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Optimization of biogas production from Wastewater Treatment Plant Sludge

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ملخص

تتناول هذه الدراسة تحسين مردود إنتاج الغاز الحيوي باستخدام حمأة محطة تصفية المياه عين بوشقيف -تيارت- ومخلفات عضوية متنوعة. استغرقت العملية البحثية حوالي 37 يومًا، وبدأت بجمع العينات من المحطات، حيث تهدف إلى تصميم مفاعل هضم في المختبر لإنتاج الغاز الحيوي. كما تم إجراء تحليل فيزيائي وكيميائي للحمأة، وتحليل ميكروبيولوجي لتحديد الخصائص المكروبيولوجية الهامة التي تساهم في عملية الهضم اللاهوائي للحمأة وإنتاج الغاز الحيوي، وتمت متابعة هذه الخصائص لتحسين أداء عملية التخمر. في هذه الظروف تم تحديد ثلاثة أنواع بكتيرية. بالإضافة إلى تجربة مزج الحمأة مع مكونات مختلفة وفقًا لتسلسل مدروس، ومراقبة كمية الغاز المنتج طوال فترة التخمر. أظهرت النتائج أن استرداد الغاز من الحمأة المستعملة كان ناجحًا، وكان أداء الحمأة المأخوذ من وحدة الهضم الأولية الأفضل بحجم 2000 مل، كما أظهرت حمأة منتجات الألبان حجمًا قدره 1600 مل، في حين بلغ الحجم 1200 مل في حالة مزج حمأة الهضم الأولية مع نفايات الحبوب. وقد سُجلت نسبٌ أقل لمزيج الحمأة من تلك الاخيرة مع وحدة الهضم اللاحقة. وعلى نفس السياق، كشف تحليل الغاز المنتج عن تركيز 13% من الميثان.

الكلمات المفتاحية: حماة، محطة معالجة المياه، تحسين، الغاز الحيوي، ركائز مختلفة.

Abstract

This study aims to improve the efficiency of biogas production by utilizing sludge from the Aïn Bouchakif wastewater treatment plant in Tiaret, along with other organic waste. The research process lasted approximately 37 days and began with the collection of samples from the treatment plant. A laboratory-scale digestion reactor was designed to produce biogas. Physicochemical analysis of the sludge was conducted, as well as microbiological analysis to determine important microbial characteristics contributing to the anaerobic digestion process of the sludge and, consequently, biogas production. These characteristics were monitored to enhance the performance of the fermentation process. Under these conditions, three bacterial types were identified. In addition to mixing the sludge with different components in a studied sequence, the quantity of gas produced throughout digestion was monitored. The results demonstrated the feasibility of gas recovery from the utilized sludge. However, the sludge from the pre-digester exhibited the highest yield with a volume of 2000 ml, while the dairy sludge showed a volume of 1600 ml, and the mixture of sludge with cereal waste yielded 1200 ml. Lower rates were recorded for the mixture of pre-digester sludge with digester sludge. Analysis of the produced gas mixture revealed a methane content of 13%.

Keywords: sludge, WWTP, optimization, Biogas, different substrates.

Résume

Cette étude vise à améliorer le rendement de la production de biogaz en utilisant les boues de la station d'épuration d'Aïn Bouchakif – Tiaret- et autres déchets organiques. Le processus de recherche a duré environ 37 jours et a commencé par la collecte d'échantillons des stations, avant de concevoir un réacteur de digestion en laboratoire pour produire du biogaz. Une analyse physiquo-chimique des boues a été réalisée, ainsi qu'une analyse microbiologique pour déterminer les caractéristiques microbiologiques importantes contribuant au processus de digestion anaérobie des boues et par conséquent, la production de biogaz. Ces caractéristiques ont été surveillées pour améliorer les performances du processus de fermentation. Dans ces conditions, trois types bactériens ont été identifiés. En plus de l'expérience de mélange des boues avec différents composants selon un ordre étudié, et en surveillant la quantité de gaz produite tout au long de la digestion. Les résultats ont montré que la récupération de gaz à partir de boues utilisées était possible. Toutefois, les boues prises du pré-digesteur ont donné le meilleur rendement avec un volume de 2000ml, et les boues des produits laitiers ont montré un volume de 1600ml, tandis que le mélange des boues avec les déchets de céréales a donné 1200ml. Des taux inferieurs ont été enregistré pour le mélange des boues du pré-digesteur avec celles du digesteur. L'analyse du mélange des gaz produits a révélé un taux de 13 % de méthane.

Mots clés : Boues, STEP, optimisation, Biogaz, différents substrats.

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List of Abbreviations

- **AFNOR:** Association Française de Normalisation
- **BOD5:** Biochemical Oxygen Demand
- **BP:** Baird- Parker
- **BSI:** Biome Solar Industry
- **C6H8O2N2S:** Sulphanilamide
- **C10H7NHCH2, 2HCI:** Phenolphthalein
- **CH4:** Methane
- **COD:** Chemical Oxygen Demand
- **CO2:** Carbon dioxide
- **EC:** Electrical Conductivity
- **G:** Gram unit
- **H3PO4:** Orthophosphoric acid
- **Hg2SO:** Mercury sulphate
- **2H2SO4:** Sulfuric acid
- **K2Cr2O7:** Potassium dichromate
- **LB:** Luria Bertani
- **LV:** Liver broth
- **Mg/ L:** Milligram unit / litter
- **Mixture A:** Pre-digester + dairy sludge
- **Mixture B:** Pre-digester+ cereals waste
- **Mixture C :** Pre-digester+ digester
- **ML:** Millilitres unit
- **MS / cm:** Millisiemens / Centimetre
- **MW:** Megawatt
- **N-(1-naphthyl):** Ethylene diamine chloride
- **NF:** Norme Française
- **NH4+:** Ammonium
- **Nm:** Nanometre unit in meters
- **NO_2^-:** Nitrite

- **NO_3^-:** Nitrate
- **pH:** Hydrogen Potential
- **RCM:** Reinforced Clostridia Medium
- **SM:** Suspended Matter
- **TSC:** Tryptone Sulphite Cycloserine
- **TSI:** Triple Sugar Iron test
- **WHO:** World Health Organisation
- **WWTP:** Waste Water Treatment Plant

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Introduction

Introduction:

Over the past few years, Algeria has implemented initiatives related to sustainable development. It has fully and actively engaged in the negotiation process and in various consultations and decisions related to sustainable development goals at the national, regional, and international levels. Algeria has also joined the consensus for the adoption of the Sustainable Development Goals platform outlined in the 2030 Agenda for Sustainable Development. The platform aims to promote sustainable economic growth, protect the planet from the consequences of climate change, and preserve the same opportunities and natural resources for future generations. In line with this, Algeria has embarked on the path of renewable energy by adopting a sustainable development program since 2011. The program aims to install renewable power of around 22,000 MW by 2030 to address air pollution by reducing CO2 emissions of 193 million tons, mainly produced by fossil fuels that represent almost all of Algeria's total primary energy consumption. Regarding biomass energy, the Ministry of Energy and Mines has launched a program to achieve an energy capacity of 1000 MW. (Ministry of Energy and Mines., 2019).

Algeria has recently shown interest in Biogas production by sludge is a promising alternative for meeting energy needs in Algeria. Where Sludge management is becoming a major challenge in Algeria due to its increasing production and polluting impacts on the environment. A study conducted in Algeria found that biogas production from sludge has the potential to not only mitigate environmental impacts but also produce renewable energy. (Rachmawatie et al., 2022) another study revealed that the use of anaerobically digested sludge for biogas production is a viable option in Algeria Where it produced electricity. A pilot project has been conducted at the Hassi Bounif landfill site in the Oran province, which aims to produce biogas that can be used as a source of energy while simultaneously reducing pollution. The idea of generating usable energy while reducing pollution is attractive to both industrial and agricultural sectors and it remains so. The success of anaerobic bioreactors in wastewater treatment can be attributed to several advantages they have over traditional treatment methods. These advantages include low sludge production, low investment and maintenance costs, minimal space requirements, production of valuable biogas, and high adaptability to effluents loaded with organic pollution, as noted by (Lettinga., 1995). However, many of these anaerobic bioreactors use active biomass in the form of

dense granules with a diameter typically between 0.5 and 2 mm. This particular form provides sludge with advantageous properties such as sedimentation speed, biomass retention in the system, solidity, and good separation between granular sludge and treated effluent (Hulshoff Pol et al., 2004).

Methanization is an anaerobic digestion or methanogenic fermentation that transforms organic matter into compost, methane, and carbon dioxide through a complex microbial ecosystem that operates in the absence of oxygen. Methanization enables the elimination of organic pollution while consuming little energy, producing few sludges, and generating renewable energy: biogas (Prévot., 2000).

The biogas generated through methanization is a flammable mixture primarily composed of methane (CH_4) and carbon dioxide (CO_2). This biogas is a renewable energy source because it originates from organic materials of plant or animal origin, which have short renewal cycles. Methanization proves to be an effective tool for reducing organic pollution and producing energy. In fact, the first attempts at biogas production in a digester date back to the 1930s with rustic but reliable processes such as batch digesters and methanization pits, which are widely used in countries searching for energy alternatives to combustion wood (Zhang., 2011). It has also been proven that the addition of bacterial culture has been shown a big enhance biogas production more than AT seed sludge and sewage sludge additions achieving a 215% reduction in volatile solids. Moreover, the use of various types of inoculate such as activated sludge and palm oil mill effluent sludge has been reported to increase methane yields by up to 35% (Dabrowski at AF., 2019). Indeed, not all substrates used in methanization yield the same amount of biogas for the same amount of input. Each material has an associated methanogenic potential that indicates the maximum volume of methane that can be produced from one ton of raw material this has been noted by (Bensmail and Touzi., 2012). In addition, Preliminary preparation of substrates can increase biogas production, efficiency in agricultural and municipal settings located at municipal wastewater treatment plants. With the added benefit of producing hygienist digestive, that can be used as an organic fertilizer. However further research is needed to determine the optimal conditions for biogas production from sludge in Algeria including factors such as temperature, pH and substrate concentration (Baudez et al., 2011). As that, the growing understanding of the role of factors such as nutrients and

trace elements, and the effect of metabolic intermediates and final products opens up excellent prospects for process control.

Sewage sludge is a waste product generated during the treatment of domestic or industrial wastewater. It is composed of water and dry matter, including pollutants and heavy metals. Despite this, sewage sludge is rich in organic matter and contains useful nutrients such as nitrogen and phosphorus, making it useful in agriculture, as noted by (Adler.,2002). In addition, Methanization makes it possible to eliminate organic compounds and allows the station to be more or less autonomous in energy. (G Letting. A.V.L.,1995).

The production of biogas from sewage sludge, household and agricultural waste has been a promising alternative since the 18th century when Alessandro Volta identified methane in marsh gases. Developed countries have been exploiting the phenomena of methanization ever since. In the 19th century, England developed the first digesters that used biogas for lighting. In the 1920s, Germany developed a continuous digestion system that supplied several cities' municipal gas networks. In Algeria, two facilities were established at the CDER in Bouzaréah in 1986 and 2001, but no digesters have been developed since (Bensmail., 2014).

The biogas can be used in several ways for energy production. There are three main pathways: thermal energy, electrical energy, and biofuel (Demeyer et al., 1980). In terms of thermal energy, the biogas combustion heat can be used for producing hot water, steam at medium or high pressure, or for process ovens. Generally, the gas pressure needed to supply gas appliances is low: 20 to 100 mbar. Thermal valorisation usually requires nearby outlets, such as external consumers of the production site (industries, district heating networks...) or internal uses. In wastewater treatment plants, part of the produced biogas is generally used to maintain the digester at the fermentation temperature (37 or 55 °C). This internal consumption of the process represents about 15 to 30% of the production. Concerning electrical valorisation, biogas can be used to feed a gas engine (or a turbine) to produce electricity. When electricity is produced alone, it is most often exported to the public network. Cogeneration produces both electricity and heat. The heat can be used for heating digesters, drying digestive or hay, producing hot water, or supplying domestic heating. For the "biogas engine" solution, it will require desulfurization and dehydration, and

their performance will depend on the motor specifications. In the case of the steam turbine solution, a simple filter to remove suspended particles in biogas will be enough at the inlet of the compressor. The boiler will be equipped with smoke tubes that can withstand biogas fumes, even with a high content of sulphur dioxide, chlorides, or fluorides (Demeyer et al., 1980).

In addition, the starchy waste materials have fermentation potential that can be utilized for non-food applications such as the production of bioethanol. This could present an interesting economic and technological opportunity since ethanol production has rapidly expanded worldwide due to its various applications in the chemical and pharmaceutical industries, as well as its use as a substitute fuel. This type of fuel has the advantage of not contributing to the accumulation of greenhouse gases responsible for ozone layer degradation. In 2000, 20 billion litters of this biofuel were produced, with a potential to reach over 100 billion by 2010 (Kacimi., 2008). The production of ethanol from starchy materials typically requires three stages: liquefaction and saccharification by amylases, followed by fermentation of the resulting must by the yeast Saccharomyces cerevisiae (Shigechi et al., 2004). However, the use of biogas as a fuel for automobiles is only the subject of a few pilot installations under optimization. For now, it is intended for the fuelling of captive fleets of vehicles from local authorities: garbage collection, public transport. Its interest is both economic and environmental, given the quality of the emissions from gas engines (Igoud et al., 2002). Thus, residual sludge appears in liquid form and with a high load of highly fermentable organic matter. These two characteristics are problematic and pose many technical problems for their disposal, including their transportation and storage, which often lead to handling problems and olfactory nuisances. This imposes the choice of a treatment option from the installation of the wastewater treatment plant (Jarde., 2003). Moreover, regardless of this the transformation of sludge into biogas (which is a green energy source) makes it possible to give value to materials previously considered harmful, or even dangerous for the environment. This also makes it possible to combat the scarcity of raw materials and fossil fuels. The wastewater treatment plant in Braunschweig, Germany, is now 100% autonomous in electricity, Thanks to the intervention of BSI Energy, a subsidiary of Veolia, which operates this site with a capacity of 275,000 equivalent inhabitants. Biological treatment of wastewater, thermophilic digestion of sludge and digestion with organic waste,

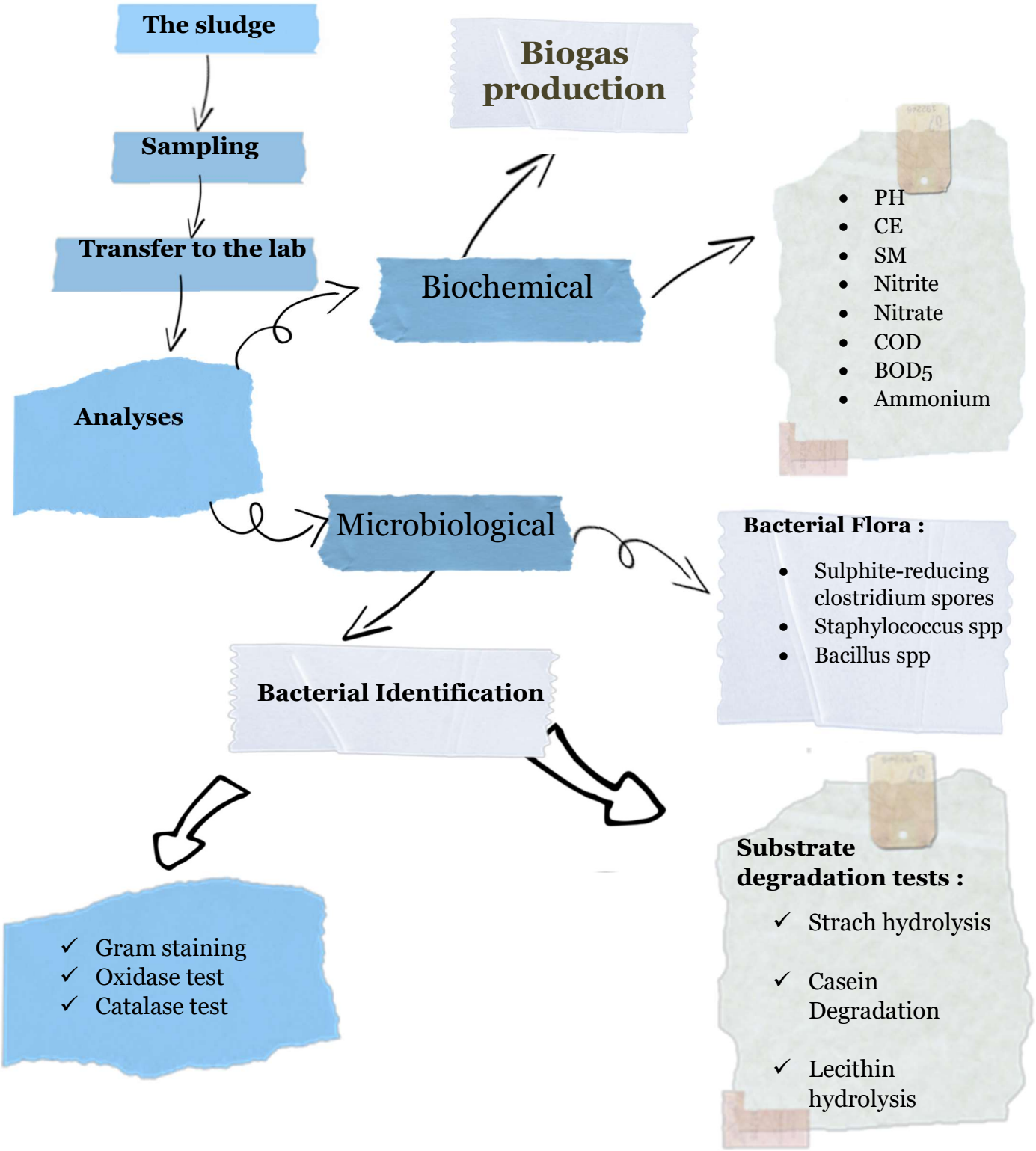
cogeneration and recovery of biogas, allowed the energy autonomy of the site. Thanks to Veolia's solutions, the wastewater treatment plant in Gresham, the fourth most important city in the state of Oregon, in the United States, has been profoundly transformed: from the most energy-intensive in the city, it has now become 92% autonomous thanks to the recovery of sewage sludge into biogas. The plant's electricity expenses have been reduced by an average of $40 to $50,000/month. (Veolia. Com., 2023).

Our research work focuses on valorising the sludge from the Wastewater Treatment Plant of Tiaret through biogas production and evaluating the impact of different substrates on biogas generation. To achieve this objective, our experiment's protocol is divided into the following sections: Firstly, we will gather samples and conduct a physicochemical and microbiological characterization to comprehend the role of specific microbial flora responsible for this biological process and its effects on sludge stability. Lastly, and significantly, we will endeavour to produce biogas by valorising the sludge and comparing the yields from various substrates.

Chapter 01

Materials and Methods

1- **Experimental Protocol:** In this research study, the following protocol is employed: conducting physical, chemical, and biological analyses on the sludge, followed by gas production. This comprehensive protocol aims to investigate and analyses the characteristics of the sludge, as well as explore its potential for gas generation.



2- Experimental Work

2.1- Objective:

The aim of our work was to investigate the production of biogas from sludge samples collected from different parts (pre-digested, digested, and clarifier) at Ain Bouchakif wastewater treatment plant. Our study also focused on analysing the physical, chemical, and microbiological properties of the sludge samples. In addition, we examined the effect of adding dairy sludge and grain waste on biogas production.

To achieve these objectives, we conducted our research at the physiology laboratory at the University Ibn Khaldoun in Tiaret. The microbiology portion of the study was carried out at the microbiology laboratory N=°01. Our work spanned from Feb,13th to April, 11th, 2023. We employed various materials and methods to collect and analyse the sludge samples and determine their properties, as well as to investigate the impact of change the different substrates on biogas and ethanol production.

2.2- Study zone:

The Tiaret Wastewater Treatment Plant (WWTP) is situated in Ain Bouchakif, which is approximately 4.5km away from Tiaret city. The plant is easily accessible by road, as it is located along the CWN°07 state path. The surrounding area is characterized by agricultural activity, and there is a Nouria valley nearby, which flows directly into the Wassel dam – DAHMOUNI-.

The wastewater that is treated at the Tiaret WWTP is transported to the facility via a network of different collectors. The plant covers an area of 9.47 hectares and has a capacity of 390,000 HE (medium load). It has been designed to treat up to 38,000 cubic meters of urban wastewater every day, with the processing based on the drying operation (Aouali et al., 2013).

2.3- Sampling:

2.3.1- Collection:

Sludge samples were manually collected from the STEP aerobics digester using a ladle on Feb, 08th,2023. The sampling was done by collecting the sludge litre by litre at a depth of over 30 cm from the surface. The sample was then collected in a 5L container, filled up to 2/3 of its capacity, and then transported to the laboratory for various analyses.

It is important to note that the sludge sample should be taken at specific points within the digester. The first sample should be taken at the point of entry of the sludge into the digester (before the start of anaerobic digestion). In addition, additional samples should be collected after the entry and in the middle of the basin's core.

Figure 1 : *Sampling of the sludge from the digester (Original photo)*

2.3.2- Transport and storage condition:

Accurate sampling and sterilization procedures are crucial when collecting wastewater samples, and it is important to take steps to prevent any contamination during transport to the laboratory. The collected sludge should also be transported in a cooler to prevent enzymatic activity from bacterial strains. Fortunately, in our case, the samples were taken on a cold day, which may eliminate the need for a cooler during transport. However, it is still essential to begin the analyses and assembly of biogas production as soon as possible to ensure the accuracy of the results.

2.4- Materials and products:

The material used consists of glassware, equipment and apparatus. It also includes a set of reagents and chemicals, which are presented in the table:

Table 1: *laboratory Materials*

Glassware	Equipment and more	Measuring device	Reagents and products
• Beaker • Conical flask • Graduated cylinder • Funnel chemical • Pipette • Watch glass • Erlenmeyer flask • Allihn condenser • Boiling flask • Dropper	• Heating magnetic stirrer and magnetic bar • Hotplate • Water bath • Oven • Desiccator	• PH meter • Analytical balance • Spectrometry	• Sulphanilamide • Phosphoric acid • Phenolphthalein • Monosodium salicylate • Sodium hydroxide • Sodium potassium tartrate • Silver sulphate • Sulfuric acid • Potassium dichromate • Distilled water • Ammonia • Orthophosphoric acid

I- Physicochemical characterization of sludge:

The physicochemical characterization of the sludge obtained from the digester was carried out by determining: Hydrogen potential (**PH**), electrical conductivity (**EC**), suspended matter (**SM**), nitrite (**NO^{-}_{2}**), nitrate (NO^{-}_{3}), the measurement of chemical oxygen demand **(COD),** biochemical oxygen demand (**BOD_5**), and ammonium (**NH^{+}_{4}**).

I.1- pH determination:

A means of expressing the hydrogen-ion concentration, and thus the acidity, of a solution (Jan and Gooch., 2020), it is measured by using a pH meter according the following protocol (Crepa., 2007).

Working method:

- ✓ We used a pH meter with a combined electrode and calibrated using a standard solution of known pH 7.4
- ✓ We prepared 03 solutions from different samples (pre-digested, digested and clarifier) to 10g of sludge are added 100ml of distilled water, the mixture is carried out by light agitation using a magnetic stirrer.
- ✓ Then centrifugation (5min, 15 turns), filters the solution.
- ✓ Note the value shown on the pH meter.

I.2- The electric conductivity determination:

Conductivity is the property of water to promote the passage of electric current. This ability to conduct an electric current is related to concentration and the nature substances (Amir., 2005).

Working method:

- ✓ Sample weight (about 10g) and blend it with 100ml of distilled water.
- ✓ Shake it for 5 min by using a magnetic stirrer.
- ✓ Immerse the conductivity probe in distilled water then in ethanol, a result of (13-14) will appears which mean the conductivity meter is calibrated so you can immure the probe directly in your sample.

I.3- Suspended matter (SM):

The suspended matter method is a technique used to measure the number of solid particles present in a liquid sample, such as water or wastewater. The method involves filtering a known volume of the sample through a pre-weighed filter paper to capture the suspended solids. The filter paper is then dried and reweighed to determine the mass of the suspended solids. The difference between the initial and final weights of the filter paper gives the mass of the suspended solids in the sample.

I.4- Nitrite Determination:

Nitrite is a naturally occurring inorganic form of nitrogen. It is relatively unstable as it is the intermediate species between ammonia and nitrate. Hence, it is usually found in very low concentrations in the environment, less than 0.1 mg/L, and its concentration does not normally exceed 1 mg/L in municipal wastewater (Real Tech.,2017).

- Reagent method of Diazotation: (Norme AFNOR NF T 90 013)

Reagents:

- ✓ Orthophosphoric acid (H_3PO_4).
- ✓ Sulphanilamide ($C_6H_8O_2N_2S$).
- ✓ N-(1-naphthyl) ethylene diamine chloride ($c_{10}H_7NHCH_2$, 2HCI) or phenolphthalein.

Preparation of the Diazotation reagent: To 80 ml of distilled water, add 10 ml of concentrated Orthophosphoric acid and 4 g of Sulphanilamide.

- After dilution, add 0.2g of N-(1-naphthyl) ethylene diamine dichloride or phenolphthalein.
- continue to 100ml with distilled water.

✓ **Nitrous nitrogen standard solution with 100mg/l:**

- Dry a quantity of sodium nitrite for 1 hour at 105°C and dissolve 0.0492g in 100ml of distilled water.

✓ **Nitrous nitrogen standard filled solution with 1mg/l:**

- Take 1ml of the mother solution in 100ml of distilled water.

Working method:

✓ Take 50ml of the sample to be analysed.

✓ Add 1ml of Diazotation reagent.

✓ Wait 10 minutes for the development of the pink colour.

✓ Perform the reading on the photometer at λ =537nm.

Calibration Scope:

The calibration curve is then used to determine the concentration of nitrite in unknown samples based on their absorbance or fluorescence intensity (Zhang et al.,2019).

Table 2: *nitrite calibration scope*

Dilute Solution 1mg/l	**0**	**1**	**2.5**	**5**	**7.5**	**10**
Distilled water (ml)	50	49	47.5	45	42.5	40
Mixed reagent (ml)	1	1	1	1	1	1
Wait 10 minutes						
[NO_2^-] in mg/l	0	0.02	0.05	0.1	0.15	0.2

I.5- Nitrate determination:

We have determined nitrates with two different methods:

I.5.1- Sulfophenic reagent method: (AFNOR NF 90 012 standard)

The determination of nitrates is done by colorimetry in the visible.

✓Preparation of the sulfophenic reagent:

Dissolve 12 grams of phenol in 144 ml of sulfuric acid (36N) in a water bath

Working method:

- Take 5ml of the sample to be analysed, evaporate it to dryness in a beaker.

- Leave to cool and add 2 ml of the sulfophenic reagent.

- Wait 10 minutes, and then add 15 ml of distilled water and 15 ml of ammonia, which develops the yellow colour.

- Complete in a 50 ml flask with distilled water.

- Take the reading on the photometer at λ = 440nm.

- Nitrate content of the sample is deduced from the calibration curve (Figure 2).

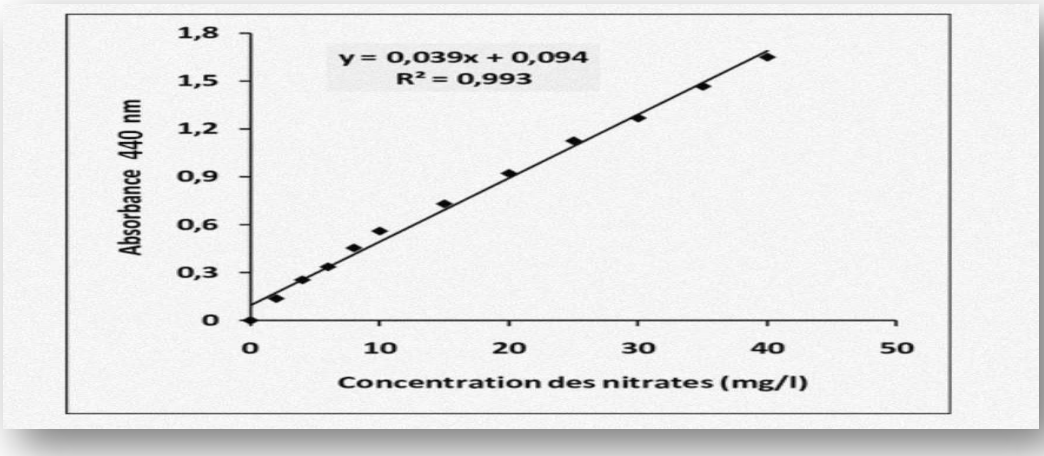


Figure 2: *Nitrate calibration curve λ = 440nm.*

I.5.2- Sodium salicylate method:

In the presence of sodium salicylate, nitrates give sodium paranitrosalicylate, coloured yellow and susceptible to spectrometric determination (Rodier et al., 2009).

Method of work:

- ✓ Take 10 ml of the sample to be analysed.
- ✓ Add 2-3 drops of 30% Na OH.
- ✓ Add 1ml of 0.5% Na salicylate. Evaporate to dryness in a water bath or in an oven at 75-88°C.
- ✓ (Do not overload or overheat for very long) allow cooling. Resume the residue with 2ml of H_2SO, then rest for 10 minutes.

- ✓ Add 15 ml of distilled water. Add 15 ml of double tartrate then pass through a spectrophotometer at 420 nm.

- ✓ the sample is deduced from the calibration curve (Figure 3).

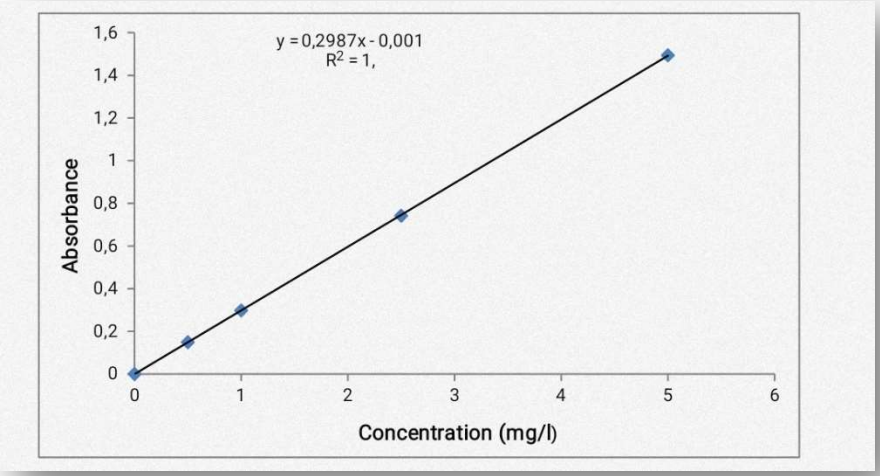


Figure 3: *Nitrate calibration curve λ = 420nm. (R. muller, 1955)*

I.6- COD determination: (chemical oxygen demand)

Chemical oxidation of reducing materials by an excess of K-CO. This oxidation takes place in an acid medium (HSO), in the presence of mercury sulphate as a catalyst (HgSO4) which can be replaced by silver sulphate. Boiling under reflux for 2 hours in a flask or tube fitted with an Allihn condenser.

Reagents:

Digestion reagent:

In 500 ml of distilled water, add in order:

- ✓ K2Cr2O7.....10.22g previously dried in an oven for 2 hours at 105°C.

- ✓ Hg2SO.....33.3g

- ✓ H2SO4.....167mL

Working method:

- ✓ Introduce 2.5 ml of the sample to be analysed into a flat-bottom COD tube
- ✓ Add 1.5 ml of potassium dichromate K2 Cr207.
- ✓ Add 3.5 ml of sulfuric acid-silver sulphate slowly and carefully, shaking the tube carefully and cooling under a stream of cold water so as to avoid any loss of volatile organic substances.
- ✓ Bring to a boil on a hot plate for 2 hours.
- ✓ Perform the reading on the spectrophotometer at λ=600 nm

Calibration Scope:

The calibration curve is then used to determine the concentration of COD in unknown samples based on their absorbance or fluorescence intensity (Apha, Awwa, Wef., 2017).

Table 3: *Data and results for the standard solution (sucrose) at 600 nm.*

Tube	**1**	**2**	**3**	**4**	**5**
Sucrose concentration (MG. L-1)	300	400	500	800	1000
Absorbance	0.0905	0.1273	0.1627	0.2471	0.3176
DOC (mg d'O_2.L^{-1}).	336.54	448.72	560.91	897.46	1121.82
F x DCO (mg d'O_2. L^{-1}).	1120.6782	1121.80	1121.82	1121.8250	1121.82

N.B: "A = 0.0003 COD", using this equation, we can reduce the value of the COD of the sludge knowing its absorbance: COD = A/0.0003".

I.7- BOD5: (Biochemical Oxygen Demand)

The DBO5 method is a classical method used to measure the amount of organic matter in water. It is based on the principle that microorganisms in water consume oxygen as they decompose organic matter. The amount of oxygen consumed over a period of five days is a measure of the amount of organic matter present in the water sample.

The classical BOD5 method includes the following steps:

- ✓ **Sample preparation:** Water samples were collected in sterilized bottles and transported quickly to the laboratory to avoid any alteration.

- ✓ **Preparation of flasks:** BOD flasks are prepared by adding nutrients and mineral salts to distilled water and adjusting the ph.

- ✓ **Inoculation of flasks:** Samples are added to the flasks and incubated at 20°C for 5 days.

- ✓ **Measurement of oxygen demand:** After incubation, the oxygen demand is measured using a Winkler titrator or a BOD analyser.

Calculation of BOD5:

BOD5 is calculated by subtracting the remaining dissolved oxygen in the flasks after 5 days of incubation from the initial amount of dissolved oxygen.

$$\mathbf{BOD5} = (\boldsymbol{L0} - \boldsymbol{L5})\boldsymbol{F} - (\boldsymbol{B0} - \boldsymbol{B5})(\boldsymbol{F} - \mathbf{1})$$

The results:

Results are interpreted based on the environmental standards in effect for water quality. It should be noted that this method is considered classical because it has been used for a long time to measure water quality and has been replaced by faster and more accurate methods such as continuous BOD5 and optical BOD5.

I.8- Ammonium Determination NH_4+:

During the mineralization of organic matter, organic nitrogen is converted into ammoniacal nitrogen, which undergoes subsequent nitrification, where it is transformed into nitrates and nitrites through the action of nitrifying bacteria such as Nitrobacter and Nitrosomonas spp.

Protocol for Ammonium Assay:

Sample Dilution:

- ✓ Add 1ml of sludge to 9ml of distilled water, and mix thoroughly.

Ammonium Assay: The ammonium assay was performed using LCK 303 kits.

- ✓ Take 0.2ml of the sample and add it to the tube.
- ✓ Homogenize the tube and wait for 15 minutes.
- ✓ Place the tube in the spectrophotometer cuvette and read the displayed value on the screen at a wavelength of 694 nm.

The concentration of ammonium is calculated using the following formula:

$$NH_{4^+}\, mg/L = \frac{Value\ read \times Dilution\ factor}{The\ test\ portion}$$

II- Microbiological characterization of collected sludge:

II.1- Methods:

II.1.1- Samples and germs sought:

Four Mud samples, from 04 different places were investigated for the research of the following bacterial flora: spores of Sulphite-reducing clostridia, Staphylococcus spp and Bacillus spp

The four sampling points are:

1- Ain Bouchakif wastewater treatment plant (pre-digested)

2- Ain Bouchakif purification station (digesteur)

3- Dairy treatment plant of Sidi Khaled Tiaret

4- Cereal waste

II.1.2- Bacteriological analyses:

The bacteriological analyses were carried out under aseptic conditions, in front of a Bunsen burner, which provides a sterile zone of 25 cm (Guiraud., 1998).

II.1.3- Sample preparation:

Preparation of stock solution: The stock suspension is represented by the Mud sample.

II.1.4- Laboratory equipment and culture media used:

The equipment and culture media used are listed in Table

Table 4: *Laboratory equipment and culture media*

Equipment	Glassware and others	Product and culture media
• Bunsen burner • Balance • Hot plate stirrer • Autoclave • Water bath • Fridge • Pasteur oven • Ovens at 37°(incubator) • Optical microscope	• Beaker of 500ml • graduated cylinder • graduated pipette 1 to 2ml • Sterile Petri dishes • test tube • Pasteur pipettes • Tubes • glass bottle • Sterile blades • wooden pegs • A platinum handles • Rack	• Sterile distilled water • Gentian Violet • Lugol • Alcohol • Fuchsine • Disinfectant • Egg yolk emulsion • Baird Parker culture medium • liver meat culture medium • Rice starch agar medium

II.2- Sample processing:

Samples are prepared in the laboratory and must not be frozen. Direct contact with samples is performed under strictly aseptic conditions, by using sterile equipment. In the aseptic area, sample containers for microbiological analysis were prepared in front of a Bunsen burner lit 15 minutes before work and on benches pre-disinfected with a bleach solution.

II.2.1- Preparation of dilutions:

The sludge in the container was briefly homogenized by stirring before analysis. In case of a liquid product (sludge), was shake the sample carefully to distribute the microorganisms as evenly as possible. Dilutions are always performed under sterile conditions. Their method of preparation is meticulous. Prepare as many tubes as possible for diluent by aseptically pipetting 9 mL of diluent (sterile distilled water) into sterile tubes. After autoclaving at 121 °C for 20 min and carefully homogenizing the tube, collect 1 ml of the starting suspension (stock suspension -sludge-) with a 1 ml pipette and add it to the first dilution 1/10 (10^{-2}) in the tube. The pipette must not strike the tube wall or the diluent. Float and cap the tubes, homogenize the contents of the tubes by shaking and inoculate the tubes (10^{-2}) using the same principle, changing the pipette each time so as not to disturb the dilution.

In our work, we performed three dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of each sample.

II.2.2- Seeding of germs:

The seeding technique involves culturing the sample on an appropriate culture medium to promote the growth of the germs. The choice of culture medium depends on the type of microorganism being targeted. For example, for aerobic bacteria, culture media that allow oxygenation are typically used, whereas for anaerobic bacteria, culture media that are devoid of oxygen are used. The culture is carried out either in a liquid medium (1 germ or a group of germs gives rise after inoculation and incubation to 1 positive culture) or in a solid medium (1 germ or a group of germs gives rise to a colony).

Once the sample is seeded, it is incubated at a specific temperature and duration to allow the growth of contaminating germs. After incubation, bacterial colonies that have grown on the culture medium can be counted. This colony count can give an approximate idea of the number of contaminating germs present in the sample. In the latter case, seeding can be done in the mass of the agar or on the surface.

In our experience, we have used:

1. A bacterial anaerobic liquid medium
2. A bacterial solid medium

Table 5: *microorganisme culture media*

Microorganism sought	**Culture media**	**Type of seeding**	**Incubation temperature and time**
Sulphite- reducing clostridium	ML	Pipetting ‘’broth’’	at 37° for 24 hours
Staphylococcus spp	BP	Surface	at 37° for 48 hours
Bacillus spp	GA	Surface	at 37° for 24 hours

II.3- The search for Sulphite-reducing clostridium spores:

Sulphite-reducing clostridium spores are of interest in sludge research because they are anaerobic bacteria that are capable of reducing sulphite to hydrogen sulphide. In wastewater treatment plants, the presence of these spores can indicate the potential for hydrogen sulphide gas production, which is a safety concern due to its toxicity and unpleasant odour. Additionally, the presence of these spores may indicate a problem with the treatment process, as they are typically associated with the putrefaction of organic matter in the absence of oxygen. Therefore, monitoring and controlling the levels of Sulphite-reducing clostridium spores in sludge can be important for ensuring the safety and efficacy of wastewater treatment processes.

II.3.1- The purpose of researching Sulphite-reducing clostridium spores in sludge:

Is to assess the potential health risks associated with the presence of these spores. Sulphite-reducing clostridium spores can produce toxins, such as botulinum toxin, that can cause serious illness or even death. Therefore, it is important to monitor the presence and concentration of these spores in sludge, which is a by-product of wastewater treatment, to ensure that it is safe for disposal or further use. This research can also help in the development of strategies to control the growth and spread of these spores in the environment.

II.3.2- The principle of researching Sulphite-reducing clostridium spores in sludge:

Involves isolating and enumerating the spores using a specific culture medium, such as reinforced clostridia medium (RCM), Tryptone Sulphite Cycloserine (T.S.C.) or liver infusion broth. Which supports the growth of a wide range of microorganisms and is commonly used for the isolation and cultivation of anaerobic bacteria, such as clostridia and Bacteroides.

The medium that was used is that of liver broth.

II.3.3- The method for cultivation of clostridia in liver meat culture medium:

The sludge sample of the dilutions (10^{-2}) is first subjected to heat treatment to selectively kill vegetative cells, but leave the spores intact. Prepare the liver meat culture medium according to the recipe and instructions provided in the reference. The heat-treated sample is then inoculated onto the medium. This can be done using a sterile loop or needle to transfer a small amount of the culture onto the surface of the medium or by adding a small amount of the culture to the medium. Incubate the inoculated medium under anaerobic conditions at the 37° and for 24h. The anaerobic environment can be created using a specialized anaerobic chamber or by using anaerobic tubes. After incubation, examine the medium for the presence of typical clostridia growth characteristics such as opaque colonies, gas production, and spore formation. (Standard Methods for the Examination of Water and Wastewater" published by the American Public Health Association (APHA)).

II.4- Identification:

Gram staining, testing for catalase and oxidase are utilized for this

II.4.1- Microscopic appearance (Gram staining):

Gram staining is a commonly used technique for the initial identification of bacterial species, including the sulfite-reducing Clostridium. The Gram stain involves the use of crystal violet, iodine, alcohol, and safranin to differentiate bacterial species based on their cell wall composition.

To perform a Gram stain of sulfite-reducing Clostridium, the following steps can be taken:

Prepare a heat-fixed bacterial smear by placing a small amount of the culture on a clean glass slide and passing it through a flame. Flood the smear with crystal violet and let it sit for one minute. Rinse the slide gently with distilled water. Flood the slide with Gram's iodine and let it sit for one minute. Rinse the slide gently with distilled water. Decolorize the slide with alcohol until no more color is coming off, and then rinse with distilled water. Counterstain the slide with safranin and let it sit for one minute. Rinse the slide gently with distilled water and blot dry with a paper towel. Observe the slide under a microscope at 1000x magnification.

Sulfite-reducing Clostridium species typically appear as Gram-positive, rod-shaped cells under the microscope. The cells will stain purple due to the retention of the crystal violet-iodine complex in their thick peptidoglycan cell walls.

II.4.2- Biochemical identification:

In addition to morphological characteristics, identification is also based on some biochemical characteristics. (Guiraud., 1998. Freney et al., 2007).

- Gender identification:

- Catalase/ oxidase test:

- **Catalase** is an enzyme that breaks down hydrogen peroxide into water and oxygen gas. Many aerobic and facultative anaerobic bacteria produce catalase as a defense mechanism against hydrogen peroxide, which can be toxic to the bacterial cell.

To perform the catalase test, a small amount of bacterial culture is placed onto a clean glass slide or into a test tube. A small amount of hydrogen peroxide is then added to the culture. If the bacteria produce catalase, bubbles of oxygen gas will be produced as the hydrogen peroxide is broken down. The presence of bubbles indicates a positive catalase test, while the absence of bubbles indicates a negative test.

- **The oxidase test** used to determine the presence of cytochrome c oxidase in bacterial cultures. Cytochrome c oxidase is a respiratory enzyme that is involved in the electron transport chain of many aerobic and facultative anaerobic bacteria. The enzyme catalyzes the transfer of electrons from cytochrome c to oxygen, producing water as a by-product.

To perform the oxidase test, a small amount of bacterial culture is placed onto an oxidase tablet (tetramethyl-p-phenylenediamine hydrochloride). If the bacteria produce cytochrome c oxidase, the oxidase tablet will be oxidized to a purple color in the presence of oxygen, indicating a positive test. If the culture does not contain cytochrome c oxidase, there will be no color change, indicating a negative test.

NB: '' We did not conduct tests on Clostridium bacteria due to their high risk and dangerous nature."

II.5- The search for staphylococcus spp*:*

It was performed on Baird-Parker medium

Staphylococci can be present in sewage sludge or wastewater treatment plant sludge. They can be introduced into the sludge through fecal contamination from humans or animals, and their presence can be an indicator of fecal pollution. Staphylococci are also commonly found on the skin and mucous membranes of humans and animals, and can be introduced into sewage through human and animal waste. However, the presence of Staphylococci in sludge does not necessarily mean that the sludge is unsafe or contaminated, as many species of Staphylococci are harmless and some are even beneficial. The safety and quality of sludge is typically assessed through a combination of microbiological, chemical, and physical tests to ensure that it meets regulatory requirements before it is used for land application or other purposes.

II.5.1- The principle of researching staphylococcus:

To detect Staphylococcus, it is necessary to isolate it on a selective solid medium. The preferred medium in food microbiology is BAIRD PARKER agar. Other media, such as Chapman agar, which has a high Na Cl concentration (7.5%), can suppress the growth of many bacteria other than Micrococcus and Staphylococcus. (Guiraud., 1998)

In this particular case, the Baird Parker medium was used for the isolation of Staphylococcus.

II.5.2- Medium preparation:

To prepare Baird-Parker culture medium with egg yolk emulsion, melt 180ml of the base medium (BP) and add 5 ml of the potassium tellurite solution. Finally, add 10 ml of the egg yolk emulsion. Mix gently by agitation, then distribute into Petri dishes. The medium should be used within 24 hours of preparation, after the surface has dried well (solidified on a horizontal surface). Using a sterile pipette, deposit 0.1 ml of the sample and dilutions ($10^{-1}/10^{-3}$) onto the surface of the culture medium. Quickly spread the sample using a spreader (prepared with a Pasteur pipette) without touching the walls of the plate. Use a sterile spreader for each plate. Incubate the plates at 37°C for 24-48 hours (Larpent., 1997).

Staphylococcus aureus produces black colonies (due to the reduction of tellurite to tellurium), with a clear halo due to the proteolysis of egg yolk proteins, and possibly an opaque white rim (precipitation of fatty acids). Their size is 0.5 to 2mm (1 to 15 mm in 24h, 1.5 to 25 mm in 48h) with a shiny appearance. Clones of non-pathogenic staphylococci are often inhibited or irregularly developed (Guiraud., 1998).

II.6- The search for Bacillus spp:

Bacillus spp. are a group of gram-positive, spore-forming bacteria commonly found in soil, water, and organic matter. In the context of sludge, Bacillus spp. may play a role in the degradation of organic matter, which is an important step in the treatment of wastewater.

II.6.1- The purpose of researching bacillus spp in sludge:

Researching Bacillus spp. in sludge may be done for several reasons. Firstly, it can provide information on the microbial ecology of the sludge and the interactions between Bacillus spp and other microorganisms in the environment. Additionally, understanding the role of Bacillus spp. in the degradation of organic matter can inform strategies for optimizing the treatment of wastewater and sludge. Furthermore, Bacillus spp. are known to produce a range of enzymes, such as proteases, lipases, and cellulases, which can be utilized in various industrial applications. Researching Bacillus spp. in sludge may therefore have potential for biotechnological applications, such as the production of biofuels or biodegradable plastics.

II.6.2- *The principle of researching bacillus spp*:

There are several different types of media that can be used to culture Bacillus species, depending on the specific strain and purpose of the culture. Some common examples of media used to culture Bacillus include Nutrient Agar, Luria Bertani (LB) Agar, and Blood Agar, rice or potato starch agar and Cereus agar

Rice starch agar was used to isolate Bacillus bacteria in this specific case.

II.6.3- The method for cultivation of bacillus in starch medium:

To prepare the rice starch culture medium, first, melt 5g of rice and 6.66g of the GN base medium mix it with 500ml of distilled water. Mix the mixture gently by agitation and then distribute it into sterile Petri dishes. Once the surface has dried and solidified on a horizontal surface, deposit 0.1 ml of the sample and dilutions ($10^{-1}/10^{-3}$) onto the surface of the culture medium using a sterile pipette. Quickly spread the sample using a spreader that has been prepared with a Pasteur pipette. Be careful not to touch the walls of the plate. Each plate should have its own sterile spreader. Incubate the plates at 37°C for 24-48 hours (Larpent., 1997).

II.7 - *Microbiological Identification*:

II.7.1- *Macroscopic aspect*:

Consists of studying the growth, shape, appearance, contour, and colour of the colonies. The colonies of Bacillus appear as massed small creamy white colonies with a diameter of 0.5 mm.

II.7.2- *Microscopic aspect*:

Bacillus is a Gram-negative, non-sporulating, Oxidase-positive, catalase-positive, bacillus capable of multiplying in 24 hours at a temperature of 35-37°C. (ISO)

II.8- The degradation tests of the different substrates:

The goal of the degradation tests of the different substrates for the bacteria is to evaluate the ability of microorganisms to break down or decompose different types of organic matter present in the substrates. This information is important for understanding the performance of biological treatment systems, such as wastewater treatment plants or biogas production facilities, and for optimizing their operation. By measuring the rate and extent of substrate degradation under controlled laboratory conditions, researchers can determine the effectiveness of different treatment strategies and identify factors that may limit the activity of the microorganisms.

II.8.1- Starch hydrolysis:

Starch is a complex carbohydrate composed of units of D glucose. By the action of an amylase, which is saccharides, starch is hydrolysed into disaccharide sugar.

Principle:

To demonstrate the degradation of starch by bacteria.

Inoculation of starch agar:

- ✓ Using a sterilized pipette, pick up a pure bacterial colony and streak it on the surface of the agar.
- ✓ Incubate at 37°C for 24-48 hours.

Possible readings:

Cover the surface of the plates with Lugol's solution and observe the colour change. (Delarass., 2014)

- ➢ **Yellow coloration around the colonies:** starch degradation by bacteria, positive test
- ➢ **Dark blue coloration around the colonies:** presence of starch, negative test.

II.8.2- Casein degradation:

Principle: The demonstration of the proteolytic properties of bacteria.

Skim milk agar constitutes a substrate rich in proteins, mainly caseins (80% of milk proteins), as well as lactalbumins and lactoglobulins in soluble nitrogen compounds. A strain is said to be proteolytic if it presents a lytic zone diameter between 5 and 15mm (Vuillemard., 1986).

Inoculation:

Using a pipette, pick up a pure bacterial colony and inoculate the surface of the skim milk agar in separate streaks.

Possible readings:

- **Formation of a clear halo around the colonies:** casein degradation, positive caseinolytic test.

- **Absence of a clear halo around the colonies:** no degradation, negative caseinolytic test (Delarass., 2014).

II.8.3- Lecithin hydrolysis test:

Principle: The detection of the lipolytic properties of bacteria.

This test is performed on a medium made of egg yolk emulsion and allows the detection of lecithin's, lipase, and lipoproteins. Lecithinases are responsible for the degradation of lecithin or Phosphatidylcholine, a lipid from the class of phosphoglycerates.

Inoculation:

Using a sterilized pipette, take a pure bacterial colony and streak, it on the surface of Nutrient agar containing egg yolk emulsion.

Possible readings:

- **White-yellowish opaque halo with a sharp border underneath or around the colony**: Positive lecithin's test

- **No halo:** Negative lecithin's test (Delarass., 2014)

II- *Biogas production*:

The anaerobic digestion process can be optimized through several parameters, including temperature, and pH. In this work, we have tried to investigate the effects of using different substrates on biogas production yield.

III.1- Assembly of a batch-mode digester:

In order to augment the production of methane from the collected sludge for the generation of biogas, a set of five batch digesters was established. The collected sludge was sequentially added to 2000 ml Erlenmeyer flasks in the following arrangement: predigesting, digestion, pre-digested with digester, a combination of 50% pre-digested and 50% dairy sludge, and finally a combination of 75% pre-digested with 25% grain waste. The Erlenmeyer flasks were filled up to (2/3) of their capacity and connected to 2000 ml urine bags for the collection of the biogas generated.

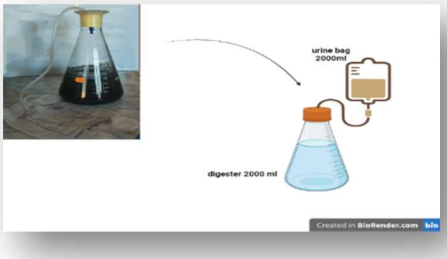


Figure 4: *Diagram and photo of the anaerobic digestion system (Original photo+ assembly)*

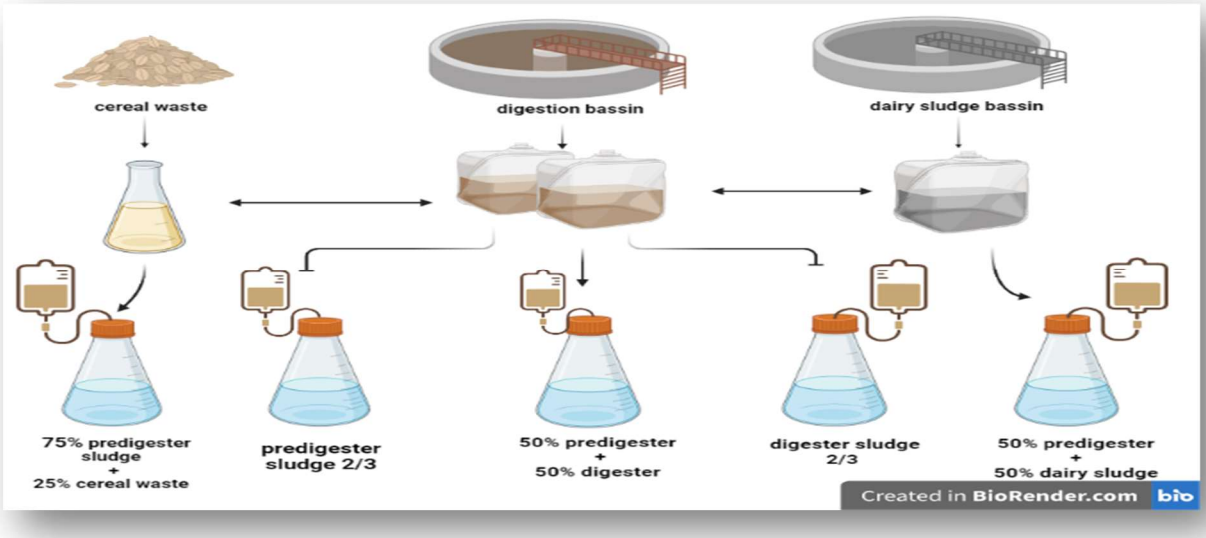


Figure 5: *Diagram of the setup for biogas production using different substrates (original assembly)*

III.2- Identification of produced biogas:

The identification of compounds in gas mixtures was initially performed using the EX-TEC® PM4 SEWERIN methane detector from SONALGAZ services of Tlemcen, should also be noted that the presence of biomethane could be tested using gas chromatography. Since gas chromatography was not available, we can choose the flame test because biomethane was the only flammable compound in the gas mixture recovered from the urine bags and the gas detector.

III.2.1- *Biogas test*:

III.2.1.1- Flame Test:

Using a syringe, extract a volume of biogas and press it around a flame.

Possible readings of the results:

- **The flame stays on the syringe:** positive test, indicating the presence of biomethane in the biogas.
- **The flame does not stay on the syringe:** negative test, indicating the absence of biomethane in the biogas.

III.2.1.2- Identification of biomethane using the methane detector EX-TEC®PM4 SEWERIN:

Extract a volume of biogas using a syringe. Release the biogas from the syringe while simultaneously drawing it in through the detector's pump.

Possible result readings:

Presence of biomethane: Display of the biomethane concentration value on the graph and an audible beep.

Absence of biomethane: The graph does not display any value, and there is no audible beep.

IV- Statistical analyses:

The manipulations were carried out in triplets. Values were expressed as means ± standard deviation.

Chapter 02

Results and discussion

I- Results and Discussions:

In this section, we will present and analyse the outcomes obtained from diverse assessments carried out to evaluate the physicochemical and microbiological attributes of the sludge collected from the wastewater treatment plant (WWTP). Moreover, we will discuss the effects of altering substrates on biogas production, and the feasibility of bioethanol production.

I.1- Physicochemical Analysis:

The results of the physiochemical analysis of the various substrates used are presented in Table N=°6.

Table 6: summary of physiochemical parameters results

Parameters		Substrates			
		Pre-digested	**Digester**	**Clarifier**	**Dairy sludge**
PH		7.90	7.96	8.13	8
CE		4.84 ms/cm	5.77 ms/cm	6.94 ms/cm	6.89 ms/cm
SM		0.5 g/l	0.6 g/l	0.2 g/l	-
NO_2^-		10.2 mg/l	12 mg/l	9.98 mg/l	-
NO_3^-	1st	0.9 mg/l	0.5 mg/l	0.4 mg/l	-
	2nd	0.62 mg/l	0.3 mg/l	0.2 mg/l	-
NH_4^-		-	1.56mg/l	-	-
BOD5		2540 mg/l	-	-	-
COD		1376,66 mg/l	1223 mg/l	1250 mg/l	-

I.1.1- pH determination:

The optimal pH range for anaerobic digestion depends on the specific population of bacteria involved. Therefore, it is a useful parameter for differentiating between the acidogenic and methanogenic phases. The pH value of the sludge before anaerobic digestion, in the digester, clarifier, and dairy sludge was found to be between 7.9 and 8. The results indicate that our samples are in the methanogenic phase, and the average value obtained is in line with the standard range of 6.5 to 8.5.

Generally, the pH optimum for each bacterial population varies. Acid-forming bacteria typically have an optimum pH range of 5.5-6.0, while acetogenic bacteria prefer a pH range close to neutrality. Methanogenic bacteria have an optimum pH range of 6.5-8.0, but can still function in slightly acidic or alkaline environments (Siegert and Banks., 2005).

I.1.2- CE Determination:

Electric conductivity is an important parameter in the assessment of sludge quality, as it can provide insight into the level of salts and other dissolved ions present in the sample. Higher levels of electric conductivity in sludge may indicate a higher level of dissolved salts, which can have implications for sludge management and disposal. (EPA., 2003).

In the case of the sludge samples analysed in this study, the measured electrical conductivity ranged from 4.8ms/cm to 6.9ms/cm. These values fall within the acceptable range for sludge intended for energy recovery according to Algerian standards, which set a maximum limit of 10 Ms/cm.

I.1.3- SM Determination:

Our study aimed to determine the suspended matter content in wastewater sludge, specifically focusing on its potential for biogas capture. The results obtained showed a suspended matter concentration ranging from 0.6 to 0.2 g/l in the sludge samples analysed. To assess the significance of these findings, we compared them to the standards outlined in the EN ISO 17353 (Sep 2004) norm. According to EN ISO 17353, the maximum permissible concentration of suspended solids in sludge depends on the designated sludge treatment and disposal methods. For instance, if the sludge is intended for agricultural use, the recommended limit is typically around 30 g/L. However, for sludge destined for landfill disposal, the recommended limit is lower, usually around 10 g/L. These results suggest that the sludge samples may be a viable option for energy recovery purposes.

I.1.4- Nitrite Determination:

The presence of nitrite in sewage sludge can have detrimental effects on both the environment and human health if not appropriately managed. One of the risks associated with high levels of nitrite is the inhibition of microorganisms responsible for producing biogas in anaerobic digestion processes, leading to decreased performance. Additionally, the accumulation of toxic intermediates can further decrease the quality of the biogas produced and impede the performance of anaerobic digestion processes.

Our recent study aimed to evaluate the nitrite levels in sewage sludge samples intended for energy recovery purposes. The results showed that the nitrite concentrations ranged from (**9.98 mg** to **12 mg** /L), which falls within the typical range of nitrite levels found in municipal sewage sludge as reported by the US Environmental Protection Agency (EPA). The typical range of nitrite levels in municipal sewage sludge is between (**1** and **50** g/L).

I.1.5- Nitrate determination:

Numerous studies have demonstrated the significant impact of nitrates on the performance of anaerobic digestion processes when present in sewage sludge. For instance, in a study conducted by Luo (2016), it was revealed that elevated nitrate levels in sewage sludge resulted in a substantial reduction in biogas production and methane content. Moreover, the accumulation of detrimental by-products such as nitric oxide and nitrous oxide further compromised the stability and quality of the generated biogas.

Our recent investigation focused on the nitrate levels within sewage sludge samples designated for energy recovery. Using the sulfophenic method, we determined that the nitrate concentrations ranged from **(0.9 mg_0.4 mg/L)**. To enhance the reliability and scope of our findings, we also employed the Sodium Salicylate method. Remarkably, the results obtained through this alternative approach fell within a similar range of **(0.6 mg_0.2 mg/L)**. These findings align with the established range of nitrate concentrations typically found in municipal sewage sludge, as outlined by the US Environmental Protection Agency (EPA). The EPA reports that nitrate levels in municipal sewage sludge commonly fall between **0.01 g/L** and **5 g/L**

I.1.6- COD determination:

The results of the analysis of chemical oxygen demand (COD) in the sludge exceeded the standard limits adopted in Algeria, with values ranging from **1376.66** to **1223.33** mg/L. The standard limit for wastewater from industrial plants in Algeria is 100 mg/L, while it is 60 mg/L for wastewater from municipal plants. National and international standards are followed for monitoring and analysing water quality. COD levels in treated water should not exceed **20** mg/L according to the WHO recommendations. The legal limits for COD levels in treated water vary across countries, with the European Union setting limits ranging from **25** to **125** mg/L based on the intended use of the water. Elevated COD concentrations in treated water indicate the presence of organic substances such as fats, oils, proteins, and carbohydrates that can lead to water pollution and ecological harm. For example, excessive growth of algae and marine plants can occur because of this pollution, which can have a negative impact on aquatic organisms.

I.1.7- BOD5 determination:

The result of **2540mg/l** of BOD5 in pre-digestion sludge samples intended for energy recovery purposes indicates a high organic content in the sludge. This high BOD5 value may be due to the presence of easily biodegradable organic matter in the sludge, such as fats, oils, and grease from food waste, which are commonly found in municipal wastewater treatment plants. High BOD5 levels can negatively affect the performance of anaerobic digestion processes, as it can lead to an accumulation of volatile fatty acids and a decrease in pH, which can inhibit the growth of methanogenic bacteria responsible for producing biogas.

According to the US Environmental Protection Agency, the typical range of BOD5 levels in municipal wastewater treatment plant sludge is between **500** and **2000** mg/l. Therefore, the BOD5 value of **2540**mg/l found in the pre-digestion sludge sample in this study is higher than the typical range. This suggests that the sludge may require additional treatment or pre-treatment to reduce the organic load and improve its suitability for anaerobic digestion for energy recovery purposes.

I.1.8- Ammonium determination:

The process of nitrification, facilitated by nitrifying bacteria, explains how ammonium is utilized. These bacteria utilize mineral carbon (HCO, CO) obtained from the oxidation of acetic acid and other generated acids as a source of carbon. Furthermore, inorganic molecules (NH) act as an energy source for the synthesis of nitrates and nitrites. In our study, the amount of 1.56 mg/l has been found. Approximatively the same result was found by Fodil, (2021) with the sludge of digestor (1.71 mg/l).

II. *Microbiological characterization*:

This study is based on the analysis of four samples of sludge from different places, including two samples (pre-digester and digester) from the Ain Bouchakif wastewater treatment plant, a sample from the digestion pool of the Sidi Khaled dairy, and a sample of cereal waste prepared in the laboratory. The targeted microorganisms were Clostridium sulphite-reducers, Staphylococcus spp, and Bacillus spp.

II.1- Research and identification of different bacterial species:

The findings of the bacterial flora investigation are outlined below:

- ✓ The colonies that developed on rice starch medium were transferred to a fresh medium (Figure 17). The Gram staining (Figure16), microscopic analysis oxidase test and catalase test aided in identifying the Bacillus spp genus (Table 7).

- ✓ The colonies that grew on BP medium were used to differentiate the Staphylococcus spp genus. The Gram staining (Figure 18), microscopic analysis oxidase test and catalase test (Table 7).

- ✓ The black-coloured colonies on the VF medium suggested a high presence of Clostridium spores (Figure 19) since all three tubes were overloaded (completely black).

Table 7: *Results of bacterial species identification.*

Bacterial species.	**Presence/ Absence.**			**Colony Morphology**	**Gram staining**	**Oxidase**	**Catalase**
Clostridium sulphite-reducers	WWTP sludge	Dairy sludge	Grain Waste	Black Colonies	/	/	/
	+	+	-				
Staphylococcus spp	+			Small, Black and opaque colonies	Gram +	-	+
Bacillus spp	+			Large, white, and opaque colonies.	Gram -	-	+

- The results obtained in our study exhibit a degree of resemblance to the findings reported by Fodil, (2021) in the Tiaret area. Staphylococci and various types of bacilli were detected in both studies, aligning with our collected samples. However, there exists a minor disparity in the specific bacterial strains identified. Consequently, it can be confidently concluded that the sample contains an abundance of diverse bacteria belonging to the same species.

N.B: "While performing our investigation, we came across a bacterium that had similar morphology and growth characteristics to Staphylococcus aureus on the same culture medium (figure 20). However, after conducting multiple classification procedures, such as the citrate test, Mannitol test, TSI test, Schubert + the Bell test (figures 21), Clotting blood test (figure 22), and the strip API ® Staph test (figure 23), we determined that it belongs to the species Staphylococcus haemolyticus. It exhibited a positive Gram stain, positive catalase test, and negative oxidase test. Additionally, the hydrolysis of various substrates test produced negative results."

II.2- Hydrolysis of different substrates:

The tests for degradation of various substrates revealed the presence of enzymatic activity in Bacillus spp species towards carbohydrates, lipids, and proteins, which is responsible for breaking down macromolecular substrates during hydrolysis. Bacillus spp species showed positive results for Strach hydrolysis, indicated by yellow staining around the colonies (Figure 4). The species also exhibited significant proteolytic activity, evidenced by a large lysis zone of up to (2 cm). Furthermore, a white halo with a diameter ranging from (1 to 2 mm) indicated the presence of lipolytic activity in Bacillus spp species around and at the boundary of the colonies. The reduced proteolytic and lipolytic activity observed is not solely dependent on the species' performance, but it also relies on the carbon source selection, pH of the medium, temperature, and substrate concentration. Optimizing these conditions for each species allows for achieving optimal enzymatic activity (Cammack et al., 2013).

Our results are comparable to those reported by Fodil, (2021) in the Tiaret region for starch hydrolysis and lipolytic activity, but they exceed theirs for proteolytic activity. These findings assist us in providing an appropriate environment for these bacteria, ultimately leading to an increase in biogas production.

Figure 6: Strach hydrolysis test (Original photo)

Figure 7 : Degradation test (Original photo)

III. *Biogas production*:

Optimal selection and preparation of feedstock play a crucial role in maximizing biogas production. High_ energy organic materials such as animal manure, crop residues, food waste, and energy crops with high biomass content can be used as feedstock proper shredding or chopping of feedstock can increase the surface area, facilitating faster and more efficient digestion.

In our study the monitoring of biogas production involved measuring the volume of biogas generated throughout the digestion period. The analysis presented in (Figure 8) illustrated a gradual rise in biogas volume, particularly noticeable from day 03 for the sludge samples. The pre-digested sludge displayed a 30% increase, while Mixture A exhibited a 15% increase. Additionally, the sample containing Mixture B experienced a 5% increase in biogas production, indicating the initiation of the methanogens phase.

By the final day of digestion (day 7), there was a remarkable surge in biogas production (refer to figure 9), resulting in the following volumes:

Pre-digested sample: 2000 ml (considered as 100%).
Mixture A: 1600 ml (80%).
Mixture B: 1200 ml (60%).
Conversely, the samples containing the digester alone or the Mixture C displayed a significantly low biogas volume of only 1% on day 7, attributable to the limited input of organic matter.

Siboukeur's study (2011) corroborated these findings, demonstrating that biogas production continued until day 37 but gradually declined thereafter. The decline progressively weakened until the study's culmination on the final day (day 60) due to organic matter depletion and an imbalance in the medium.

The sludge from primary settling, called "primary sludge", is more heavily loaded with organic matter than the sludge from clarification, called "biological sludge". is the production of biogas by digestion of mixed sludge (primary sludge + biological sludge) is optimized compared to that obtained solely by digestion of biological sludge (Zhang, 2011).

Also, it is possible to optimize the production of biogas by degrading the sewage sludge before digestion by hydrolysis (in particular thermal and ultrasound). The degraded sewage sludge releases a greater quantity of organic matter and thus allows greater biogas production.

Furthermore, co-digestion involves blending different types of feedstocks with complementary characteristics to enhance biogas production. Combining substrate with varying carbon-to-nitrogen (C/N) ratios can help balance the nutrient content and improve the overall digestion process. For example, mixing manure with food waste or agricultural residues can enhance biogas yield (El Mashad and Zhang, 2010).

Figure 8: Biogas volume day 03 (Original photo)

Figure 9: Biogas volume day 07 (Original photo)

III.1- Identification of biogas:

III.1.1- Flame test:

In our specific case, the failure of ignition can be attributed to a significant imbalance in the ratio of carbon dioxide to methane. This disparity is considerably higher than what has been observed in previous studies (figure 7). As a result, the conditions necessary for combustion to take place are hindered and combustion does not occur.

Figure 10: *flame test by (FODIL.,2021)*

III.1.2- Gas detector results:

The Swerin gas measurement device, provided the following precise results for our gas analysis:

Table 8: *Biogas components results*

Biogas components	The percentage
CH_4	13%
CO_2	75%
N_2	02%
O_2	01%
Others	< 01%

Conclusion

Conclusion:

Biogas is a renewable energy that has the advantage of being storable and not dependent on climatology. It is produced through the anaerobic digestion of organic matter, such as animal manure, agricultural waste, sewage, and food waste. Biogas is primarily composed of methane (CH_4) and carbon dioxide (CO_2), and it can be used as a fuel for heating, electricity generation, or as a transportation fuel. The purpose of this research is to obtain a large yield of biogas, and this is through the use of different substrates to be involved in the process of anaerobic digestion, resulting in the emission of methane, which is the basic for the composition of biogas.

The obtained results can be resumed as follow:

1) **Physio-chemical analysis:** Measuring various parameters at the beginning, during, and at the end of the experiment allowed for the temporal delineation of different stages of methanization and monitoring of organic matter consumption during this biological process. Consequently, it was observed that, based on the availability of organic matter at the end of anaerobic digestion and the continuous production of biogas, the experiment could be extended beyond 37 days if other parameters allowed for it. These findings, especially through the notable increase in BOD_5 followed by a decrease in COD, indicated self-regulation of the digester. Based on these results, we can conclude that this sludge is suitable for utilization in energy sources.
2) **Microbiological characterization:** Microbiological analysis revealed the presence of a bacterial flora consisting of Staphylococcus spp, Bacillus spp, and spores of Sulphite-reducing clostridium species during the hydrolysis stage. Tests assessing the degradation of different carbohydrate substrates (starch), lipid substrates (lecithin), and protein substrates (casein) by these bacterial species yielded satisfactory results, particularly for Bacillus. However, the remaining bacterial species showed a decline in activity, indicating a deterioration in their enzymatic functions. Nevertheless, their role in substrate degradation during the hydrolysis stage cannot be completely disregarded.

3) **The biogas yield** was studied using different substrates and the biogas yield was (pre-digestion) with 100%, (Mixture A) by 80%, (Mixture B) by 60%, (Mixture C) with 10% and (digestion) by 2%.

4) **The biogas identification:** The test conducted to assess biogas production was deemed successful, despite the limited quantity obtained. The presence of combustible biomethane at a concentration of 13%, as confirmed by the gas limiter, was detected along with several other gases, notably carbon dioxide constituting 75% of the gas composition. This significant proportion of carbon dioxide surpassed levels observed in previous studies, resulting in the failure of methane ignition. Nonetheless, these findings remain highly satisfactory, serving as a testament to the efficient functionality of the digestion process, as the ultimate objective is the production of biogas, with methane being the primary target compound.

It is important to note that the implementation of the method adopted in this work or the other methods used to enhance the efficiency of biogas production, should be tailored to specific local conditions, available resources, and the desired scale of biogas production. Regular monitoring, process optimization, and continuous improvement are key factors in maximizing biogas production efficiency.

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Annexes

Annexe 01:

I- Ain Bouchakif wastewater treatment plant:

The plant has a 38,000 m3/day treatment capacity. Where Wastewater is conveyed by gravity and treated using the activated sludge process in an intensive small surface technique outside. The plant relies on a combined sewer system that collects domestic and industrial wastewater as well as rainwater in the same pipes, known as the "all-to-sewer" system.

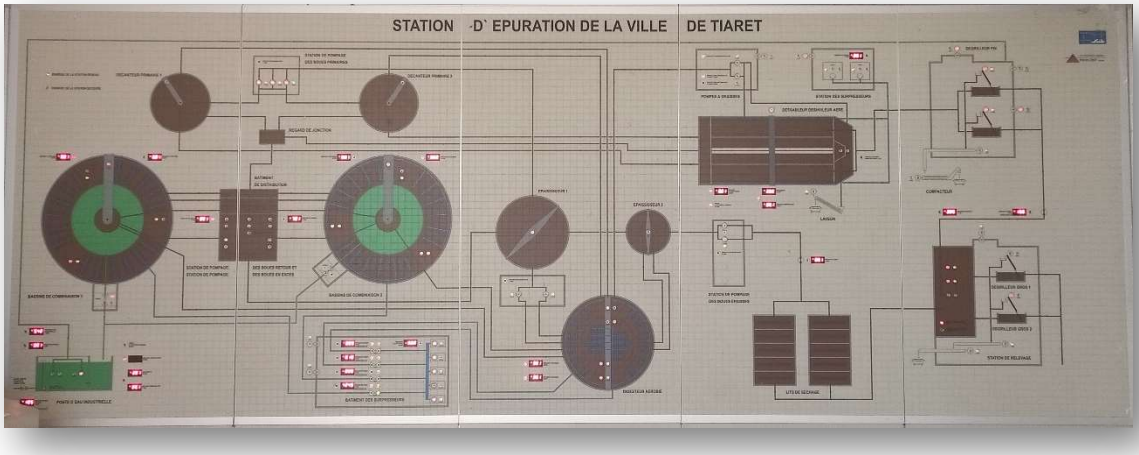

Figure 11: *A schematic diagram of the process of wastewater treatment at the Ain Bouchekif plant (Original photo)*

I.1- Treatment Stages:

The treatment process at the plant involves four phases:

I.1.1- Pre-treatment phase:

Comprises the following four steps:

I.1.1.1- Screening:

The wastewater enters the station and passes through screens with a diameter of 10cm and a depth of 5 meters to remove all solid debris and minimize the load on the lifting station.

I.1.1.2- Coarse screening:

This step eliminates large waste. The wastewater passes through screens with a diameter of 8 cm. The waste is then collected on a conveyor belt and deposited into a bin. Afterwards, it is transported to a landfill or incinerated.

Figure 12: *coarse screening*
(Original photo)

I.1.1.3- Lifting system:

There are three pumps, two of which work simultaneously and the third is a backup. The pumps transport the water to the fine screen. Moreover, after this step they will measure the water's BOD with a nanometre.

I.1.1.4- Fine screening:

This step consists of screens that are inclined and finer than the previous ones. The water is pumped through screens with a diameter of 1 cm, which eliminates any waste greater than 1 cm. A conveyor belt to a compactor, where the liquid phase is separated and recovered, then transports the waste.

I.1.1.5- Screening:

The engineers at this station have built a sieve with a diameter of 1 to 2 mm. The sieve is immersed and rotates, keeping all the sand and waste that is larger than 1 mm. After all the collected waste is transferred to the washer.

Figure 13: *the sieve*
(Original photo)

I.1.2- Oil and Sand Separator:

In this step, pumps are installed, including grease pumps and aeration pumps that inject air into the bottom of the tanks. There are four compartments:

The first two compartments are for sand removal. In addition, the remaining two compartments are for oil removal, using pumps that inject air to bubble the water and create movement in the tanks. As we all know, oils and fats have a lower density than water, so they will naturally float to the surface, where a scraper scans all the oils and fats. Meanwhile, the sand and gravel settle at the bottom of the tank. Then, we pump all the sand and gravel to the washer to minimize the volume, while the fats are directed to the digester for treatment.

Figure 14: *Oil and sand separator*
(Original photo)

I.1.3- Primary treatment phase:

In this stage, two settling basins are used, which are combination basins. According to the debit, for example, when the flow rate is very high in winter, we are obliged to use both basins. On the other hand, when one of the clarifiers is out of service, we use the second one. In this clarifier, a clarifier rotates to remove suspended solids. There is a large basin and a small basin, with the difference being in the depth, as the height of the small basin is higher than that of the large basin.

I.2- Secondary treatment phase:

In this stage, chemicals are added, but this is not done in this station.

I.3- Third treatment phase: "Biological treatment"

In this phase, the station relies on the activated sludge process. All conditions are met for the normal development of microorganisms, including dissolved oxygen, air injection into the basins at a rate of 0.2-2 mg/L, the presence of organic matter, nutrients, a pH of 6.5-9, and a temperature between 18°C and 35°C for the bacteria to grow and fix pollutants. The bacteria then develop in the form of biological flocs that can be separated from the treated water by settling.

I.4- Sludge treatment:

The sludge and remaining water are recovered and recycled, then transferred to the lifting station or to the settling tank. It is preferred to transfer them to the lifting station to increase the contact of bacteria with organic matter, thus increasing elimination efficiency. The sludge is initially composed of water (99%), fresh, highly fermentable organic matter, and dissolved or insoluble mineral matter. Two methods are used for this treatment phase: thickening and dehydration.

The extracted sludge is sent to the thickener to reduce its volume. In this thickener, another clarifier is used to increase the elimination of the aqueous phase. Then, the sludge is sent to the digester to stabilize it either biologically or chemically.

Biologically: by the presence or absence of oxygen (anaerobic digestion).

After the thickening phase, which eliminates 60 to 85% of the water, and the stabilization phase, the sludge treatment is completed by dehydration to eliminate the maximum residual water. Two types of processes are generally used: mechanical and thermal processes. During the drying phase, the sludge behaves in a plastic and sticky manner for dry matter contents of about 50%, which requires adjustments to techniques and equipment.

Note:

- Polymer resin is mixed with the sludge to coagulate and facilitate the separation operation.
- The sludge is transferred to the perforated belt filter and then to the press filter for filtration.

Figure 15: *drying beds (Original photo)*

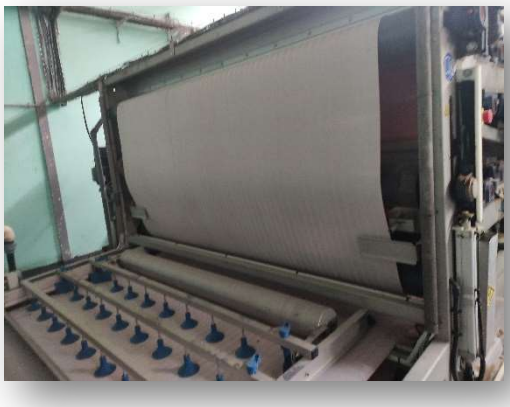

Figure 16: *the perforated belt filter and the press filter for (Original photo)*

Annexe 02:

II- The Main bacterial species responsible for substrate degradation:

the main bacterial species responsible for the hydrolysis of substrates this table is based on the work of (M.A. El-Mashad and Z. Zhang., 2010).

Table 9: *the main bacterial species responsible for the hydrolysis of substrates*

Bacterial space	Type de bactérie	Substrats cibles
Espace extracellulaire	Bactéries extracellulaires	Protéines, glucides, lipides
Espace péritrophique	Bactéries fixées sur les particules	Cellulose, hémicellulose, pectine
Espace intracellulaire	Bactéries intracellulaires	Amidon, sucres, protéines
Espace des protéases	Bactéries protéolytiques	Protéines
Espace des amylases	Bactéries amylolytiques	Amidon
Espace des cellulases	Bactéries cellulolytiques	Cellulose

Table 10: *Main bacterial species responsible for the degradation of substrates in the hydrolysis stage (Garcia, from Moletta., 2011).*

Substrate	Mesophilic Bacteria	Thermophilic Bacteria
Cellulose	**Acetivibrio cellulolyticus, Acetivibrio cellulosolvens, Bacteroides succinogenes, Bacteroides cellulosolvens, Butyrivibrio fibrisolvens, Cillobacterium cellulosolvens, Ruminococcus albus, Ruminococcus flavifaciens, Clostridium aldrichii, Clostridium cellulobioparum, Clostridium cellulolyticum, Clostridium chartatabidum, Clostridium lochheadii, Clostridium longisporum, Clostridium paradoxum, Clostridium populeti,**	**Anaerocellum thermophilum, Clostridium stercorarium, Clostridium thermocopriae, Clostridium cellulosi, Clostridium thermocellum, Clostridium thermopapyrolyticum**

	Clostridium celerecerscens, Clostridium cellulofermentens, Clostridium cellulovorans, Clostridium josui, Clostridium lerntocellum, Clostridium papyrosolvens, Clostridium polysccharolyticum, Clostridium termitidis	
Hemicellulose	**Bacteroides ruminicola**	**Clostridium thermobutyricum, Clostridium thermocopriae, Clostridium thermocellum**
Pectin	**Clostridium butyricum, Clostridium multifermentans, Clostridium felsineum, Lachnospira multiparus**	**Acetomicrobium faecalis, Clostridium thermohydrosulfuricum, Clostridium thermosccharolyticum, Clostridium thermocellum, Clostridium thermolacticum, Clostridium thermosulfurogenes**
	Bacillus spp, Clostridium	**Acetomicrobium flavidum, Clostridium**

Starch	**butyricum, Lactobacillus spp, Pseudomonas spp, Bacteroides spp, Clostridium spp, Micrococcus spp, Succinomonas amylolytica**	**fervidus, Clostridium stercorarium, Clostridium thermocopriae, Clostridium thermolacticum, Clostridium thermobutyricum, Clostridium thermohydrosulfuricum, Clostridium thermopalmarium, Clostridium thermosccharolyticum**
Lipids	**Anaerovibrio lipolytica, Syntrophomonas spp, Bacillus spp**	**-**

Table 11: *Main bacterial species responsible for the degradation of substrates during the acetogenesis step (Garcia, from Moletta., 2011).*

Metabolites	Bacterial Species
acetate	Acetivibrio spp, Acetoanaerobium noterae, Acetofilamentum rigidum, Acidominobacter hydrogenoformans, Clostridium acidiurici, Clostridium formicoaceticum, Clostridium magnum, Eubacterium limosum, Peptococcus glyconophilus, Syntrophococcus sucormutans, Acetobacterium spp, Acetobacterium flavidum, Acetobacterium ruminis, Clostridium aceticum,

	Clostridium ijungdahlii, Clostridium mayombei, Pelobacter spp, Sporomusa spp
Acetate, Butyrate	Acidaminococcus fermentans, Butyribacterium methylotrophicum, Eubacterium spp
Acetate, Butyrate, Ethanol	Clostridium spp
Acetate, Propionate	Anaerovibrio lypolytica, Propionibacterium spp, Selenomonas spp
Acetate, Ethanol, Lactate	Lachnospira spp, Lactobacillus spp
Acetate, Lactate	Bifidobacterium spp
Acetate, Lactate, Formate	Ruminococcus spp
Lactate	Lactobacillus spp, Streptococcus spp, Leptotrichia buccalis

Succinate	Anaerobiospirillum succinoproducens, Succinovibrio dextrinosolvens, Succinomonas amylolytica
Butyrate	Butyrobivrio spp, Roseburia cecicola, Fusobacterium spp
Acetate, Propionate, Butyrate, Valerate, Caproate	Megasphaera elsdenii
Metabolites	Bacterial Species

Annexe 03:

III- Preparing different culture media for microbiology work:

III.1- Preparation of culture media (Starch agar, BP, VP):

III.2 Preparation of meat liver culture medium (LV): Composition of VF culture medium:

- Base medium..... 48g/l
- Meat liver base..... 30g
- Glucose..... 2g
- Starch 2g
- Agar 11g

Supplements:

Complete agar: base medium plus

- sodium sulphite 5% 50ml
- ammonium iron alum 5% 10ml

Instructions:

- Add 2.88g of VF dehydrated medium to 60ml of distilled water and boil under agitation in a beaker.
- Transfer the medium to a flask and autoclave at 121°C for 20 minutes.

III.3- Preparation of nutrient agar (GN):

- Composition of GN culture medium..... 28g/l
- Meat peptone 5g
- Meat extract 1g
- Yeast extract 2g
- Sodium chloride..... 5g
- Agar: 15g

Instructions:

- Add 5.04g of King a dehydrated medium to 180ml of distilled water and boil under agitation in a beaker.
- Transfer the medium to a flask and autoclave at 121°C for 20 minutes.
- Pour the medium into Petri dishes and let it cool with the lid semi-open.

III.4- Preparation of Baird-Parker (BP) agar: Composition of BP culture medium:

- Base medium 33g/l
- Tryptone 10g/l
- Meat extract..... 5g/l
- Autolytic yeast extract..... 10g/l
- Sodium pyruvate..... 10g/l
- Glycine..... 12g/l
- Lithium chloride.....5g/l
- Agar 20g/l

Supplements:

Complete agar: base medium plus

- potassium tellurite 3.5% 3ml
- egg yolk emulsion..... 47ml

Instructions:

- Add 5.94g of BP dehydrated medium to 180ml of distilled water and boil under agitation in a beaker.
- Transfer the medium to a flask and autoclave at 121°C for 20 minutes.
- Add 3ml of egg yolk emulsion and 0.5ml of potassium tellurite to the medium when it has cooled down, and mix slowly to avoid the formation of foam.
- Pour the medium into two Petri dishes and let it cool with the lid semi-open.

III.5- Preparation of starch agar: Composition of starch agar

- Peptone 5g/l
- Starch..... 10g/l
- Agar..... 10g/l

Instructions:

- Add 0.9g of peptone, 1.8g of starch, and 1.8g of agar to 180ml of distilled water in a beaker, and boil under agitation.
- Transfer the medium to a flask and autoclave at 121°C for 20 minutes.
- Pour the medium into Petri dishes and let it cool with the lid semi-open.

III.6- Preparation of skimmed milk agar: Composition of skimmed milk agar:

- Nutrient agar base
- Complete agar
- Skimmed milk 10ml

Instructions:

- Add 5.04g of GN dehydrated medium to 180ml of distilled water and boil under agitation in a beaker.
- Transfer the medium to a flask and autoclave at 121°C for 20 minutes.
- Add 1.8ml of skimmed milk with a sterile pipette and homogeny.

Annexe 04:

IV- Biogas production

IV.1- Bacterial species involved in biogas production:

Table 12: *some additional bacterial species involved in biogas production*

Substrate	Bacterial Species	Metabolites	Reference
Cellulose	Clostridium cellulolyticum	Acetate, hydrogen, carbon dioxide, ethanol	(Kashyap et al., 2015)
Cellulose	Fibrobacter succinogenes	Acetate, hydrogen, carbon dioxide, succinate	(Zhang et al., 2018)
Protein	Peptostreptococcus anaerobius	Hydrogen, carbon dioxide, acetate, propionate, butyrate	(Tian et al., 2013)
Protein	Desulfotomaculum reducens	Acetate, propionate, butyrate, carbon	(Oliveira et al., 2015)

		dioxide, hydrogen sulfite	
Lipid	Methanosarcina mazei	Methane, carbon dioxide, acetate	(Zheng et al. 2016)
Lipid	Syntrophomonas wolfei	Hydrogen, carbon dioxide, acetate	(Sousa et al., 2013)

IV.2- The composition of biogas:

The composition of biogas can vary depending on the source and process used to produce it. However, in general, biogas is primarily composed of methane (CH_4) and carbon dioxide (CO_2), with small amounts of other gases such as hydrogen sulphide (H_2S) and nitrogen (N_2).

Biogas can be produced from a variety of organic materials, including agricultural waste, food waste, and wastewater. The composition of biogas varies depending on the feedstock and the process used to produce it. Typically, biogas contains 50-70% methane, it has a high-energy value (802kJ/mol) and a high heating value (25 times that of CO_2). 25-45% carbon dioxide, it is a non-corrosive gas that does not have any energy value. For valorisation in a boiler or cogeneration engine, it is kept. For valorisation as fuel biogas or for injection into the gas grid, it must be removed by purification systems. Also, small amounts of other gases such as hydrogen sulphide that is a gas, which is produced during the catabolism of sulphur-containing amino acids. It is a dangerous gas as inhalation of it can be lethal. This gas, during its combustion, generates hydrogen sulphide that damages the valorisation elements (boilers or engines). Moreover, nitrogen. (Roland Wengenmayr and Thomas Bührke., 2012).

Siloxanes can also be found in biogas, which are silicon derivatives that typically come from the degradation of cosmetic products. Landfill biogas (ISDN) is often rich

in siloxanes, while agricultural biogas is free of them. Siloxanes can be very harmful to biogas valorisation elements because they vitrify at high temperatures in the heat exchangers of biogas boilers or in the cylinders of cogeneration engines.

Water vapour (H2O) is also present in biogas. Generally, at saturation in raw biogas, it can damage biogas valorisation elements (solenoid valves, regulators, burners, etc.) when it changes to a liquid state.

- Here is an expanded table showing the composition of biogas from (Roland Wengenmayr and Thomas Bührke., 2012) and (Arthur Wellinger, Jerry Murphy, and David Baxter., 2013), including additional components:

Table 13: *the composition of biogas*

Component	Percentage Range
Methane (CH4)	40-75%
Carbon Dioxide (CO2)	20-60%
Nitrogen (N2)	0-10%
Hydrogen Sulfide (H2S)	0-3%
Oxygen (O2)	0-2%

Ammonia (NH3)	0-1%
Water Vapor (H2O)	Saturated
Siloxanes	trace amounts

IV.3- The Steps of anaerobic digestion process:

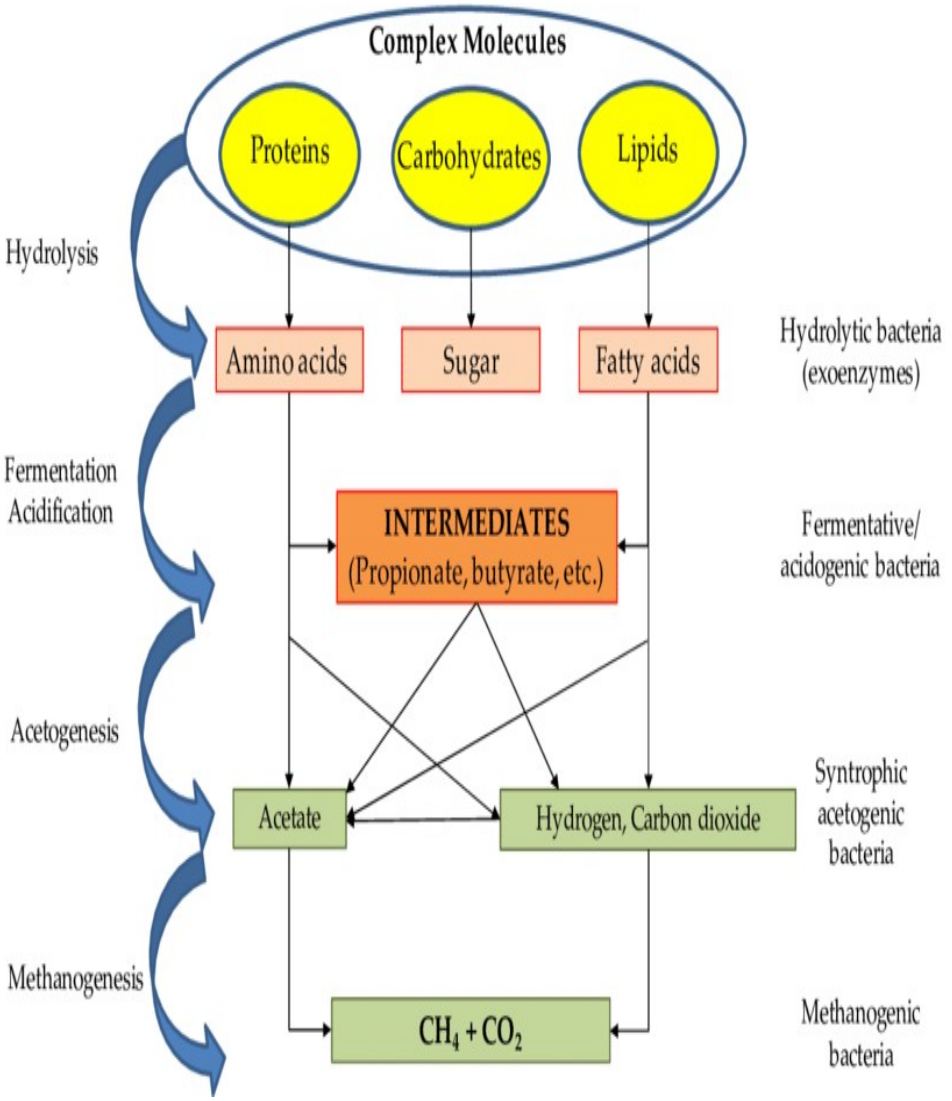

Figure 17: *Phases of the anaerobic digestion process*

Photos:

V- Photos of experimental work

Figure 18: *digester basin of dairy sludge (Original photo)*

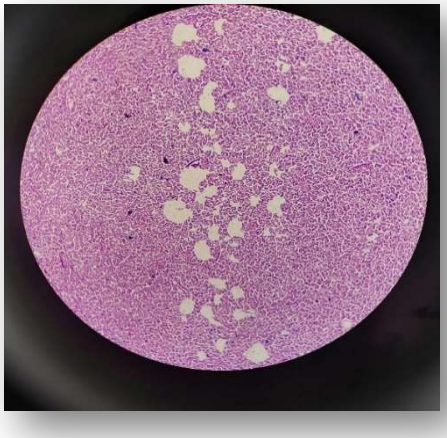

Figure 19: *Bacillus gram staining×100 (Original photo)*

Figure 20: *the seeding of bacilli (Original photo)*

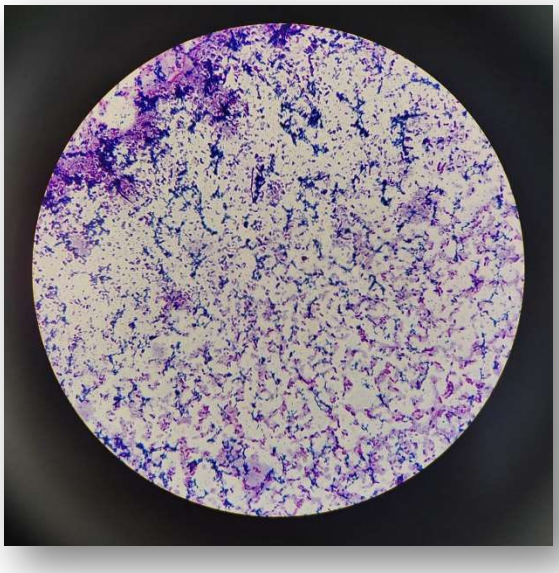

Figure 21: *staphylococci Gram staining×100 (Original photo)*

Figure 22: *clostridium results (Original photo)*

Figure 23 : *staphylococcie morphologie (Original photo)*

Figure 24: *chemichal tests*

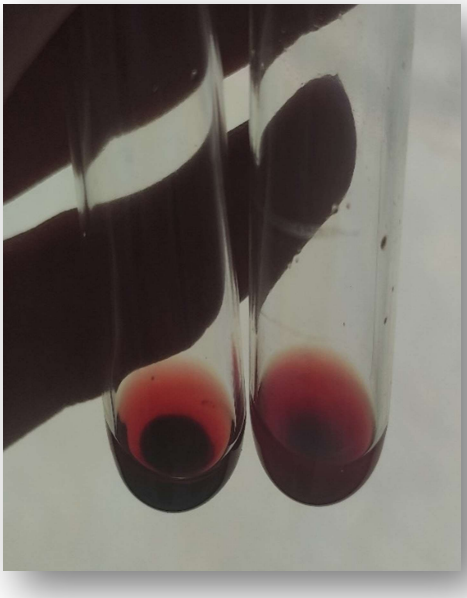

Figure 25: *blood clotting test (Original photo)*

Figure 26: *staph's gallery (Original photo)*

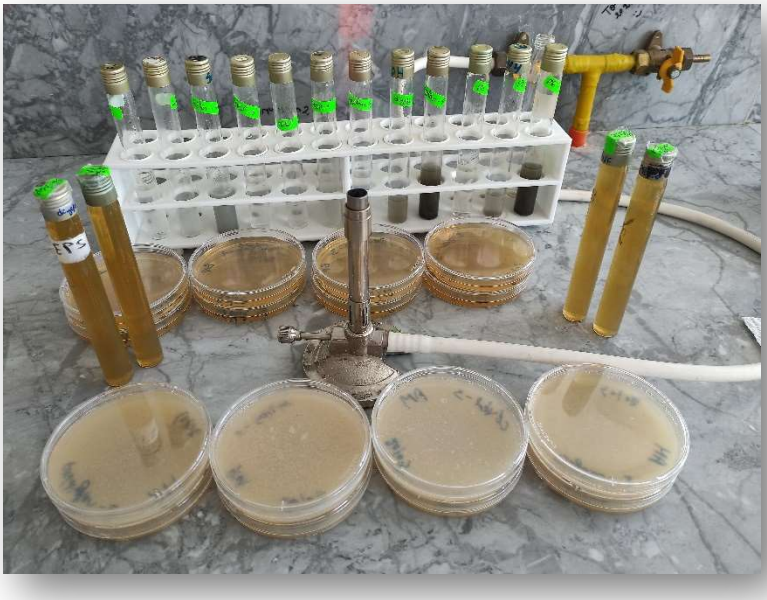

Figure 27 : *microbiological products (Original photo)*

Figure 28 : *SM materiels (Original photo)*

Figure 29 : *COD materiels (Original photo)*