Laboratory contaminating bacteria of acidophilic extremophiles: Polymerase chain reaction (PCR) characterization

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Abstract

Some bacteria can survive in conditions in which even extremophiles cannot survive. In this study, the conditions of contamination of mine-waste extremophiles with other bacteria was studied on the laboratory scale. At the first step, the acid-producing extremophile bacteria were isolated from mine tailings and characterized using a biochemical protocol. The extremophiles survived at the pH from 0 to 8.5 and temperature from – 70 °C to 90 °C. After the complete growth and isolation of active colonies of the acidophilic bacteria in solid medium, their pollution possibility were examined in the laboratory. The characterization of contaminating microorganisms was performed through polymerase chain reaction (PCR) and 16s rRNA gene sequencing. The polluting bacteria were isolated from the acid-producing bacteria using a nutrient broth liquid medium in a sterilized condition for 1 week, which reached an anaerobic condition after a while. The significant growth of acidophilic bacteria in an anaerobic condition required the 9K medium containing $Fe_2(SO_4)_3$ and elemental sulphur. The results showed that the contaminating bacteria of extremophiles included Bacillus cereus (strain 1), Bacillus sp. (TS3) and Bacillus oryzae-corticis (WJB138), enduring the anaerobic conditions in a nutrient broth medium.

key words

Acid-producing extremophile bacteria

Isolation

Extremophiles' contaminating bacteria

Laboratory environment

PCR

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1. Introduction

The extreme environments mean the conditions with a harsh life opportunity for any microorganisms and living beings [1]. The 'extremophile' organisms explain microbes that live in the environmental niches that can be called as 'extreme' compared with the standards of most living microorganisms. These environments are commonly identified by extremes of pH, temperature, salinity, hydrostatic pressure, dessication, redox potential and ionizing radiation[2]. The acidophilic extremophiles can be used in the bioleaching of copper and other heavy metals from sulphide ores [3]. They also are employed for the environmental treatment of polluted sulphide sources in minewastes [4]. The acidophilic extremophiles have the potential to dissolve the trace elements or inhibit their discharge into the aquatic ecosystem according to the adjusted conditions at the start of the process [5].

Bacillus cereus, as a Gram-positive bacteria [6], is a familiar cause of food poisoning disease [7]. It is widely found in the environment [6], and has been commonly isolated from various foods, including vegetable, cooked rice, meat produce and pasteurized milk [8]. Also, bloodstream infections (BSIs) generated by B. cereus take place among hospitalized sicks [7]. The microorganisms of different ecosystems can be the main decomposers of macromolecules. In previous studies, Bacillus sp. strain M1 was introduced as an effective decomposer of high molecular weight-polycyclic aromatic hydrocarbons (HMW-PAHs). Such microbes not only utilize organic matter like proteins and sugars as their nutrition resources but also can degrade toxic organic contaminations that are present in the environment.[9]. According to the report by [10], Bacillus sp. Strains CS-1 and CS-2 also represented the ability to alkali lignin degradation. Bacillus oryzaecorticis is a new moderately halophilic that was isolated by Hong et al. (2014) aerobic from rice husks. They also reported its strain R1(T) as a Gram-stain-positive, rode and endospore-forming [11].

A technique for the characterization of microorganisms is polymerase chain reaction (PCR), proposed by [12] and has been converted into a fundamental method in molecular biology [13]. In recent years, different molecular protocols have been presented using PCR for the characterization of microorganisms, including the amplification of 16S rDNA sequences from extracted genomic DNA. [14] presented a biochemical protocol for the separation of extremophiles from the soil and their characterization. According to their report, the mine soil bacteria survived during the extreme conditions of the high concentrations of chemical reagents in the mineral processing plant. The acidophilic bacteria could live at alkaline pH for a long time from the mineral processing plant to the tailings dam.

In this work, the survival and growth of the laboratory microorganisms were investigated in the culture medium of acidophilic extremophiles bacteria. The PCR analysis was used to identify the polluting bacteria of the extremophile bacteria. The conditions in which only contaminating bacteria could survive in the presence of mine extremophiles were studied. The results confirmed the necessity of the special culture medium for each microorganism, especially extremophiles. In this study, the details and procedure of PCR analysis were explained. Our findings showed the special conditions for the growth or contamination of a specific bacteria. Also, the aerobic and anaerobic situations for the proliferation and activity of acidophilic extremophiles were studied.

2. Materials and methods

2.1. Isolation of acidophilic extremophile bacteria

In this work, the contamination of three extremophile bacteria in the laboratory was studied. The pure colonies of the extremophile bacteria were isolated from the Sarcheshmeh tailings sample using the solid culture medium [14] to study the possibility of extremophiles contamination in the laboratory environment. The color of the extremophile bacteria was yellow (sample 1), red (sample 2) and white (sample 3) in the nutrient agar solid medium (Fig. 1). For the preparation of the solid medium, 2.8 gr nutrient agar was dissolved in 100 mL of deionized water using a 250 mL Erlenmeyer flask. Then, the solution was sterilized at 121 ºC by autoclave for 15 min to provide a sterile solid medium. The final pure colony of the three strains was achieved by streak cultivation in a solid nutrient agar medium under the sterile condition in a laminar hood around a flame. The solid media were placed into an incubator at 32 °C for 48 hours. The pure colonies cultivated in the solid medium were preserved at 4 °C as a microorganism bank reservoir.

For polymerase chain reaction (PCR) tests, the

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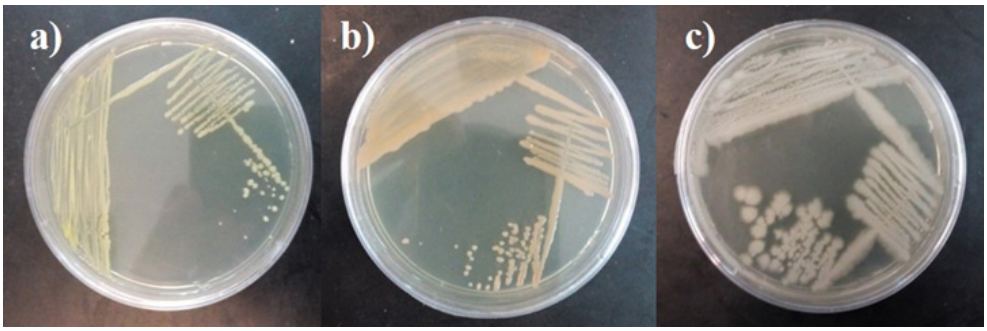

Fig. 1. The isolated colonies of the acidophilic extremophile bacteria (a) yellow strain (sample 1), (b) red strain (sample 2)[14], (c) white strain (sample 3).

fresh colonies of each strain were prepared by the sub-cultivation of the microorganism bank reservoir in a new solid medium. The fresh colonies were 24-hour colonies.

2.2. Contamination of purified colonies

The parafilm of solid medium plates were opened in the laboratory environment after the complete growth of their colonies for 48 hours (Fig. 2). It could contaminate the pure colonies of extremophiles with laboratory microorganisms. The extremophiles can survive in a variety of harsh conditions. The possibility of extremophile bacteria contamination in the laboratory was a desired topic in this paper. Therefore, the polluting microorganisms was isolated in a liquid culture medium and characterized by PCR analysis.

2.3. Enrichment and isolation of contaminating bacteria

A single colony of each of samples 1, 2 and 3 was picked up from the fresh colonies plate using a loop under the sterile condition around a flame. These colony was cultivated in an Erlenmeyer flask containing 100 mL of sterile cooled nutrient broth solution. Then, the sterile culture medium containing pure colonies was placed on a shaking incubator at 32 °C for 1 week. The anaerobic conditions prevented the proliferation and survival of acidophilic bacteria, and only the contaminating bacteria from the laboratory environment survived and grew. The sterile nutrient broth solution containing a huge number of microorganisms amplified during 1 week was used for PCR analysis.

2.4. Cultivation of extremophiles in anaerobic conditions

The cultivation and growth of extremophiles were investigated in an anaerobic environment at the laboratory scale. The anaerobic condition was prepared in a 250 mL Erlenmeyer flask using a 50 mL syringe. The syringe was used to empty the air of the Erlenmeyer flask. In this context, the upper edge of the flask was covered by a piece of parafilm, and the Erlenmeyer air was emptied via the syringe. Finally, the surface of the parafilm was covered by an aluminium sheet. Fig. 3 indicates the steps of preparation for an anaerobic condition. The culture medium was a 9K medium containing ferric sulphate and elemental sulphur.

The acidophilic extremophile bacteria can nourish from Fe^{3+} ions and 9K mediums salts. It helps the bacteria to survive and proliferate. This condition has never been employed for the bioleaching of acid-producing bacteria based on our knowledge

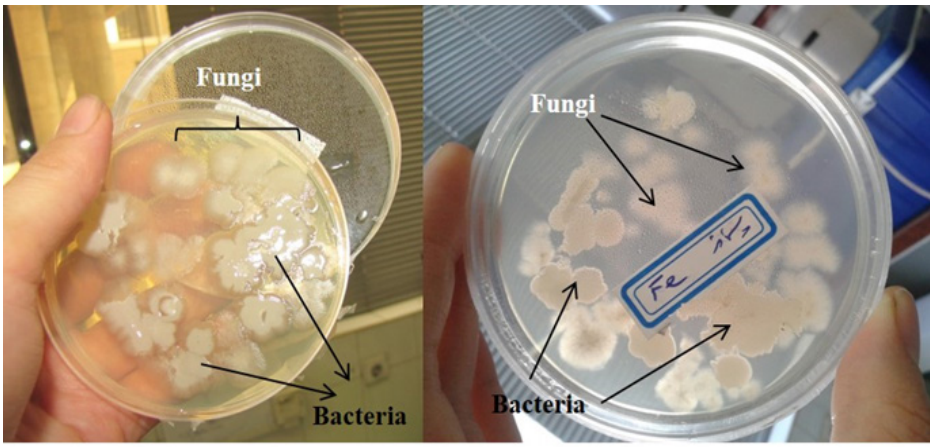

Fig. 2. The cultivated extremophile with white colonies in the nutrient agar solid medium [14].

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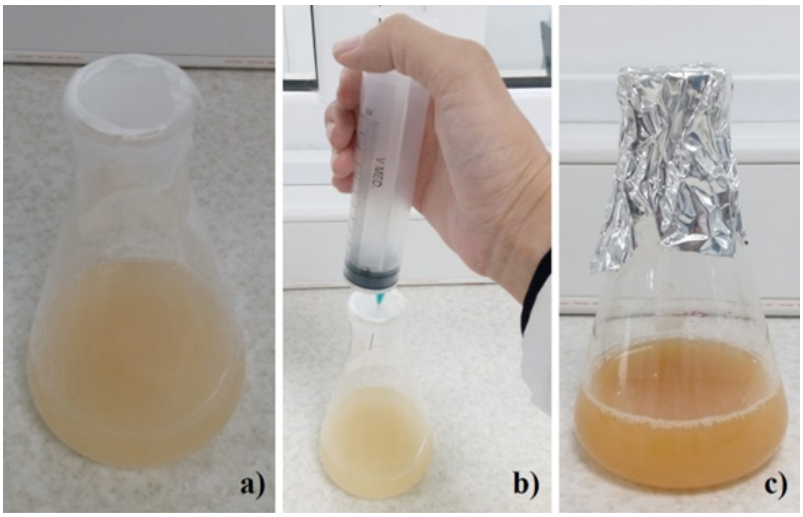

Fig. 3. The preparation of an anaerobic condition in the Erlenmeyer flask on the laboratory scale (a) covering the upper edge of the flask with a parafilm (b) emptying the air of the Erlenmeyer with a 50 mL syringe (c) coating the parafilm by an aluminium sheet.

due to the need for extra facilities for bioleaching in this situation.

2.5. Characterization of isolated bacteria

The characterization of bacteria using PCR technique generally includes different steps: 1) DNA extraction, 2) detection of extracted DNA using electrophoresis, 3) amplification of 16s rRNA gene by PCR, 4) sequencing the amplified gene, 5) determination of the microorganism according to the PCR results (Ilieva et al., 2011). Each of the steps performed for this study and the results will be presented in the next sections.

2.5.1. DNA extraction

The first step of the bacteria characterization was the extraction of microorganisms' DNA using a specified protocol. In this context, the details of the extraction through the Cinacloon company protocol (2016) were as follows:

1. The DNA extraction kit was brought to room temperature before use (due to the preservation in the refrigerator). The lysis solution was warmed to 37 °C for 10 minutes and gently stirred.

2. The 100 µL of the fresh colonies solution was mixed with 400 µL of the lysis solution and vortexed for 15 to 20 seconds. At this step, the solution should be thoroughly homogenized.

3. In the third stage, the 300 µL of precipitation solution was added, vortexed for 5 seconds, and then centrifuged at 12000 rpm for 10 minutes.

4. The solution above the tested sample was slowly emptied by emptying it on a paper towel. It should take 2 to 3 seconds. If we have several samples, we must be careful about contamination between samples.

5. One mL of wash buffer was added to the solid remaining at the end of the microtube. It was mixed using a vortex machine for 3 to 5 seconds and centrifuged at 12000 rpm for 5 min.

6. The wash buffer was drained completely, and the remaining solid was dried at the end of the microtube for 5 minutes at 65°C (until the entire drying).

7. The 50 µL of the solvent buffer was added to the dry solid, gently stirred and placed at 65°C for 5 min. The slow pipetting of the solution was performed (repeatedly pulling up the solution using a sampler and emptying) to wash the wall of the microtube from any residue.

8. The biomass of bacteria was precipitated using a centrifuge at 12000 rpm for 30 seconds (DNA extraction protocol, Cinacloon company).

The solution above the solid contained pure DNA. The DNA extraction can be analyzed using electrophoresis with agarose gel. The details of the continuation of characterization process are discussed the following sections.

2.5.2. Electrophoresis and test components

The electrophoresis device is used for the detection of the extracted DNA or the amplified gene from DNA. The presence of DNA in the electrophoresis gel is associated with band formation. No band formation in the examined sample means no extraction of DNA or no amplification of the desired gene. Generally, electrophoresis is a process in which the movement of molecules occurs and is observed due to an electrical current in a special gel (Fig. 4). The molecules move in an elec-

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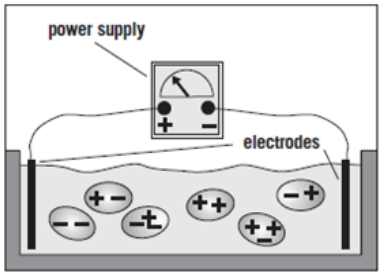

Fig. 4. The arrangement of electrons in the electrophoresis process [15].

tric field depending on their charge, shape and size and are fixed at a point of the electrophoresis gel. This characteristic of the electrophoresis device is employed for molecular separation. The process is simple and relatively fast. It is widely used for proteins and nucleic acids. Additionally, it can be employed for sugars, amino acids, peptides, nucleotides and other simple ions. The applied voltage causes the different types of molecules move with different speeds [15]. The electrophoresis device and its components are shown in Fig. 5.

The components of the electrophoresis device include the electrophoresis tank, buffer solution (generally TAE short for Tris-acetate-EDTA or TBE short for Tris-borate-EDTA), electrophoresis gel, current power source and electrodes. The direction of current in the electrophoresis device is from negative to positive [16].

A tray and a comb were used to cast the electrophoresis gel containing the well (Fig. 6). The agarose gel consisted of agarose powder in a buffer. Agarose is a natural linear polymer that is extracted from seaweed. After dissolving agarose in a buffer through boiling and cooling, a gel is formed. Agarose gel is the most common medium for the separation of medium and large-sized nucleic acids [17]. In the electrophoresis test to determine the band related to DNA extraction and 16s rRNA gene amplification, 1 % (wt/vol) of agarose was dissolved in the buffer [18]. The amount of agarose required for the preparation of agarose gel depends on the dimensions of the tested part. Agarose concentration is expressed based on the amount of agarose to buffer volume (weight to volume). The gel concentration is usually in the range of 0.2 to 3%. The lower the concentration of agarose, the higher the speed of DNA transfer. Generally, if the goal is to separate large DNA fragments, a small concentration of agarose should be used. In the case of the isolation of small parts of DNA, a high concentration of agarose is needed. The size of the band corresponding to the 16s rRNA gene is equal to 1500 bp [17]. Therefore, based on Table 1, 1 % of agarose is suitable for gel preparation.

2.5.2.1. Steps of electrophoresis test

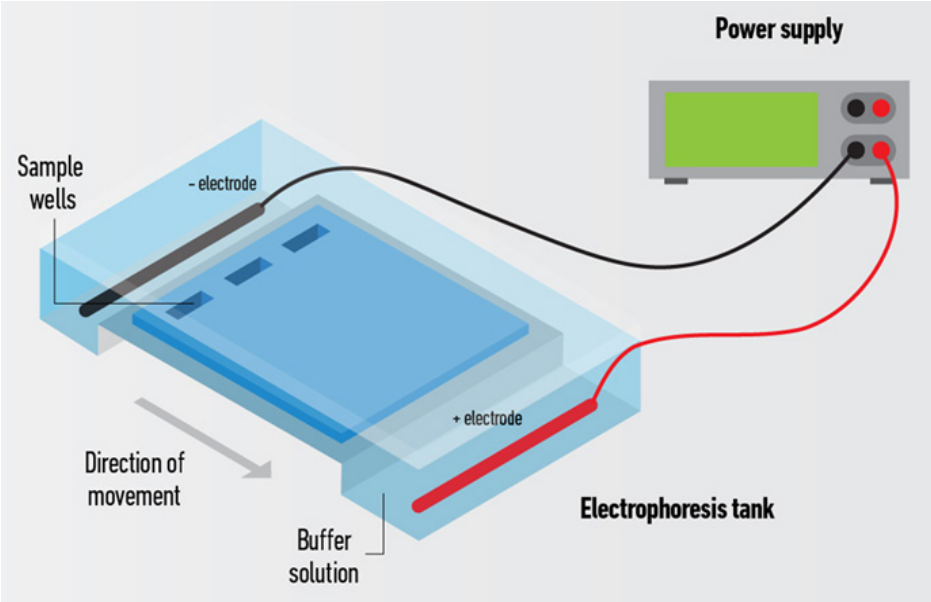

Fig. 5. The electrophoresis device; the view of the machine in the laboratory, device components and current direction [16].

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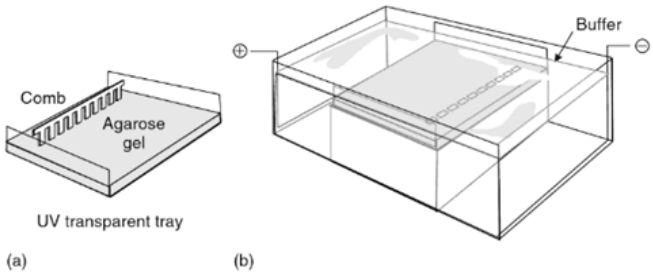

Fig. 6. Preparation of electrophoresis gel (a) special mold and comb to create wells, (b) location of gel inside the electrophoresis tank (Westermeier, 2016).

1) Preparation of agarose gel

The different stages of agarose gel casting in the electrophoresis equipment are schematically shown in Fig. 7. Agarose powder was dissolved in a buffer (TAE or TBE) [15]. Then, the combination of agarose powder and buffer was placed inside the microwave device [17], until the elimination of agarose particles inside the solution. The hot agarose solution was poured into a special mold for this purpose. A number of wells can be created on the agarose gel inside the mold by installing a special comb in this context. The purpose of creating these wells was to load the DNA into the gel. The addition of ethidium bromide to the initial gel solution could also help to increase the quality of the process. However, this method is not recommended due to the remarkable toxicity of ethidium bromide [15]. After hardening, the gel was placed horizontally inside the electrophoresis tank [17].

Generally, the transfer rate of molecules decreases with the enhancement in the concentration of a gel. Therefore, the separation of smaller DNA molecules will be easier. An increase in voltage enhances the speed of DNA molecules movement. However, the increase in the voltage is associated with a lower resolution of the bands. It enhances the possibility of gel melting. The maximum voltage for our studied electrophoresis was 100 V and the recommended voltage for bands separation was 70 V [17].

Table 1 The different concentrations of agarose gel for different DNA dimensions (Magdeldin et al., 2012).

No.	Agarose concentration (%)	Range of DNA dimension (bp)
1	0.2	5000-40000
2	0.4	5000-30000
3	0.6	3000-10000
4	0.8	1000-7000
5	1	500-5000
6	1.5	300-3000
7	2	200-1500
8	3	100-1000

2) Mixing DNA with loading buffer

Before loading the DNA into the electrophoresis gel, DNA (2 to 5 µL) was mixed with a loading buffer (1 µL). The loading buffer contained a high-density substance (to help the DNA to be placed in the gel well) and one or two loading substances (which can move in the gel length along with the DNA and make the path of DNA movement in the gel visible). In addition to providing an excellent environment for analyzing the molecular dimensions of DNA, agarose gel also helps to purify DNA [17]).

3) Loading the DNA by loading buffer

After placing the gel in the electrophoresis machine, the DNA and loading buffer will be loaded into the well using a sampler (0.5 to 10 µm) (Fig.

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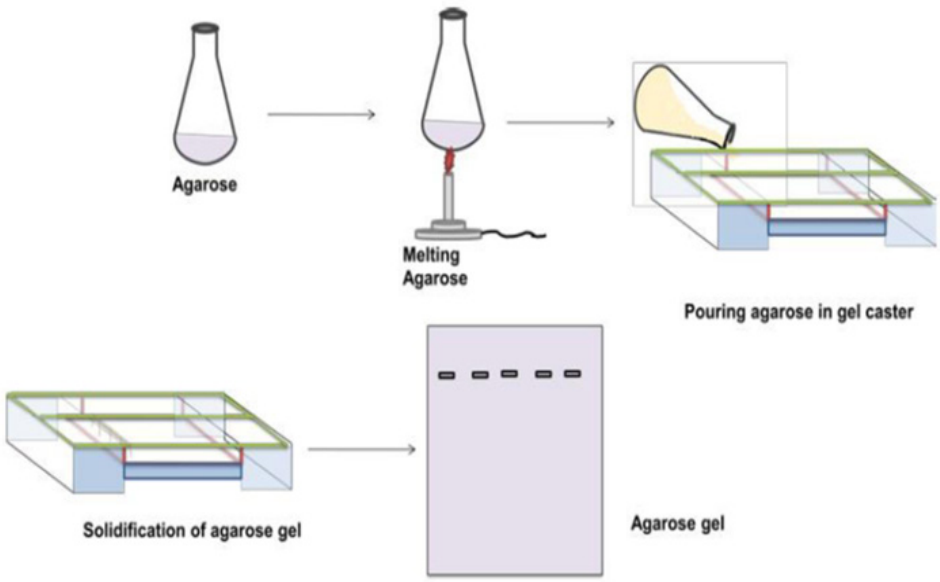

Fig. 7. The different steps of casting of the agarose gel for the horizontal electrophoresis device (Joy et al., 2015).

8)[17]. Each of the tested bacteria DNA mixture was loaded into a well.

In addition to DNA, an indicator solution called ladder was loaded into the gel to determine the DNA dimensions in the length of the gel [19]. One µL of the ladder was loaded after DNA into the next well.

4) Run of the electrophoresis test

After providing an electric current in the device, the movement of the mixture containing DNA in the direction of the gel was observed. The direction of DNA movement was from the negative to the positive pole (Fig. 9). The test took 0.5 to 1 hour [20].

5) Gel staining and DNA visualization

Since proteins in most cases and nucleic acids in all cases cannot be seen directly, the agarose gel must be modified in a way that the protein or nucleic acid can be observed. The method in this field is staining [15]. There are different methods for observing the isolated DNA species on an agarose gel. Among different methods, silver staining

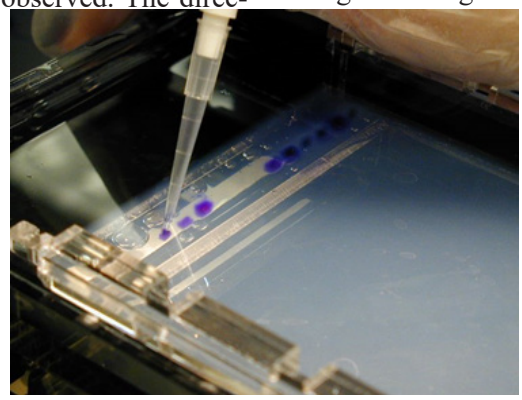

Fig. 8. The loading of a mixture of DNA and a special dye into the agarose gel well; the gel placed in the electrophoresis machine (the protocol by School Work Helper).

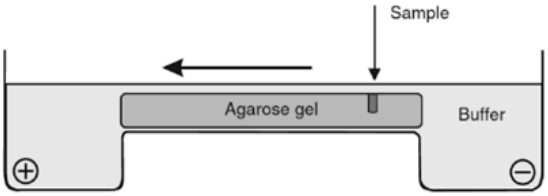

Fig. 9. Agarose gel setup for isolation of 16s rRNA gene (Westermeier, 2016).

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and ethidium bromide staining can be mentioned. In this study, the ethidium bromide technique was used. In the silver staining method, silver particles are utilized. This method is an efficient technique to observe the DNA band. However, its prices have resulted in the use of the ethidium bromide method. Ethidium bromide is very toxic, and this method must be performed using appropriate gloves that prevents skin contact with the solution [21]. The staining with ethidium bromide takes 10 min. Generally, it can be said that the electrons of the aromatic ring of the ethidium bromide molecules will be activated by exposure of UV light. The energy will be released to return the electrons to a stable state. This energy makes the DNA stained with ethidium bromide visible [22].

The next step after gel staining is the decolorization step by water [23]. This stage is aimed is to better observe DNA by removing the ethidium bromide color from the areas that are not linked to DNA. Then, the gel is placed inside a special device named gel doc (Fig. 10). The desired gel bands and ladder can be seen at this stage using UV radiation [24].

The gel was removed from its tray and then exposed to the UV light. It is most generally carried out by a gel documentation system [25]. In this context, the steps of gel documentation device (Bio-Rad, Hercules, CA, USA) performance are as follows:

1. Placing the stained gel inside the device (in which all parts of the gel can be seen by the camera of the device after turning on the UV light),

2. Selecting the camera of the installed software from the top menu,

3. Opening a window including the capture option,

4. Selecting the stop option to fix the image,

5. Observing the image of extracted DNA.

2.5.3. PCR tests

In the PCR method, a specified gene of the DNA is amplified in excessive numbers. PCR is called a DNA copying machine. The general concept of the process is simple to understand. However, PCR is a complex process with a large number of reactions [26]. The reason for naming this technique is the performance of process reagents on the DNA polymer chain (Fig. 11) [27].

Fig. 10. Gel documentation system for observing stained bands on gel [11].

The PCR test is performed for 16s rRNA gene amplification and the detection of this gene by a pair of primers and a master mix [28]. A critical point in conducting a PCR experiment is the performance in sterile conditions. In this condition, the test environment, which includes a microtube, should contain only the reagents and DNA of the desired bacteria. The type and amount of factors in the PCR test were according to Table 2. The table shows the compounds used for each DNA sample.

The PCR test should show the positive results for the extracted DNA of the studied samples. It was considered as a positive control [29]. Therefore, the positive control must contain a test target and verify that the PCR worked. A negative control test also was performed, containing 1 µL of sterile distilled water instead of the DNA. The negative control was used to examine the contamination of the PCR analysis environment. In this context, the electrophoresis analysis could be carried out to observe the correct performance of the PCR tests.

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Table 2 The type and amount of factors for the PCR tests (units in μL).

Final volume	Sterile distilled water	DNA	Primer F	Primer R	Master mix
20	7	1	1	1	10

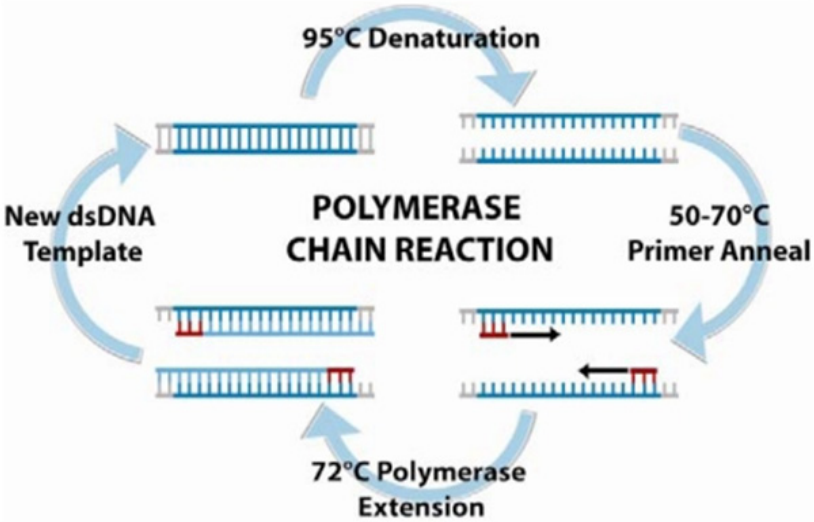

Fig. 11. The three steps of amplification process in the PCR analysis [24].

The condition required for the PCR results' correctness was 1) the visible bands of the extracted DNAs, 2) no band visible for the negative control test. Accordingly, the PCR test consisted of two steps:

1. The preparation of a sterile solution for PCR,
2. Loading the sterile solution in the PCR machine based on the designed program in this context.

Similar to the protocol by [30], the program of the PCR analysis in our experiments was as follows (Fig. 11):

1. Initial denaturation step at 95 °C for 10 min,
2. Denaturation step at 95 °C for 45 seconds,
3. Annealing stage at 65 °C for 45 seconds,
4. Extension step at 72 °C for 90 seconds,
5. Final extension stage at 72 °C for 10 min.

Steps (2)-(4) were repeated 30 times before starting stage 5.

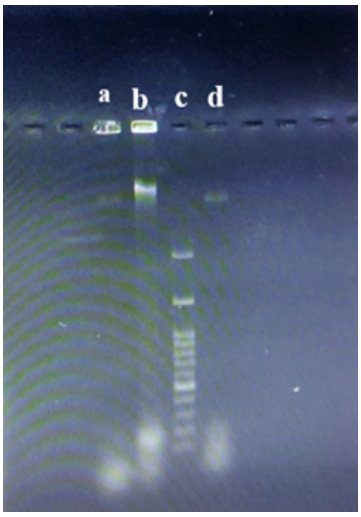

Fig. 12. The agarose gel for the extracted DNA of three bacteria samples (a) sample 1, (b) sample 2, (c) ladder, (d) sample 3.

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3. Results and disscusion

3.1. Extraction of bacteria DNA

After the extraction of DNA, the electrophoresis test was performed for the three samples to ensure DNA extraction. The visibility of the bands on the gel demonstrated the success of DNA extraction. Fig. 12 shows the bands generated on the electrophoresis gel due to the extraction of DNA samples. The bands' numbers (a)-(d) were, respectively, contributed to the bacteria number 1, bacteria number 2, ladder and bacteria number 3. These results were required before PCR tests, identifying the strain of isolated bacteria in nutrient agar medium.

3.2. Electrophoresis for PCR products

The solutions of amplified genes obtained from PCR experiments were investigated for PCR correctness. In this step, the visible bands of samples 1, 2 and 3 were desired. The results indicated that the PCR tests performed for the three samples were successful (Fig. 13). The bands A, B and C were related to the sample DNA, negative control and ladder, respectively. The negative control tests represented that the DNA samples did not contaminate with additional microorganisms. The results of this step also ensure us for the sequencing stage of the study, reported in the next section.

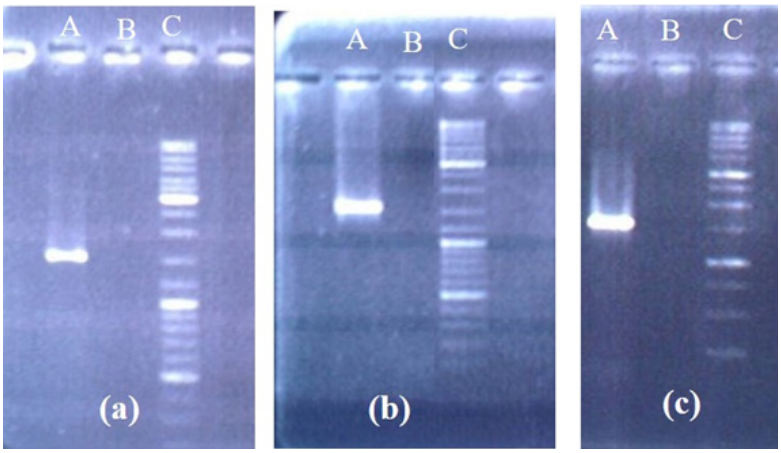

Fig. 13. The results of electrophoresis analysis for PCR test (a) sample 1, (b) sample 2, (c) sample 3 (A: sample DNA, B: negative control, C: ladder).

3.3. Sequencing results

The PCR test products were sent abroad for the determination of sequencing of the 16s rRNA gene and final characterization of the bacteria. The text files of bacteria sequencing were uploaded to the website of National Center for Biotechnology Information (NCBI). These bacteria included Bacillus cereus (strain 1), Bacillus sp. (TS3 strain) and Bacillus Oryzaecorticis (WJB138 strain). The curve of the bacteria sequences could be utilized to ensure the correctness of the sequencing tests. If the curves showed the specified and distinct peaks, the results were accurate and correct. These curves were observed for the characterized bacteria.

3.4. Extreme conditions for acidophilic extremophiles

The extremophile bacteria showed the different morphological properties and colors in nutrient agar solid medium. The acid-producing bacteria can endure a wide range of pH and temperature, respectively, from 0 to 8.5 and from – 70 °C to 90 °C. According to the literature [31], these bacteria could be assumed as extremophiles. Furthermore, the isolated extremophiles were cultured in a 9K medium and pH 2 and decreased the solution pH from 2 to 1.2 after 30 days. They tolerated the extreme changes in pH, temperature and toxic elements concentrations. The extreme variations in the pH and temperature were, respectively, from 0 to 8.5 and from – 70 °C to 90 °C. Furthermore, they survived in the presence of the high concentrations of heavy metals and trace elements. According to the biochemical protocol suggested by [32], the isolated bacteria were characterized as acidophilic extremophiles.

3.5. The activity of extremophiles in anaerobic conditions

The results of PCR experiments represented that acidophilic extremophiles could not grow in nutrient broth medium. However, they showed a steady proliferation in 9K liquid medium containing $Fe2(SO_4)_3$. The activity of the extremophiles was controlled by the significant change in the culture medium color and bacteria population. Fig. 14 shows the variation in color of culture medium

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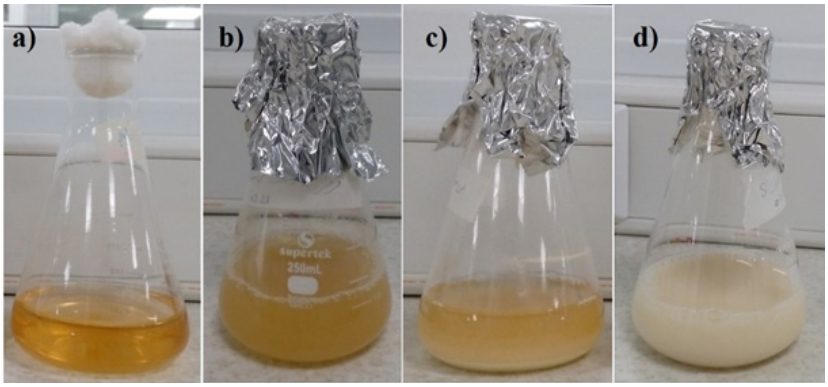

Fig. 14. The steps of change in culture medium color during the reduction of Fe3+ (a) primary culture medium without elemental sulphur, (b) after 2 days, (c) for 5 days, (d) after 7 days.

because of the reduction of Fe^{3+} during the experiment.

The ferric ions present in a medium convert the medium color into reddish. At the first of the experiment, the initial culture medium was red-brow, changing to a colorless medium due to the consumption of ferric ions. The variation in the color of the culture medium demonstrated bacteria activity. The bacteria number was in the range of 107 to 108 during the test, showing the growth and activity of the extremophiles.

4. Conclusions

In this study, a laboratory condition was investigated in which the different bacteria from acidophilic extremophile bacteria could grow and represent more activities. The results showed that acidophilic extremophiles could not proliferate in an anaerobic medium in the absence of mineral salts after 1 week. Furthermore, the anaerobic growth of extremophiles was desired in a 9K medium containing ferric ions, reducing Fe^{3+} ions and oxidizing the elemental sulphur. The analysis of the polymerase chain reaction was used to characterize the contaminating bacteria of extremophile bacteria in the experiment environment. The characterization indicated the polluting bacteria included Bacillus cereus, Bacillus sp. and Bacillus Oryzaecorticis. Additionally, the solid nutrient agar could lead to the complete isolation and final purification of colonies of mine-waste soil extremophiles. Bacillus species indicated a condition in which could grow in return for extremophiles in the absence of a specialized medium.

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