinetics and can be described by the Monod equation:

$$\mu = \mu\max \frac{S}{Ks+S} \quad (2.4)$$

Where the (μ) represented as the specific growth rate of the microorganisms, μ_{max} is the maximum specific growth rate of the microorganisms, S is the concentration of the limiting substrate for growth, and K_s is the half velocity constant that when the value μ/μ_{max} =0.5. Moreover, the biological meaning and experimental determination of growth parameters were expressed by K_s and μ. This model still very popular because of its elegant simplicity and also play an important role in the history of microbial growth kinetics as a first encouraging example, when the theoretical development based on mathematical formalisms came before novel experimental designs [44-45].

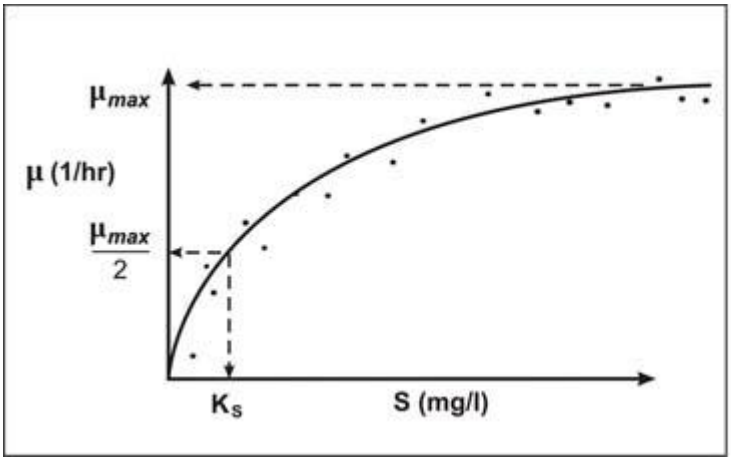
The most important biological implication of this theory was the discovery of the negative feedback between microbial growth rate and concentration of substrate in the bioreactor to establish steady state.

In addition to, Monod model is more focused on the concept of controlled cell biosynthesis, which implies the purposeful manipulation of microbial culture to optimize the

productivity by selecting the most appropriate flow rate and by changing the composition of medium in continues flow bioreactors (Figure 2.3.) This figure explains the Monod equation observing the μ_{max} which considered the maximum specific growth rate and K_s is the substrate concentration [48-49].

$$1/\mu = \frac{Ks}{\mu m}\ \frac{1}{S} + 1/\mu m \qquad (2.5)$$

K_S and μ_m values can be determined from the plot of $1/\mu$ versus $1/S_o$ yields a linear line with a slope of K_S/μ_m and y-axis intercept of $1/\mu_m$. The specific growth rate depends on the concentration of the limiting nutrient, which can be a carbon source or other factors needed by the microorganisms for growth. When substrate is used as the main substrate in the medium in the presence of higher concentrations of inhibitory (toxic) substances such as other pollutant, microbial growth becomes inhibited, and growth rate depends on inhibitor concentration. The following non-competitive inhibition model describing toxic component inhibition may be written as follows, and can be applied to the growth profile of yeast cells [47, 50].



$$\mu = \mu_{max}\left(\frac{S}{S+K_S}\right) \qquad (5)$$

Figure 2.3. Monod equation fit to observed data [45].

2.6. Isolation Technique

The diversity of the hypersaline microorganisms have seen widely distributed for biotechnological applications. Since alkaliphiles are highly blooming in alkaline pH, it need are up to 33% NaCl for their optimum growth. Alkaliphiles have been isolated mainly from neutral environments, such as soda lakes and soda desert. Soda lakes are intermittent saline lakes that contain sodium carbonate/bicarbonate (soda) and sodium chloride that usually are found in lakes and desert regions. Furthermore, the soda lakes have been explored worldwide for the isolation and characterization of mainly haloarchaea and anaerobic phototrophic bacteria. In moderate halophiles area, the isolations are usually specified by means of mixture phenotypic tests like Gram reaction, motility, growth at various salt concentration, cell morphology, and catalase and oxidase production. All these isolations are optimally growing in media that containing between (5-30% w/v) of total salt. The isolation of Haloalkaphilic was studied either in nutrient agar or nutrient broth, also by knowing the rate of salt and substrate. Theoretically, the isolation media are usually contained; NaCl, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, KH_2PO_4, $(NH_4)_2SO_4$, trace element solution (containing; Mn', Mo, Cu, and Zn) and different dilutions of water and soil samples were added. The incubation of bacterial growth appears and diluted for ten times then plated on complete medium agar glucose, peptone and yeast extract which resulting colonies were purified by repeated streaking on media broth then the isolated sample can be stored.

2.7. Haloalkaliphiles enzymes

Through some of the researches and studies for alkaliphiles, it is shown that many types of enzymes have been discovered which exhibit unique properties in various respects. Alkaliphiles are known to have a specific type of enzymes known as hydrolysis. Hydrolysis enzymes are widely distributed in bacteria and eukarya. They are able to tolerance high salt, pH, temperature and extreme conditions. Halophilic microorganisms have a specific type of enzymes, the most common one known as Hydrolysis enzymes. Hydrolysis enzymes are widely distributed in bacteria and eukaryotes. They have the ability to tolerate high salt and temperature and other extreme conditions. By different types of circumstances, the enzymatic activities of Haloalkaphilic bacteria are divided into three categories according to their activities.

The first category defined as intercellular enzymes, which is not exposed to the external salt concentration. Secondly, enzymes are membrane-bound activities that characterized by transporting proteins and salt to the outer habitat and intracellular environment. The third enzymes are called by truly extracellular enzymes that exposed to the external salt concentrations. The activities of halophilic enzymes are exposed to appear in extreme condition consequently led to having a wide variation of halophile. Halophiles (including haloalkaliphiles) that producing extreme enzymes, they assumed to have a significant role in biotechnological processes via the interference with bioremediation and biodegradation.

Hydrolytic enzymes have numerous types of enzymes like amylase, proteases, DNase, inulinase, pectinase, pullulanase, xylanases, cellulose and lipases [51-55]. Hydrolyses enzymes were obtained for further study due to their industrial usage. Such as the Lipolytic enzyme is isolated and detected by screening for zones of hydrolysis. The cleavage of these esters was measured by phosphate-buffer. The amylolytic activity was determined by using a medium of soluble starch. While DNase activity was isolated by using DNase test agar. In several types of hydrolysis enzymes that produced by Haloalkaphilic, bacteria have the ability to tolerance to extreme conditions such as organic solvent, salt content, and high temperature making them exploited wherever transformation in order to survive under harsh environment [56-60]. As result, all of these halophiles enzymes are able to function in high salt concentration while a few of them become active. But even so, they are useful for catalysis under a harsh condition in industrial processes, additionally they are becoming more advantageous in aqueous and terrestrial enzymology due to the solvent stability among these enzymes [58, 61-64].

2.8. Anaerobic Systems by haloalkaliphiles

Biological processes considered as one of the most suitable choices in wastewater treatment through their low operation cost, less chemical addition requirement and zero polluted production. Anaerobic processes are known as an attractive biological process that has a high tolerance to the varying of influent water quality and low sludge production with low energy consumption. Anaerobic technique applied with absence of oxygen which leads to converting organic pollutants to carbon dioxide and methane gas which use as a source of alternative energy.

Several types of anaerobic microorganisms are applied in wastewater treatment by working together for converting organic material into biogas. Biogases usually consist of 30% carbon dioxide (CO_2), 70% methane (CH_4), and some residual fractions of gases like H_2 and H_2S. In classical anaerobic processes, H_2S, which is formed by sulfate reduction, easily seeps into the cell and produce a toxic effect to the bacteria. In this case, Haloalkaliphile conditions are able to reduce this toxicity of H_2S compared to the natural pH condition. Anaerobic treatment could be more expensive and sustainable technology for the treatment of many of these saline wastewaters. The using of Haloalkyphile in an anaerobic system has to give us many benefits including, low sulfide and sulfate toxicity, low production of COD, and H_2S^- containing biogas and reduced need for pH control. The biogas is collected and disposed of at the top of the reactor, separately from the partially purified water and the sludge. Furthermore, an extra purification phase Haloalkaliphiles microorganisms have proven how can degrade under anaerobic condition in order to implemented after anaerobic purification. The amount that produced from different biogas and contaminants in saline solutions was observed in marine sediments and has been characterized during the last two decades [9, 65-67].

Indeed the biological suspension in submerged anaerobic membrane bioreactors appears to have a high concentration of suspended soiled and soluble polymeric substances, due to the membrane selectivity and their accumulation onto the membrane surface and in the membrane pores. Such conditions are already difficult for survival of the aerobic Haloalkaliphiles with high energy metabolism, in contrast to they can grow under anaerobic conidian with low energy generating efficiency.

In addition, the bioenergetic constraint of high salt and high pH seems to be a unique challenge. These types of microorganisms are predominantly represented by haloarchaea with few exceptions for extremely halophilic bacteria. Although anaerobic treatment can typically reduce the amount of chemical oxygen demand by as much as 90% based to Barker 1999 research. However, in most of industrialized countries, COD usually still too high to meet the discharge standards [45, 68].

2.8.1 Specific advantages and disadvantages

There are some advantages and disadvantages in an anaerobic system that could be partially or totally impact on the treatment processes. As a result, these characteristics and the specific problems must be in advance in order to choose the most appropriate type of reactor for a successful wastewater anaerobic treatment as will briefly be discussed below:

Advantages:

1- Biogas formation; where organic pollutant converted into biogas with high energetic value by allows the energy needed to operate the water purification system to be fully or partly treated.

2- High loading ratio

3- The volumetric load (COD load per m_3 active volume per day) in an anaerobic reactor is typically much higher than aerobic wastewater purification.

4- Low sludge production

5- Sludge generation in an anaerobic reactor is less than in an aerobic system.

6- This system is fairly simple in terms of complexity.

7- In countries that have the priority in the removal of organic pollutants through discharge control, an anaerobic treatment considered a very cost-effective in domestic wastewater.

Disadvantage:

1- No nutrient removal, due to aerobic condition need for nutrient removal.

2- Unlike aerobic purification, incomplete degradation of the organic compound

3- Most efficient purification in the mesophilic range as an example between 30-37 ^{o}C whereby the influent must be heated in most cases.

4- Less efficient system in reducing toxicity and inhibition ratio.

5- Risk of odor problems.

6- About 60-75% of the biogas consists of methane produced by anaerobic systems, which is a greenhouse gas with an adverse impact approximately twenty times more than carbon dioxide.

haloalkaliphilic microorganisms considered one of biological treatment for saline wastewaters in addition to anaerobic treatment that has a great role in this environment from seafood processing and ion-exchange deal with salty wastewater (1.5-15% of NaCl) [65-67].

2.8.2. Chemical oxygen demands (COD) and sulfate removal

The chemical oxygen demand test known as (COD) is typically used in organic compound measurement that found in pollutant water (wastewater, river or lakes). However, the anaerobic treatment can typically reduce COD amount through anaerobic microorganism's as much as 90% w/w by converting organics into methane gas which is a valuable energy. Among the microorganisms in bioreactors which include bacteria, archaea, eukaryote, and virus, bacteria play important roles in wastewater treatment processes [66, 68-72].Several applications are coordinated towards the removal of organic pollution in wastewater, such as slurries and sludge. As shown in Figure 2.4 how the organic pollutants are converted by anaerobic microorganisms (haloalkaliphiles) to a gas containing methane and carbon dioxide which known as "biogas".

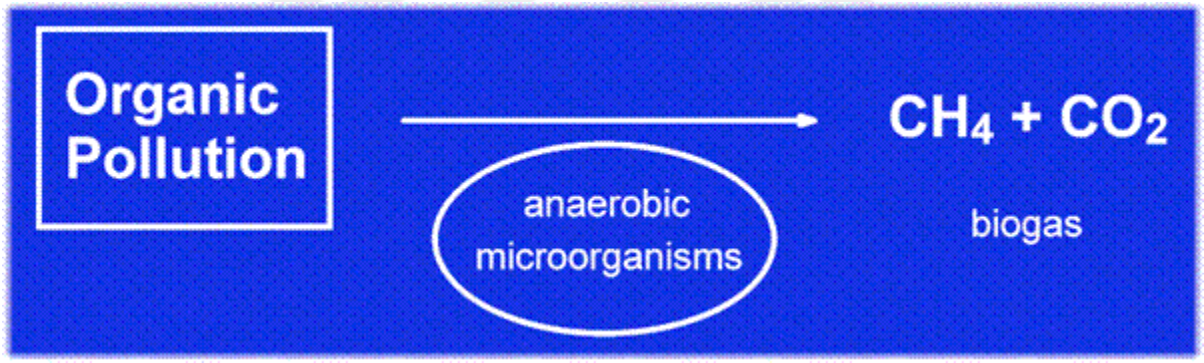


Figure 2.4. Conversion of organic pollutants to biogas by anaerobic microorganisms [66].

The main reason for choosing anaerobic reactor type depends on the composition of the habitat that contains several types of effluent. This treatment system can be possible only if the characteristics and specific problems for each individual wastewater are known in advance. Anaerobic technologies are not just utilized to remove the bulk of COD, it can also use for the biodegradation or biotransformation of toxic priority pollutants. As well as, anaerobic bioremediation can take place in bioreactors to treatment the industrial wastewater. A complex microbial community of anaerobic is feasible to maintain in bioreactors [65-66].

There are some factors that limiting the resident COD in the effluent that especially found in the development countries [70]. The effects of different COD and SO_4^{2-} on the Sulfate-reducing Bacterium (SRB) were investigated in model experiments. SRB typically present in soda lakes that considered as the most active element cycle in this environment, also it specializes in the consumption of inorganic electron donors, such as H_2, form, and thiosulfate. Several studies have shown the diversity, activity, and abundance of SRB in sediment samples and enrichment cultures from a range of (hyper) saline soda lakes. The most interesting physiological trait of the novel Haloalkaliphilic SRB isolates was their ability to grow lithographically by disputation of thiosulfate and sulfide. All isolates were obligate alkaliphilic with optimum pH around 9.5-10 and moderately salt tolerant. On the basis of phylogenetic, genotypic and phenotypic characterization of the novel soda Lake SRB isolates mostly affiliated with members of the order *Desulfovibrionales* and family *Desulfobacteraceae*. The majority of the methane was produced by acetoclastic at high ratios of COD and $SO4^{2-}$. Only at low COD and SO_4^{2-} ratios SRB species such *Desulfovibrio* which plays a significant role in ethanol degradation, and all SRB were found to be oxidizers at both high and low COD/SO_4^{2-} [73-76]. In addition to, high sulfate removal efficiency affected by the ratio of COD and SO_4^{2-} are also assessed in the Haloalkaliphilic sulfate-reducing bioreactor.

2.8.3 Total organic carbon (TOC)

The term TOC normally refers to the measurement of organic molecules and contaminants in purified water. It considers an analytic technique that helps organizations to understand whether the water they are using is pure enough for their processes. All water, no matter how pure, contains some carbon materials. Many of these materials are introduced into the water from the water source or from materials and systems during purification and production [77]. The value of total organic carbon has not been assigned a limiting value or criterion but can be considered as a cautionary warning for action under unusual circumstances. This measure concludes that the sum of all chemically oxidizable organically bound compounds present in water. TOC is an important indicator of the degree of organic contamination. The determination of TOC is used to detect and study environmental and wastewater pollution.

Recently, the nitrogen compounds or TN (total nitrogen) measurement has received a lot of attention because they are responsible eutrophication. Nitrogen and carbon can be effectively removed from synthetic wastewaters using coupled aerobic and anaerobic filters, where methane production and de-nitrification will be encouraged to take place in the anaerobic filter [78-80].

2.9. Haloalkaliphiles applications

Alkaliphiles have made a great impact in industrial applications since the rediscovery of alkaliphilic bacteria. They considered as unique microorganisms with high potential for microbiology and biotechnological exploitation. These microorganisms are usually seen in hypersaline environments. Alkaline enzymes such as proteases, amylase, and cellulose have been playing an important role in biotechnological application due to the ability to reduce the cost and paved the way for its use in large quantities in foodstuffs, chemicals, and pharmaceuticals [52-56]. Moreover, they interfere in the production of β- carotene, vitamins, and natural nutritional supplements. One of the most widely used of halophiles is the treatment of hypersaline wastewater by degrading organic pollutant of saline environments. Halophilic microorganism's applications can be classified into a number of categories. For example, exploitation of properties of a specific compound produces by certain types of halophiles which enable them to tolerate high salt concentration in their medium (ectoines, glycerol, and others). The characteristics of alkali usually results from the synthesis or uptake of compatible solutes in the cytoplasm, leading to osmotic balance strength of the medium, and preventing the water loss.

A diverse community of halophiles including Archaea, lactic acid bacteria and another representative of domain bacteria has shown different yeasts. H.B. are more versatile and enzymatic diverse than Archaea have. These enzymes have shown to produce biodegradable plastics, protein and lipid vesicles and microbial [81-84]. On the other hands, alkaliphile microbial act as bioenergetics by maintaining pH homeostasis in the exceedingly alkaline environment where has been progressively examined over the most recent two decades. They show a significant ability to keep the internal pH of the cytoplasm much lower than external around 10 to11.

Alkaliphiles are assumed a very good general genetic resource for such applications as a production of signal peptides for secretion and promoters for hyper-production of enzymes [7, 52]. However, anaerobic purification is implemented in various sectors. This technique is regularly used to reduce the high cost of aerobic waste purification by partially breaking down the organic load and converting into biogas. Anaerobic processes are also frequently used to ferment aerobic sludge and fluid organic waste [66]. These theories have shown us that alkaliphilic bacterial possess the potential to provide many opportunities for biotechnology For example, after an alkaline pretreatment the operating cost is reduced using halo-alkalinity microorganisms in reducing pH adjustment to maintain bio-fermentation at natural pH conditions. It is also has been contributed in the alternative to the formation of methane. Because of the high pH conditions, the toxicity of volatile fatty acids decrease and this allows the bioreactors to operate in high organic loads. At the same time, at alkaline pH, carbon dioxide appears in form of carbonate and causes a decrease in the amount of CO_2. Alkaliphilic bacteria also have advantages in the reduction of sulfate [69, 85].

3. MATERIAL AND METHOD

3.1. Collecting the sediment samples

The collection technique was done by taking H.B. sample from the bottom sediment of Van lake in Turkey. The Sediment samples were taken with the help of the Hydro-Bio bottom sampler according to the method that reported by. Sludge samples were collected in 1000 ml sterile glass jars on aseptic conditions. Coordinates of the sampled stations were also be recorded by GPS as shown in table 3.1. Samples were either observed immediately or maintained for several days (10–15°C) in glass bottles for further study.

Van lake considered as the largest soda lake and fourth-largest terminal lake in the world, located on East Anatolian Plateau in Turkey. It has a volume of 576 km^3, surface area of 3522 km^2 and maximum depth of 450 m Figure 3.1 with salinity reaches to 21.7% and a pH of 9.81 [86, 87].

However, the calcium concentration is very low, where varies between 4-5 mg/L. with ED-1950-UTM-Zone-38N a total of 11 stations were identified. In order to reflect the general characteristics of Van lake, station locations are chosen as distant as possible.

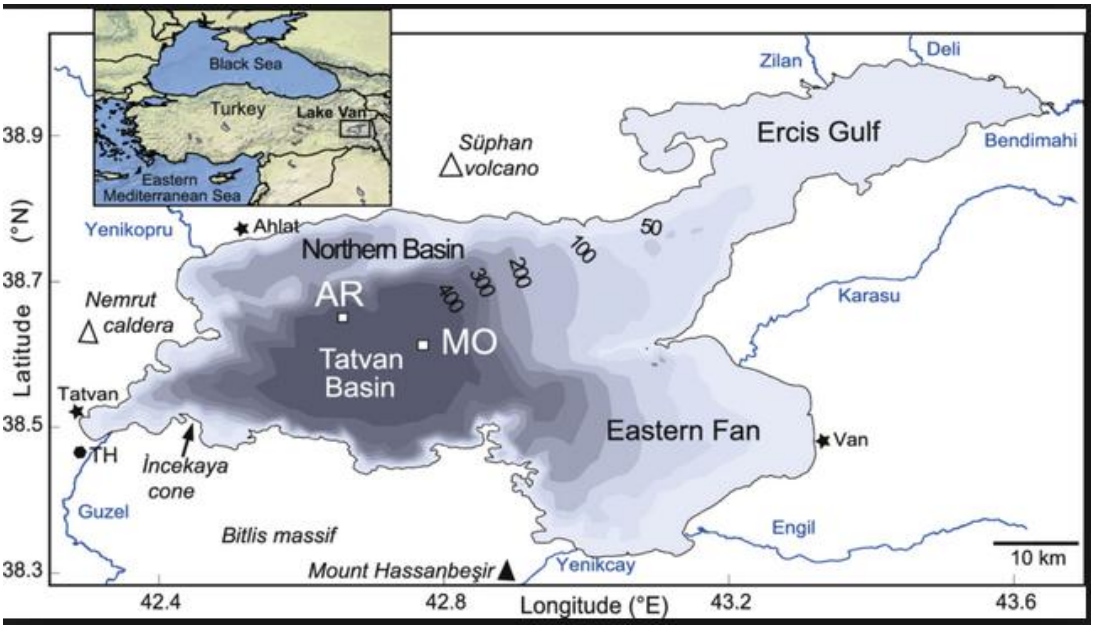


Figure 3.1. A location map for the studied area.

Table 3.1. Coordinates of Van lake sediment sampling sites.

Location No.	X	Y
1	35^{o} 17’	42^{o} 63’
2	33^{o} 57’	42^{o} 46’
3	32^{o} 25’	42^{o} 45’
4	31^{o} 01’	42^{o} 62’
5	26^{o} 96’	42^{o} 65’
6	27^{o} 79’	42^{o} 87’
7	30^{o} 26’	42^{o} 95’
8	35^{o} 74’	43^{o} 14’
9	36^{o} 06’	43^{o} 17’
10	38^{o} 15’	43^{o} 10’
11	34^{o} 32’	42^{o} 71’

3.2. Isolation and identification of novel haloalkaliphilic anaerobic bacteria

The isolation of the novel strains which been presented in this study was preserved for a total 4 months period of time. In the enrichment of haloalkaliphilic anaerobic bacteria, DSMZ 742 medium was used [86]. The composition of the haloalkaliphilic anaerobic growth medium was calculated by (mg/L): NaCl 10 g; Na_2SO_4 3.0 g; Na_2HPO_4 0.2 g; KCl 0.2g; NH_4Cl 1.0 g; Metal solution (H_3B0_4, 6.0mg; $CoCl_2.6H_20$, 12.0 mg; $CuCl_2.2H_20$, 1.5 mg; $MnCl_2$. $4H_20$, 10.0 mg; $NiCl_2.6H_20$, 2.5 mg; Na_2Mo0_4 x $2H_20$, 2.5mg; $ZnCl_2$, 7.0mg) 10ml ; Resazurin 0.5 mg; $NaHCO_3$ 15 g; Na_2CO_3 10 g; Yeast extracts, 1.5 g; vitamin solution 10 ml; Na-formate 5.0 g; Na_2S. $9H_2O$1g. All chemicals were of analytical grade, and solutions were prepared with sterile deionized water.

All components except bicarbonate, carbonate, yeast extract, vitamin solution, Na-format and $Na_2S.9H_2O$ were dissolved in distilled water and the medium was made anoxic by application of nitrogen gas for 30-45 minutes in anaerobic vessel. The pH of the medium was adjusted to 9.5-10 by adding bicarbonate and carbonate to the medium. The prepared growth medium (9 mL) was transferred to sterile Hungate tubes (in presence of nitrogen gas). These tubes are normally designed for obtaining and maintaining anaerobic culture conditions.

The growth medium was autoclaved separately (at 121 ˚C for at least 20 min) for the sterilization and also to prevent the contamination of the samples with different microorganisms during the experiments. After the sterilization, growth media was supplemented with yeast extract, vitamin solution, Na-formate and Na_2S. From these, a total of 1 gr sediment were transferred to sterile Hungate tubes containing 9 mL of growth medium as well under anoxic conditions to enrich the sulfate reducing Haloalkaliphilic anaerobic bacteria. Dilutions of 10^0 to 10^6 were then made. All these processes were carried out in anaerobic chamber which present in the Department of Environmental Engineering of Firat University (as shown in Figure 3.2).

Figure 3.2. Nitrogen-dry box (anaerobic chamber).

For the isolation of the H.B., DSMZ 742 medium that mentioned above is added to 2% bacteriological agar before the sterilization while the medium was distributed to the petri dish after the sterilization. Hungate tubes were seeded in duplicate on two petri dishes for each tube containing 1 g of sediment and diluted. The reactor was kept under anaerobic conditions for the growth of anaerobic bacteria for a period of 7 days at 30 °C till the color of media changed to blackish gray.

Only the tubes in which growth was observed were selected for a second round of isolation by streaking on agar plates. Individual grown colonies were transferred to gas tight serum bottles (120 mL) containing 50 mL of growth media under an anoxic conditions, the cultures were grown at 30 ◦C on an orbital shaker incubator at 100 rpm constant shaking rate for 72 h. After incubation, the microorganisms that developed in the petri dishes with dilution ratios of 10^{o} to 10^{2} were transferred to the liquid medium, 1ml was added to the petri dishes again and incubation was continued at 30 ^{o}C for 72 hours as shown in Figure 3.3. In order to obtain pure cultures, sowing was continued by drawing on petri dishes, while the completion of the incubation period was kept in the permanent chamber. All the bacteria that been selected were had the same dilution ratio after incubation.

Furthermore, the samples were taken and analyzed for determination of bacterial species. At the end of the incubation, the microorganisms on the petri dishes were taken to the liquid medium. The microbial Haloalkaliphile in the petri dishes with the same first dilution rate was added to the tube that contains liquid medium.

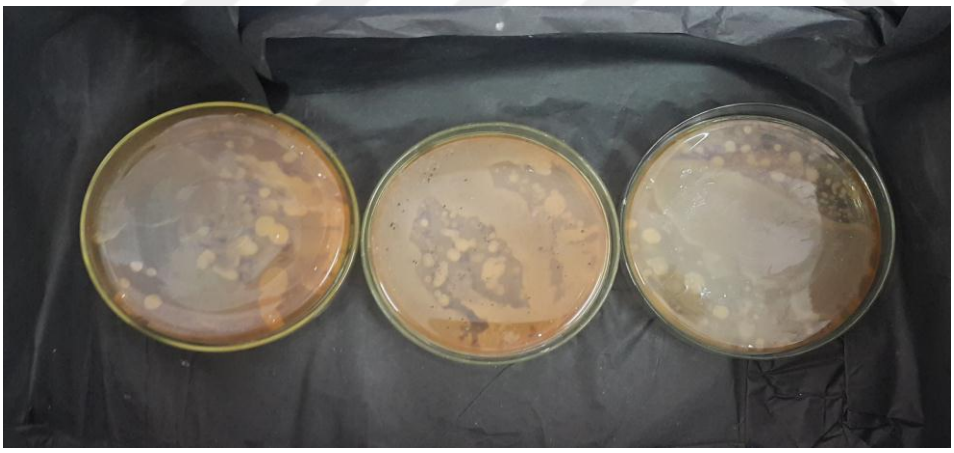


Figure 3.3. Halophilic anaerobic bacteria strains in petri dishes.

The sterile pipette is a small tube, often with an enlargement or bulb in the middle. It has usually used for transferring or delivering measured quantities of the liquid (solution). Pipette was used during the procedure by the anaerobic chamber (known as Nitrogen-Dry Box) that used to minimize or remove the risk of possible contamination Also sulfate and COD removal were examined. The incubated liquid cultures were then refrigerated at 4 ^{o}C to be used for further analysis.

3.3 Adaptation of haloalkaliphilic microorganisms to Na_2CO_3 and $NaHCO_3$

The adaptation of microorganisms to organic and inorganic compounds is an important process to increase microbial activity. The duration of the exercise varies from a few hours to a few weeks, depending on the nature of the vaccine used [88]. In this research, a series of experiments were carried out for determining sodium carbonate and sodium bicarbonates at different concentrations in order to find the best concentration so that Haloalkaliphilic anaerobic bacteria can be activated. The microorganism's acclimation in sodium carbonate, and sodium bicarbonates was carried out step wise.

The effect of Na_2CO_3 and $NaHCO_3$ concentration on Haloalkaliphilic microbial growth was one of the topics. Once Na_2CO_3 concentration was kept constant, DSMZ 742 medium was prepared with $NaHCO_3$ concentration that adding to 90 ml of serum bottles as 0, 5, 10, 15, and 20 g/L, respectively. Furthermore, the DSMZ742 medium was prepared with present of Na_2CO_3 concentration as 0, 1, 5, and 15 g/L respectively, and 90 ml is distributed to 250 ml serum bottles and sterilized in autoclave operating at 121 ^{0}C at 1 atm pressure. After sterilization, the 10ml of liquid culture were incubated at 30 ^{0}C for 48 hours in an orbital incubator running at 100 rpm. The pH values were measured by taking samples at different time intervals. A specific volume of samples was filtered through a 0.22 μm membrane filter paper using a vacuum filtration apparatus and microorganism dry weight analysis was performed also the COD, sulfate and total organic carbon (TOC) analysis.

3.4 Applying different carbon sources

To investigate the effect of different carbon sources of the carbon source Na-format, several tests sequences were applied. These sources include; 5g/L glucose, 5g/L acetate, 5g/L glucose + acetate, 5ml methanol, 5ml ethanol, 5ml methanol + ethanol where each of these chemicals was added to sterile 250ml serum bottles containing 90 ml of growth medium. After sterilization, 10ml of liquid culture was added to each and incubated at 130 rmp at 30°C for 120 hours in an orbital incubator. For the first 3 series, samples were taken at deferent time intervals.

For the other series at t= 0 and 96 h time intervals. Furthermore, microorganism dry weight, COD, sulfate, and TOC analyzes were made. As a result of the above test, acetate and ethanol have been selected since the optimal activity of Haloalkaliphile microbial was observed when these carbon sources are used.

3.5. Batch systems

Our experiments were performed by the batch system under anaerobic conditions using glass serum vials (120 mL). Moreover, a 50 mL of modified growth medium with pH around 9.5-10 was added in the vials for batch tests. The bottles were sealed with cotton stoppers and aluminum crimp seals incubated at temperature 30 °C under optimum condition.

Haloalkaliphilic anaerobic bacteria that used in batch systems were cultivated in closed serum vials through standard procedures for growth medium [86]. Thereafter, the vials were sealed and 5 mL inoculum of bacteria cultivated in growth medium was added by a sterile pipette. The effect of various carbon sources was studied using the growth medium amended with different carbon sources, like Na_2CO_3, $NaHCO_3$, ethanol, and acetate. The different carbon sources were amended in such a way that total carbon content was approximately 2.58 % from the various carbon sources. The Na_2CO_3, $NaHCO_3$, ethanol, and acetate 10.0 g/L, 15.0 g/L, 5 ml/L and 5 g/L, were added respectively.

3.6. Analysis for various parameters

Ten milliliters of the samples were withdrawn and filtered out by using 0.22μm of filter paper. The filtration process is carried out by pouring the sample onto the paper and then the air pump is activated so that the solution will be filtered. All the chemical analyses were carried out by the standard methods for the examination of growth medium. The pH was electrochemically measured by ORION 3 STAR, pH, Benchtop model. The COD was measured according to Standard Methods. The TOC concentration were determined through combustion of the samples at 680 °C using a non-dispersive IR source (TOC-VCPN- model, Shimadzu) by non-purgeable organic carbon method. The sulfate in the diluted sample was measured using UV spectrophotometer Hcich-loge DR 6000[89, 90].

All the experiments were duplicated, for checking the reproducibility of the results. The maximum experimental error was below 2%, and the average values have been reported. Each measurement was made with three repetitions, and the average of three results was obtained. The removal efficiency each of COD, TOC and sulfate were calculated as follows;

$$\text{Removal\%} = \frac{Ci - Cf}{Ci} x100 \quad (3.1)$$

Where; C_i is initial COD, TOC or sulfate concentration (mg/L) and C_f is the final concentration (mg/L). The dry weight of the microorganism was determined and calculated after the cell pellet had been dried at 80 ^{o}C for 24 h.

The microorganism concentration is calculated as:

$$D = \frac{Cf - Ci}{V} * 1000 \quad (3.2)$$

Which D considered as concentration weight of microorganism, C_i weight of filter paper before we put the microorganism, C_f is the dry weight of microorganisms, V represent the total volume of the solution in serum bottle that we used in these experiments in mg/L. to make the calculation g/L we multiply it to 1000.

3.7. Microbial analysis

3.7.1. PCR based molecular techniques

Polymerase chain reaction (PCR) and denature gradient gel electrophoresis (DGGE) methods were used to detect the isolated bacterial species. PCR is a technique that used to measure the amount of DNA. It can be located on almost any liquid or surface where DNA strands may be disposed. On other hand, DGGE known as one of the most widely applied methods for human gene detection at mutations point. The mutations are located to limited regions of the gene by DGGE and subsequently identified by sequence analysis.

In order to determine the microbial species that isolated from the specimens, each of the mentioned methods were used at different points in the study. The samples from the colonies were taken from each petri dish and subjected to DNA extraction. DNA extraction was performed with Ultra Clean Soil DNA isolation kit. The 16S rRNA genes of the microbial species in the extracted samples were amplified using the TECHNE/TC-512 brand PCR device. The base sequence of the primers used in the PCR process is given in Table 3.2. PCR products obtained in order to control the success of the assay which stained with ethidium bromide and run at 100V for 30 min with loading 1%(w/v) of agar gel.

Table 3.2. The base of polymers sequences that applied in the process.

Primer Name	Base Sequence
Forward (GC-BacV3f)	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAGGGGGGGCCTACGGGAGGCAGCAG -3'
Forward (BacV3f)	5'-CCTACGGGAGGCAGCAG -3'
Reverse (907R)	5'- CCGTCAATTCMTTTGAGTTT -3'

3.7.2. Denature gradient gel electrophoresis (DGGE)

In PCR, the amplified target genes were subjected to denaturing gradient gel electrophoresis (DGGE). DGGE treatment was performed by using INGENY Phor L-2 device. A gel with a gradient of 35-60% in the assay was prepared and the DNAs were run in 1XTAE buffer solution. This process was carried out at 60 ^{o}C and 100 V for 16 hours. At the end of DGGE process, the DNA fragments of each microorganism were separated from each other. Then the sequence on the DGGE gel were cut with sterile scalpel under UV light and transferred to the PCR tube. The obtained DNAs were subjected to PCR again and species that detected used as result of sequence analysis.

4. RESULTS AND DISCUSSION

Many researches were investigated about anaerobic processes and their some advantages compering to aerobic treatments in terms of their low sludge production, low energy requirement, and high production of methane gas where recently been used as bioenergy resource. However, the presence of high salinity in wastewater has been observed as inhibitory for conventional anaerobic treatment which has a high ability to degrade organic material and removing toxic sulfate.

PCR was used in order to identify the 16s DNA of the bacterial population isolated from Van lake. It is very important to change the optimum conditions such as salt concentration, carbon sources for the removal of COD and sulphate by anaerobic haloalkaliphilic bacteria.

4.1. Microbial ecology

Polymerase chain reaction (PCR) and denature gradient gel electrophoresis (DGGE) methods were used to determine the types of bacteria to be used in this project. Due to this purpose, analysis of the bacterial species present in 10 different samples was carried out. The agarose gel images of the PCR products that preformed after nucleic acid extraction are given in (Fig 4.1.) The resulting agars gel image has shown that the PCR process is successful for all samples. The PCR products that loaded into the acrylamide gel in the DGGE system and the mixed DNA fragments were separated in the denaturant gel under electric current. The separated DNA fragments were amplified using same PCR product and checked by agarose gel electrophoresis as shown in (Fig 4.2.).

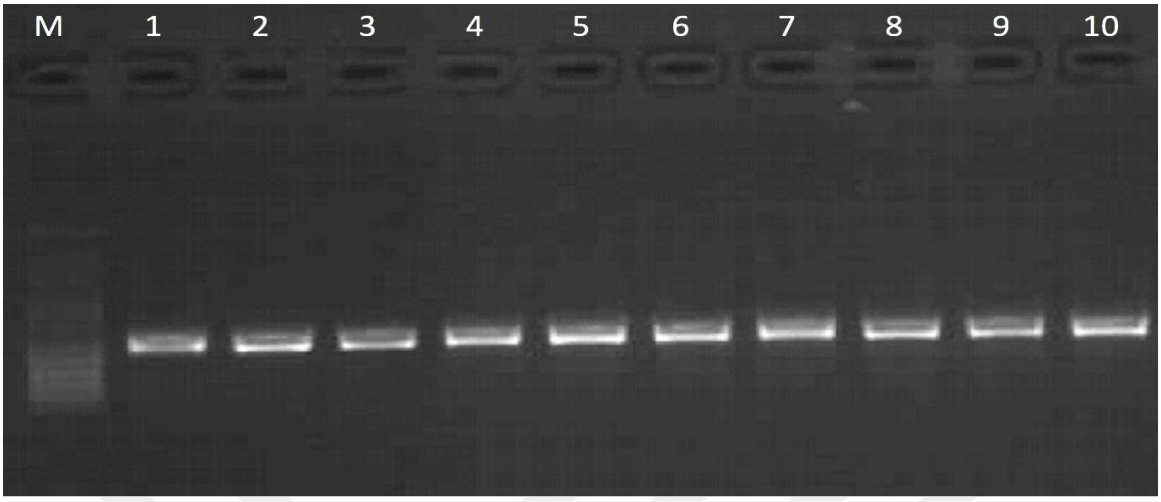


Figure 4.1. Agarose gel image of PCR amplified products before DGGE.

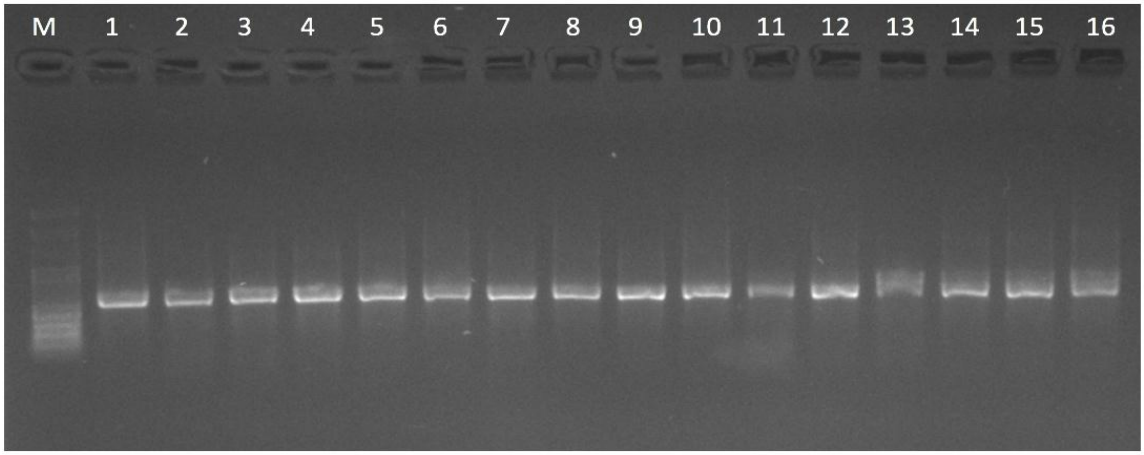


Figure 4.2. Agarose gel image of PCR amplified products after DGGE.

In the DGGE gel image of each sample, the horizontal sequencing of numbered representing different microbial species that given in (Fig 4.3). As a result of planting in petri dishes, 16 different bacterial species detected from the samples. The succession analysis of the PCR products have been detected for each sequence revealed gene bank access numbers of the sequence succession where microbial species represented by these series. Percent similarity and organism classes are given in Table 4.1 together with the band numbers. As shown in figure 6 how the bacteria are detected by florogenic tree.

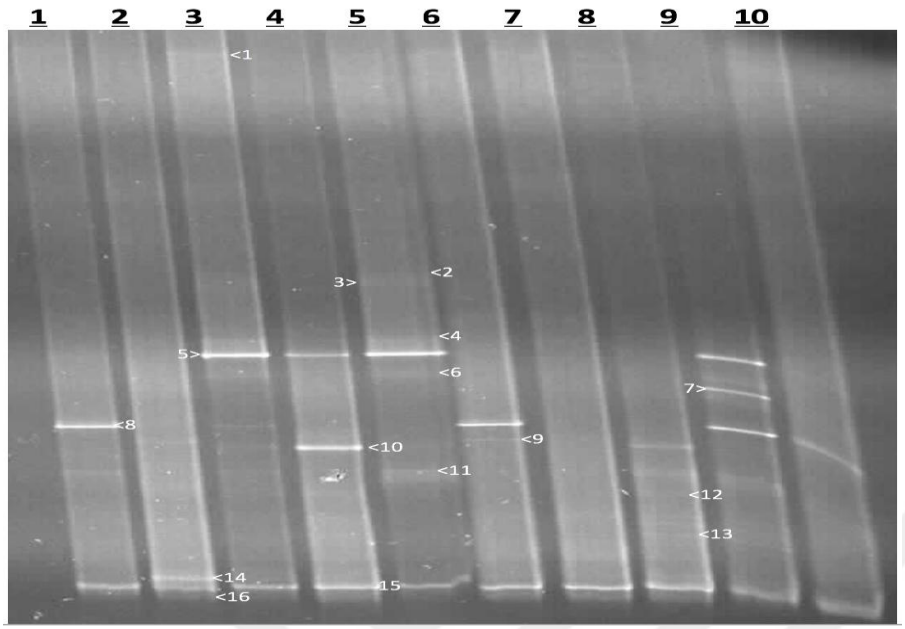


Figure 4.3. DGGE image of insulated species.

Table 4.1. Colonies of species that detected in isolated

Band	Microorganisms species	Identity (%)	Accession number	Reference
1	*Proteobacteria bacterium*	99	MH061186	AUTHORS Chen,H. TITLE Phylogenetic Studies of Halophiles in Yuncheng Salty Lake and The Application in Sewage Treatment
2	*Pseudomonas sp.*	95	KJ174591	AUTHORS Zhang,D., Berry,J.P., Zhu,D., Wang,Y., Chen,Y., Jiang,B., Huang,S., Langford,H., Li,G., Davison,P.A., Xu,J., Aries,E. and Huang,W.E. TITLE Magnetic nanoparticle-mediated isolation of functional bacteria in a complex microbial community JOURNAL ISME J 9 (3), 603-614 (2015)
3	*Sphingobium sp*.	74	GQ287635	AUTHORS Whang,K., Kim,K. and Han,S. TITLE Isolation of exopolysaccharide producing bacteria from Angelica gigas rhizosphere soil
4	*Taibaiella koreensis*	91	NR_133876	AUTHORS Son,H.M., Kook,M., Kim,J.H. and Yi,T.H. TITLE *Taibaiella koreensis sp*. nov., isolated from soil of a ginseng field JOURNAL Int. J. Syst. Evol. Microbiol. 64 (PT 3), 1018-1023 (2014)

5	*Aliidiomarina sp.*	99	KX449149	AUTHORS Sisinthy,S., Mantri,S. and Gundlapally,S.R. TITLE Description of Aliidiomarina sp. from Lonar Lake, Buldhana District of Maharastra, India JOURNAL Unpublished
6	*Fluviicola sp.*	99	KT262951	AUTHORS Rangel,C., Queiros,D., Serafim,L.S., Rossetti,S. and Lemos,P.C. TITLE PHAs from industrial waste by applying the three-step process JOURNAL Unpublished
7	*Anaerobranca sp*.	98	KR349724	AUTHORS Quemeneur,M. TITLE Isolation of novel anaerobic alkaliphilic microorganisms from new Caledonian springs JOURNAL Unpublished
8	*Alkalimonas sp*.	99	JF937428	AUTHORS Shi,W., Takano,T. and Liu,S. TITLE Isolation and characterization of novel bacterial taxa from extreme alkali-saline soil JOURNAL World J. Microbiol. Biotechnol. 28 (5), 2147-2157 (2012)
9	*Terrimonas ferruginea*	97	NR_113718	AUTHORS Nakagawa,Y., Muramatsu,Y., Miyashita,M., Sugimoto,M., Yoshino,M. and Kamakura,Y. TITLE NITE Biological Resource Center (NBRC) JOURNAL Unpublished
10	*Alkalimonas delamerensis*	99	EF423727	AUTHORS Joshi,A.A., Kanekar,P.P., Borgave,S.B., Kelkar,A.S., Sarnaik,S.S.and Shouche,Y.S. TITLE Bacterial diversity of Lonar lake JOURNAL Unpublished
11	*Uncultured Comamonas sp.*	99	JF745002	AUTHORS Zhang,Y. TITLE Microbial population dynamics in a bioaugmented membrane bioreactor JOURNAL Unpublished
12	*Thermomonas sp.*	99	KY212537	AUTHORS Dahal,R.H. TITLE Thermomonas sp. S-48 sp. nov., isolated from forest soil JOURNAL Unpublished
13	*Uncultured gamma proteobacterium*	81	HE856467	AUTHORS Uria,N. and Mas,J. TITLE Electron transfer role of different microbial groups in microbial fuel cells harbouring complex microbial communities JOURNAL Unpublished
14	*Halomonas alkaliphila*	99	KU561610	AUTHORS Remmas,N. and Ntougias,S. TITLE Extremophiles from mature leachate JOURNAL Unpublished
15	*Halomonas sp.*	99	CP016490	AUTHORS Wu,S., Chen,C., Gan,R., Wang,L. and Chen,S. TITLE The complete genome of Halomonas sp. GFAJ-1 JOURNAL Unpublished
16	*Halomonas hydrothermalis*	99	MF928274	AUTHORS Changjian,L. and Qiu,L. TITLE Distribution of Petroleum Hydrocarbon and Variation of Microbial Community in Sediments of Dalian Sea Area

From the table, we found that the most microbial species grow under alkaline conditions are bacterial group number: 8 (*Alkalimonas sp.*), no: 10 (*Alkalimonas delamerensis*), no: 14 (*Halomonas alkaliphila*), no: 15 (*Halomonas sp*.) and no: 16 (*Halomonas hydrothermalis*) Phylogenetic tree of determined species and types of classes to which the isolated species belong given in (Figure 4.4 and 4.5.) respectively.

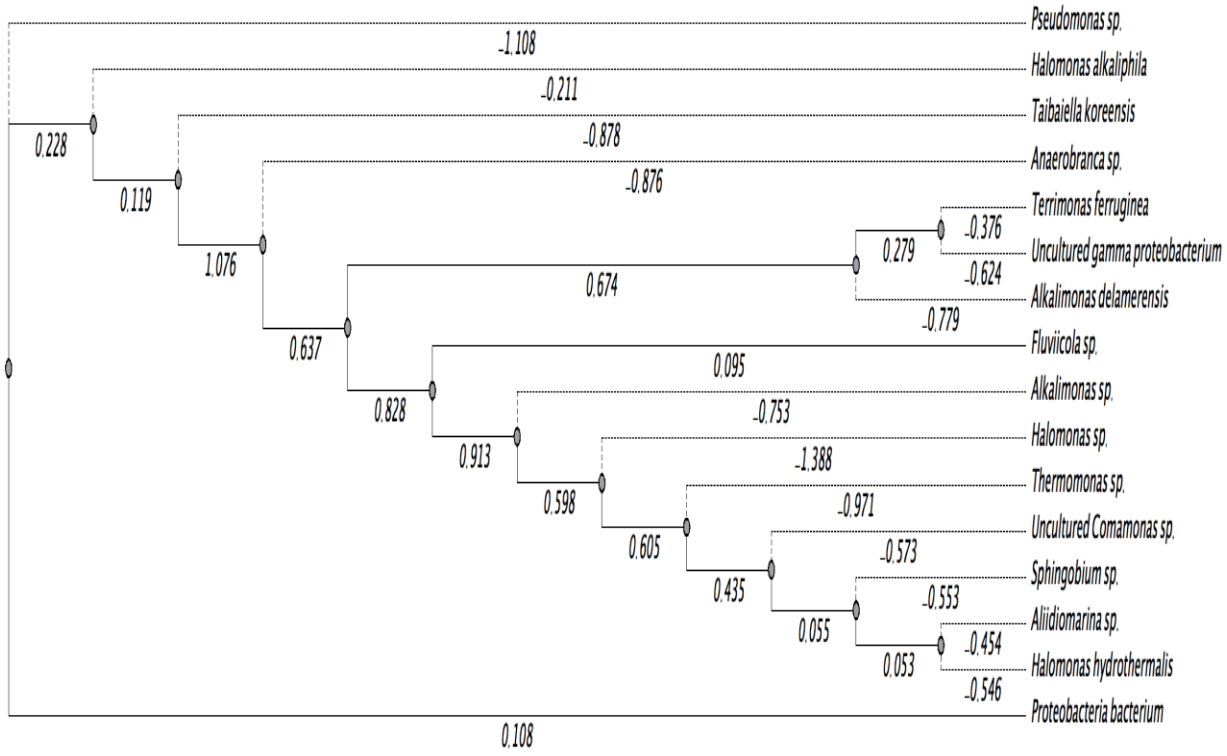


Figure 4.4. Phylogenetic tree of determined species.

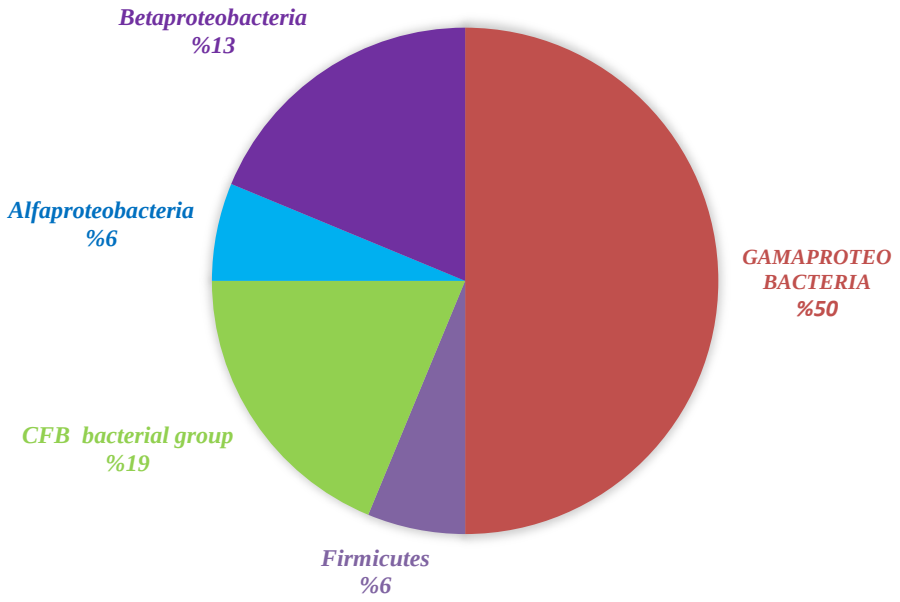


Figure 4.5. Relative abundance of bacterial community composition.

According to Figure 4.5, a significant percentage of microorganism classes that isolated were found to be in the bacterial species are class of *Gamaproteobacteria* (50%). Besides, CFB group bacteria (19%), *Betaproteobacteria* (13%), *Alfaproteobacteria* (6%) and *Firmicutes* (6%).

4.2. Effect of temperature on the growth of microorganisms

One of the important parameters affecting the growth rate of microorganisms is temperature. In Fig 4.6 shows the final biomass concentrations plotted against temperature. The temperature is normally influence on H.B growth through alkaline environments was observed between 20-40 ^{o}C. Makzum 2016 showed that the highest specific growth and thiosulfate removal of *Thioalkalivibrio versutus* occurred in the alkaline state (pH 10.5).

The optimum salt concentration for thiosulfate removal was 2.5 % w/v and 5 % NaCl and specific growth rate elevated 2.5% w/v. It was also specified that this strain thrives occurred in 37 ºC and at 35 and 37 ºC higher removal of thiosulfate [91]. In another investigation, Zhilina 2005 a novel alkaliphilic, sulfate-reducing bacterium was isolated from a syntrophic acetate-decomposing community enriched from samples of the soda lake.

The temperature range for growth was 15-40 ^{o}C, with an optimum of 35-38 ^{o}C. The pH range for growth was 6.7-10.3, with an optimum of pH 8.0-9.0. [92]. In our study, the optimum growth of H.B. has been examined at different concentrations of carbon sources. The optimum temperature for bacterial growth was determined as 30 ^{o}C, which considered an important parameter that impact on the activity of H.B. It can be particularly influential on the enzyme systems of microorganisms. At low temperatures, the binding of other pollutant to microorganism is by passive uptake. The metabolic yields of magnesium, potassium, phosphorus and carbon decrease at lower temperatures resulting in reduction of their energy yields, which, in turn, results in lover biomass concentration. Above the optimum temperatures, bacterial enzyme activity decreases with temperature because of enzyme denaturation. High temperature makes inhibition effect on the growth and death of microorganism by which the growth of the microorganism decreased with increasing temperature. The changes of microorganism concentration are mainly depending on the temperature, as shown in Figure 4.6 and 4.7.

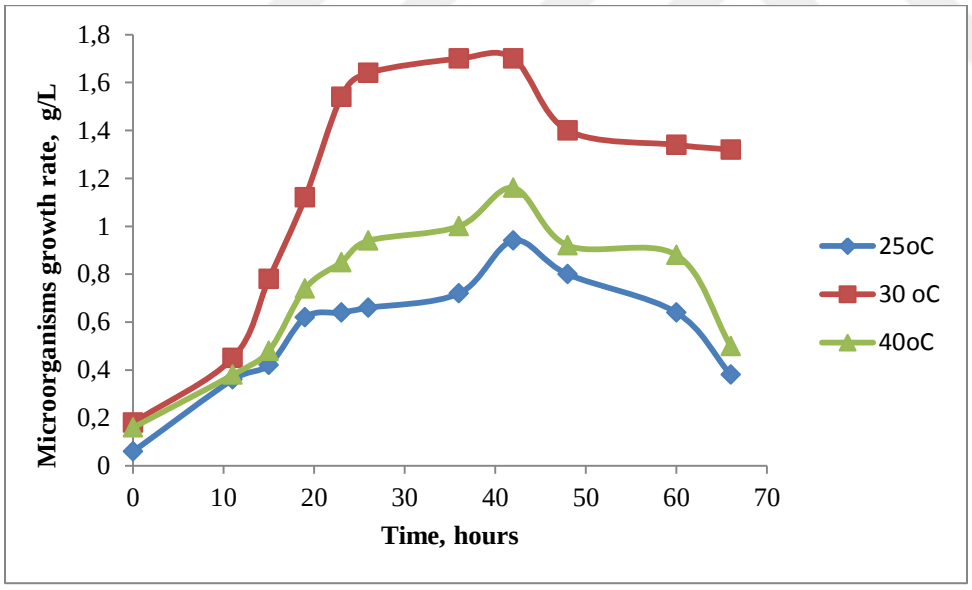


Figure 4.6. Variation of microorganism concentration at different temperatures (pH= 9.74; X_o= 10 mL; NaCl= 5 g/L; Acetate= 5 g/L; $NaHCO_3$= 15 g / L; Na_2CO_3= 10 g/L; V_R= 100 mL; 130 rpm).

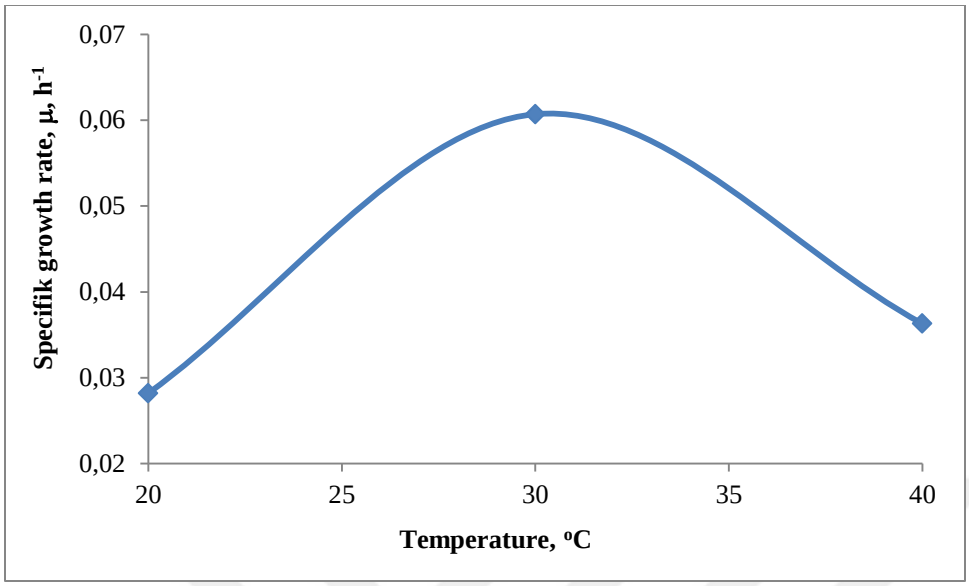


Figure 4.7. Effect of temperature variation on specific growth rate (pH= 9.74; X_o= 10 mL; NaCl= 5 g/L; Acetate = 5 g/L; $NaHCO_3$= 15 g / L; Na_2CO_3= 10 g/L; V_R= 100 mL; 130 rpm).

4.3. $NaHCO_3$ and Na_2CO_3 Impact

Through all saline lakes, sodium carbonates and sodium chloride were the dominating salts, has been showed in 2005 the Sorokin's study that both $NaHCO_3$ and Na_2CO_3 their proportions are significantly varied from soda to extreme hypersaline lakes [93]. The initial metal salts concentration plays a major role in the growth properties of H.B. where the initial $NaHCO_3$ concentration in an experiment set was increased in the range 0-20 g/L while the initial Na_2CO_3 concentration was kept constant 10 g/L for each experiment set at pH 9.74 (Fig 4.8). As presented in (Fig. 4.8 and Table 4.2) , microorganism concentration increased with increasing initial $NaHCO_3$ concentration up to 15 g/L in the growth medium. The growth inhibition was determined at 20 g/L of $NaHCO_3$.

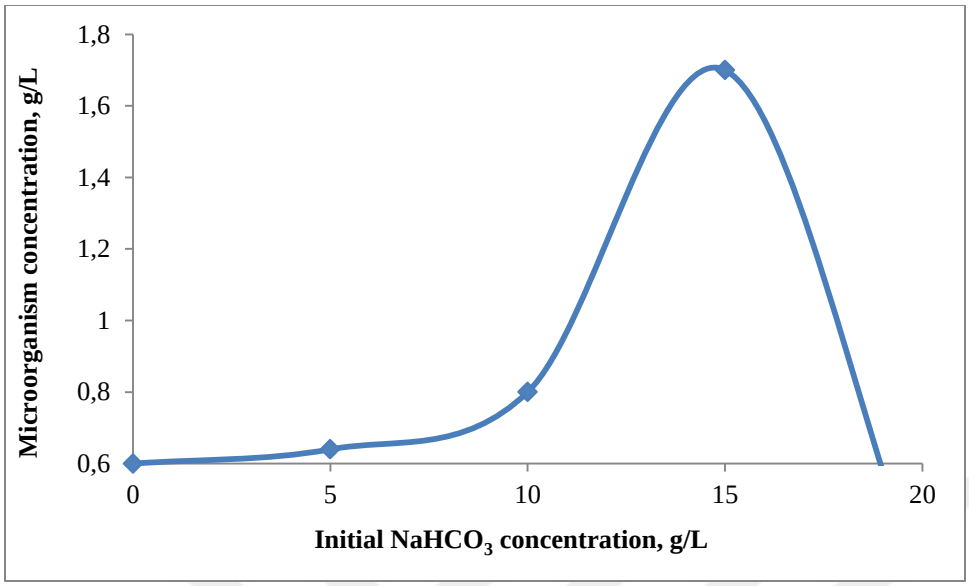


Figure 4.8. Microorganisms growth ratio g/L at different $NaHCO_3$ concentrations (Na_2CO_3 = 10g; pH= 9.74; X_o = 10 mL; NaCl = 5 g/L; Acetate= 5 g/L; V_R = 100 mL; KH = 130 rpm).

Table 4.2. COD; sulfate; and TOC's removal efficiencies value in the presence of $NaHCO_3$ concentration and Na_2CO_3 stability.

$NaHCO_3$ Concentration, g/L	COD,%	Sulfate, %	TOC, %
0	0.89	9.13	56.86
5	14.61	20.25	75.98
10	19.22	21.74	89.6
15	72.72	63.39	92.08
20	3.93	20.97	82.85

The improvement of $NaHCO_3$ concentration has an inhibitory effected on the growth of microorganism. The concentration of $NaHCO_3$ was kept constant at 15 g/L.

While in the Na_2CO_3, the concentrations were changed to 0, 1, 5, 10, and 15 g/L respectively. Microorganism's concentrations and removal efficiency of Sulfur, COD, and TOC have been applied when the 15 g/L $NaHCO_3$ and 10 g/L Na_2CO_3 been taken as an optimum condition for having the highest H.B. grow as given in Figure 4.9, Table 4.3.

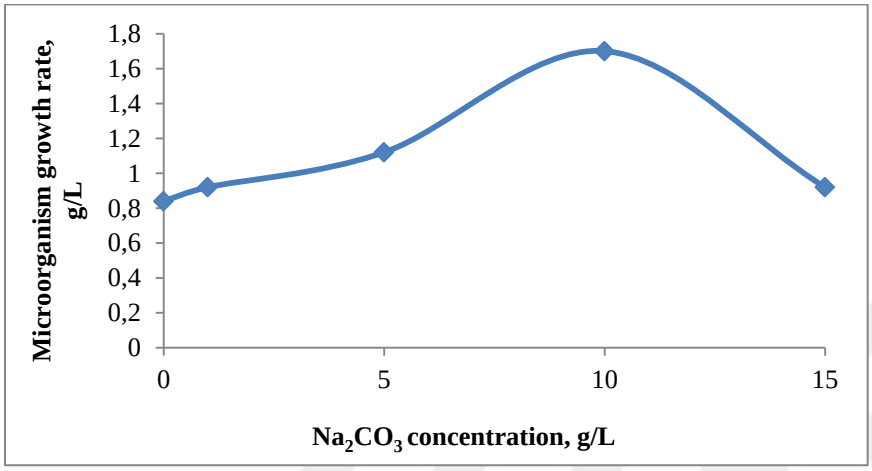


Figure 4.9. Microorganisms growth ratio g/L at different Na_2CO_3 concentrations ($NaHCO_3$= 15g/L; (pH= 9.74; X_o= 10 mL; NaCl= 5 g/L; Acetate= 5 g/L; V_R= 100 mL; K_H= 130 rpm).

Table 4.3 COD; sulfate; and TOC's removal efficiencies value in the presence of Na_2CO_3 concentration and $NaHCO_3$stability.

Na_2CO_3 Concentration, g/L	COD, %	Sulfate, %	TOC, %
0	0.56	28.685	62.93
1	14.04	30.54	71.65
5	18.53	30.16	87.27
10	72.72	63.39	92.08
15	2.25	28.2	98.4

According to the data of the above table; and the microorganism's concentration improvement, COD, sulfate, and TOC removal were also been increased.

4.4. Effect of different carbon sources

The initial substrate concentration plays a major role in the growth of H.B. The substrate concentration is usually present in the environment at certain values. Low concentrations are limited, while high concentrations are inhibition effects. This type of inhibition is usually a non-competitive inhibition. At this stage of the study, different carbon sources were chosen to replace the Na-formate by keeping Na_2CO_3 and $NaHCO_3$ concentrations constant. Glucose, acetate, glucose +Acetate, methanol, ethanol, and methanol+ ethanol were chosen as the carbon source in the batch system. The effect of each carbon source was investigated separately. For this purpose, 5g/L glucose, acetate, 5g/L glucose+ acetate, methanol, ethanol, and methanol+ ethanol were added to the medium instead of Na-formate. The results were obtained as shown in Table 4.4, COD, sulfate, and TOC removal have been observed in present of acetate. Low bacterial activity was observed in glucose presence. Best temperature that we've identified for the highest blooming of microorganisms was 30 ^{o}C with pH around 9.74- 10.1. In the later stage, the impact of ethanol, methanol, and ethanol+ methanol were also investigated by adding 5ml/L for each of them respectively. In Table 4.4, it has been proved that methanol and methanol +ethanol mixture was not a convenient choice for the medium content comparing to ethanol solution.

Table 4.4. COD, sulfate, and TOC's removal efficiencies value in the presence of different carbon sources.

Carbon Source	COD, %	Sulfate, %	TOC, %
Glucose	11.14	30.08	51.20
Acetate	72.72	63.39	92.08
Glucose +Acetate	32.22	_28.43	19.28
Methanol	94.09	9.55	35.39
Ethanol	96.66	3.72	59.83
Methanol+ Ethanol	95.16	2.62	66.80

Xiao and Roberts (2010) explained how methanol considered an external carbon source and electron donor for some studies through an anaerobic process which was able to remove some materials in saline habitat [94]. Moreover, other research like the Zhao and Shulin (2014) were also been seeking for Haloalkaliphilic culture and how impacts on COD and SO_4^{-2} removal using ethanol. As well as, they chose acetate to be used as an indicator for high sulfate reduction at 5g/L of total broth. They claimed that acetate concentration for sulfate removal efficiency was high compering to glucose that shows a low bacterial growth through using it. Usually the pH of this bacterial tends to be alkaline about 9.5 or more and temperature reaches about 37 ^{o}C [4]. In this study acetate was selected as the most suitable carbon source for COD, sulfate, and TOC removal.

Same results are been seen in our experiment where H.B. at optimum growth when NaCl was obtained at 5.0 g/L which considered the main source of salt in our broth. In addition to, Na_2CO_3 and $NHCO_3$ play an important role to provide high salinity in their growth medium were the highest specific growth rate was observed at 15 and 10 g/L respectively. Ethanol has been acts as the sole electron donor for the bacterial growth. Methanol was more appropriate for sulfate reduction and sulfide oxidization where been applied in our study at 5 ml/L concentration of total bacterial culture.

4.5. Changing of acetate concentration and its impact

In Zhilina 2005 studied explained how the amount of acetate in the medium is changing in order to study the influence each of microorganism's concentration, COD, sulfate, and TOC removal in the effluents that contain the only acetate, the concentration of acetate was high when the sulfate removal efficiency is high, and vice versa [95]. Moreover, Zhao and Shulin (2014) reviews reported that acetate improvement can be a good indicator of high sulfate reduction where maximum concentration of acetate was observed at 0.11 M by using Haloalkaliphilic bioreactor [4]. A series of experiments in this thesis were carried out with optimal concentrations of acetate that selected as a carbon source to achieve COD, sulfate and TOC removal at optimum conditions of 2.5, 5 and 7.5 g/L, respectively.

As shown in Figure 4.10 and 4.11, the Haloalkaliphilic bacterium concentration increase with increasing initial acetate concentration (S_o) up to 5 g/L and then did not change further because of the beginning inhibition effect of acetate. At different initial acetate concentrations, the bacterium was observed and approximately fixed during the first 36-42 hours after the linear

change. From the plot of $1/\mu$ versus $1/S_o$ obtained, the values of μ_m and K_s were determined as 0.062 h^{-1}, 1.678 g/L, respectively. The straight line obtained in this plot indicated that Monod-type kinetics is valid. The plots of $1/\mu$ versus $1/S_o$ obtained in the presence of increasing acetate concentrations showed that the inhibition obeyed the Monod model.

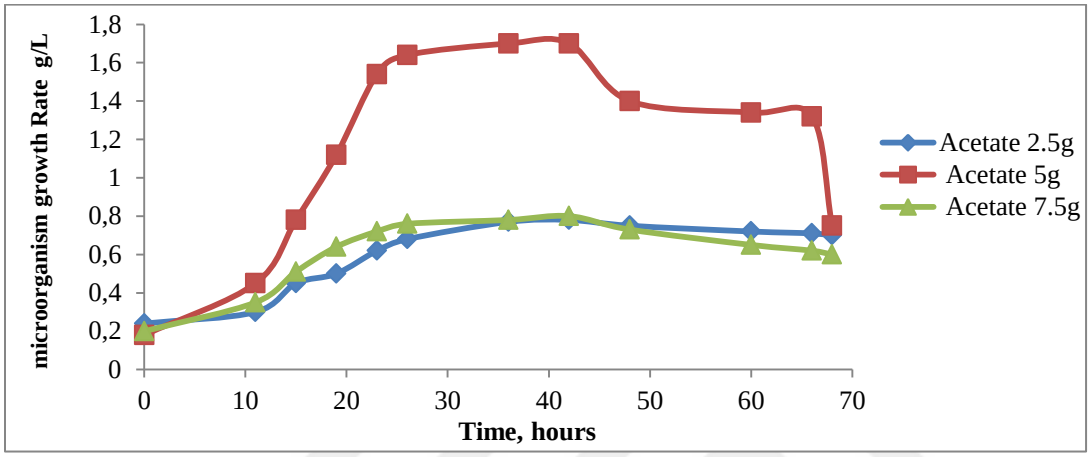


Figure 4.10. Variation of microorganisms concentration at different acetate concentrations (pH=9.74; X_0=10 mL; NaCl=5 g/L; $NaHCO_3$=15 g/L; Na_2CO_3= 10 g/L; V_R= 100 mL; KH=130 rpm).

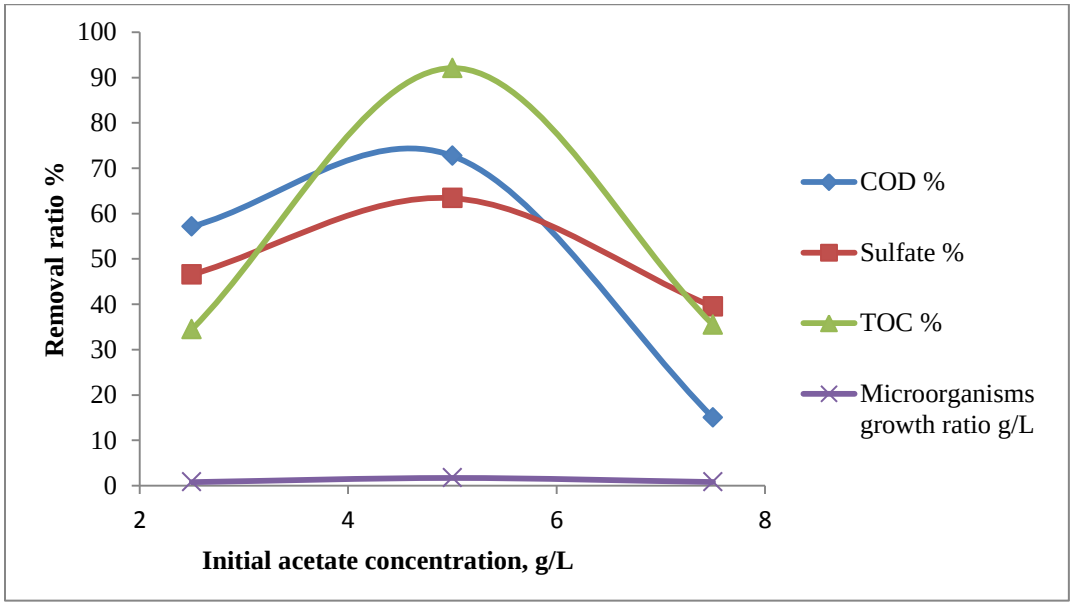


Figure 4.11. COD, sulfate, and TOC ratios at different initial acetate concentrations (pH=9.74; X_o=10 mL; NaCl=5g/L; Na_2CO_3= 10g/L; $NaHCO_3$= 15 g/L; V_R=100 mL; KH=130 rpm).

4.6. Effect of sodium chloride concentration

Xiao 2010 reported that Haloalkaliphiles are optimally able to grow at 5.0 % of NaCl concentration for biodegradation of thiosulfate [94]. Other studies Zhou 2014 showed the ratio of NaCl between 1.5-15% w/v considered the best wastewater treatment from seafood processing or textile dying. In addition, about 2% to 8% of NaCl could be highly effective for COD removal [96]. However, (5, 10, and 15 g/L NaCl) concentration on the microorganism were obtained in our study under optimum conditions includes (pH= 9.74; X_o= 10 mL; Acetate=5g/L; Na_2CO_3=10g/L; $NaHCO_3$=15g/L; V_R=100 mL; KH=130 rpm), where salt concentration has great impact on the bacterial growth as shown in Table 4.5. According to the results, the initial NaCl concentration changes effects on COD, sulfate, and TOC removal efficiency.

Table 4.5. COD; Sulfate; and TOC's removal efficiencies value in the presence of different rate of NaCl.

NaCl Concentration, g/L	COD, %	Sulfate, %	TOC, %	Microorganism Concentration, g/L
5	72.72	63.39	92.08	1.7
10	70.53	21.2	80.32	8.71
15	68.21	10.11	74.04	6.66

CONCLUSION

In this thesis, it is shown that haloalkaliphilic bacteria isolated from Van lake can be used for COD and sulfate removal in the anaerobic batch systems. The improving COD and sulfur removal by isolated H.B. was one of the main aims of this study. Haloalkaliphilic bacteria were isolated in solid and liquid form from water and sediment samples taken from Van lake. As it is well known that haloalkaliphilic bacteria are salt-loving and high alkalinity, in our study at different concentrations sodium chloride and other salts were added to the bacterial culture. Moreover, sterilized 90ml DSMZ 742 medium was filled into serum bottle and 10ml of the liquid culture was taken in the anaerobic chamber and this medium was added. The samples were taken at t=0 and t=120 hours and 0.22 μm injector was filtered and analyzed for COD and sulfate analysis. Our results has shown that the optimum condition were determined for highest growth of our bacterial growth medium concentration and alkalinity as in 5 g/L NaCl, 15 g/L NaHCO3, and 10 g/L of Na2CO3 with different concentrations of carbon sources includes 5 g / L acetate, and 2.5 ml/L ethanol at temperature 30 ^{0}C.

Through our study and previse researches, we can say that Haloalkaliphilic organisms will prove even more valuable in the future. It is important to estimate that the potential of the halo alkane bacteria has been successfully applied in several environmental and biotechnological applications. As well as, haloalkaliphilic bacteria can be widely used in the removal of COD and sulphate due to their ability to resist the denaturing effects of salts and their low disadvantages.

The anaerobic treatment can typically reduce COD amount through anaerobic microorganisms by converting organics into methane gas which is a valuable fuel. Although, anaerobic treatment differs from conventional aerobic treatment in that no aeration is applied. The absence of oxygen leads to controlled anaerobic conversions of organic pollutants to carbon dioxide and methane gas which use as a source of alternative energy for future experiences. This treatment system can be possible only if the characteristics and specific problems for each individual wastewater are known in advance. However, high sulfate removal efficiency affected by COD to SO_4^{2-} that achieved in the H.B. bioreactor was still investigated.

Hypersaline and alkaline environments can be a substitute solution for human industrial processes, like mineral ore, refining, pulp and paper, textile preparation, calcium carbonate kilns, detergent manufacture and food industry.

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CURRICULUM VITAE

My name is Shaymaa Farooq Alsaadawi. I was born in Bagdad/Iraq on date 07/08/1993. I lived in Bagdad for 6 years and then I moved to Erbil in the year 1999. I took my education in public schools Amin Zaki and Alzahraa for girls/Erbil, (2000-2011). As well as, I graduated had a B.Sc. degree in Erbil at Salahuddin University/College of Science/ Environmental Science Department, year (2011-2015). I speak four languages includes Arabic, Kurdish, English, and Turkish. Through these years, I had the passion for learning more about our environment and how we can keep natural sources less contaminated, so I decided to continue my education and getting M.Sc. degree, thus I can have skills and more knowledge about protecting our environment from recent human activities and natural disasters.