Screening And Identification Of Bacterial Isolate Producing Arbutin Glycoside And Improving Its Productivity By Mutagenesis

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KEYWORDS

glycosides, Arbutin, isolate bacterium, Vitek 2 compact, 16S rRNA, Ultraviolet mutagenesis.

ABSTRACT

From three sources of Iraqi soil, 30 bacterial isolates were obtained. after performing the primary isolation process, 12 isolates could produce the glycoside arbutin as an indication of the reddish-brown precipitate. When secondary isolation was conducted, it was found that two isolates, A7 and C10, gave the highest production of arbutin as a function of the concentration of the resulting compound, as it reached a concentration of Arbutin 5.3 µg/ml and 8.5 µg/ml, respectively. The diagnosis process was carried out for isolate C10 based on cultural and microscopic characteristics, as the nature of growth on the liquid medium was that it floated on the surface, and the cells formed a thin membrane with each other. Pellice as for the nature of growth on a liquid media, the colonies were spread out and flat, with irregular edges, a cream color, and a wrinkled surface, while the microscopic characteristics of the bacterial isolate were that they were positive for Gram stain and the cells were rod-shaped of medium length. The biochemical tests Vitec 2 results showed that the bacterial isolate belonged to Bacillus subtilis with a probability of 98%. Upon genetic diagnosis at the 16SrRNA level, it was found that the isolate belonged to the genus Bacillus subtilis by 100% compared to what is available in the NCBI GenBank and that exposure for 30 minutes at a wavelength of 257 nanometers (20w) and at a distance of (20) cm from the dish to ultraviolet radiation resulted in to improve the production of arbutin twice as much as the wild strain.

1. Introduction

Glycosides are secondary metabolites found naturally in plants,They consist of two chemically and functionally independent parts: the non-sugar part, called the aglycone, which is usually aliphatic or aromatic, and the sugar part, called the glycone, linked by a glycosidic bond, the overall effect of the glycosides depends on their components. The aglycone part mainly affects the therapeutic direction, while the sugar part increases the solubility in water and the drug's dynamic properties (Bartnik et al., 2024; Libik-Konieczny et al., 2021; Araruna et al., 2020). glycosides are known for their therapeutic properties, Putra et al.,2024 reported glucoside, chrysophanol chrysophanol-8-glucoside, and tabilities in therapy encompassing antiviral and antioxidant due to the presence of bioactive compounds. As the number of common diseases that are resistant to medications grows, along with the risks that come with chemotherapy drugs, it is more important than ever to find alternatives to these drugs (Al-Hayali et al.,2024). A lot of different plant products are used to treat different illnesses because they kill microbes very effectively (Hussein et al.,2019). phenolic compounds also have multiple biological activities, as they act as antimicrobials and act as antioxidants, as they have the ability to dismantle types of free radicals, and are considered among the most important natural antioxidants (Albuquerque et al., 2021; Ballard et al., 2019). Phenolic compounds are found in many foods and work to improve quality, It increases the shelf life of foods (Zeb, 2020). Arbutin is a glycoside compound belonging to the subclass of phenolic glycosides, structurally, it comprises hydroquinone linked to a sugar molecule (Couteau and Coiffard, 2016; Ortiz-Ruiz et al.,2015). It is obtained from different types of natural and artificial sources, including different types of plants, enzymatic processes, and metabolic engineering of microorganisms (Araujo-Andrade andGómez,2010), bacteria produce a number of compounds and enzymes that are important in food applications and useful in therapeutic nutrition, such as the enzyme L-Asparaginases (Al Zobaidy et al.,2016). Sadeghinezhad et al., (2022) stated that arbutin is derived from hydroquinone and indicated that it is safer than hydroquinone. The biosynthesis of arbutin depends on the process of transferring the glycosyl molecule to hydroquinone using glycosyl transferase enzymes as catalysts (Hazman et al., 2021; Zhu et al., 2018). the chemical structure of arbutin contains a phenolic group as an inhibitor of the enzyme tyrosinase, which causes enzymatic

browning in a number of foods, such as edible mushrooms (Zhou et al., 2017; Hong et al.,2018). Due to their great nutritional content, edible mushrooms have been used as food for over 2,000 years and are accepted by people (Chechan et al.,2017). enzyme inhibitors have recently gained attention as indispensable tools in the pharmaceutical and food industries, and arbutin is one of the competitive inhibitors of tyrosinase (Ghafary, 2019). Ultraviolet (UV) (200-280 nm) They refer to it as short-wave, totally taken up by the ozone layer and Its strong mutagenic potential is another characteristic that makes it useful for sterilization so It is distinguished by its potent ability to eradicate microorganisms (Hammok and Al-rawi,2021). (UV) mutagenesis is a technology widely used to increase bacterial mutation rates in laboratory experiments, as UV radiation can rapidly acquire adaptive traits in bacterial cells (Shibai et al., 2019). ultraviolet rays work to polymerize pyrimidines by causing many chromosomal changes, and deletion, repair, and mutation processes lead to mutation (Ibrahim, 2018). Members of the Bacillaceae family are of great interest in the medical industry due to the large antimicrobial peptides they produce as therapeutic agents. Recent findings have shown that several Bacillus bacteria are promising alternatives to conventional drugs to combat the emergence of new drug-resistant pathogens (Lajis, 2020). This genus has been found in a number of different and diverse environments with potential applications for biotechnological and industrial purposes such as biofuels, bioactive agents, biopolymers, and enzymes (Harirchi et al., 2022). The genus Bacillus is used in various forms for industrial purposes and is recognized as generally recognized as safe (GRAS), non-toxic and non-pathogenic, and what makes this genus one of the most powerful microorganisms in industry is its metabolic diversity, non-pathogenicity except for a few species, rapid growth rates, high cell density, remarkable resistance to harsh conditions and production of various metabolites and bioactive compounds (Douriet-Gámez et al., 2018; Fan et al.,2018). The purpose of this study is to isolate and identify the bacterial isolate producing arbutin glycoside from the local soil in Wasit, Iraq and Conducting biochemical tests and genetic diagnosis and improving arbutin production by physical mutation using ultraviolet rays.

2. Methodology

Materials

The ingredients used in the tests were all of reagent-grade quality (Nutrient Broth, Yeast Extract HiMedia, Mumbai, India, Arbutin Standard Compound Sigma-Aldrich, KH_2PO_4 and K_2HPO_4 BDH, Polymerase Thermo cycler Bioneer Korea, Spectrophotometer Biotech engineering England).

Media for the production of the arbutin

The production medium for the active compound arbutin was prepared according to what was stated in Liu *et al*., (2013), with some minor modifications made by the researcher of the current study. One liter of distilled water was used to dissolve the following ingredients: Yeast Extract 10g/L, Molasses 4% (w\v)) KH_2PO_4 1 g/L, K_2HPO_4 1 g/L, NaCl 2 g/L, $MgSO_4.7H_2O$ 1 g/L, (Hydroquinone(w/v)0.5%, Nutrient Broth 13 g/L, $NaHCO_3$ 1 g/L and sterilized with an autoclave for 15 minutes. minutes at a temperature of 121°C and a pressure of 15 pounds/ang². The pH of the medium was adjusted to 7.0 using sodium hydroxide at a concentration of 0.1 molar prepared previously. Also, 0.5% (w/v) of hydroquinone was dissolved in 1 ml of distilled water and sterilized alone in an autoclave. The medium was used in the isolation stage to determine the optimal conditions for arbutin production, just as the medium was used in all stages of the study.

Preparation of inoculum

Cultures no more than 48 hours old were used from the bacterial isolates under study that were selected during the isolation stage and were characterized by their efficiency in producing the compound arbutin by activating them on the liquid nutrient medium N.B , A smear is taken from the selected isolates using Loop the bacterial carrier and immerse it in a liquid nutrient medium N.B., incubated at 37°C for 24 hours. After that, the absorbance was measured at a wavelength of 600 nanometers after zeroing the

device with the Blank control solution of the medium. The curve equation obtains the required number of cells to be added to the production medium—MacFarland standard

Arbutin standard curve:

The method given by Barsoom *et al*., (2006) was followed to prepare the standard curve for arbutin. The standard compound arbutin stock solution was prepared by dissolving 50 mg of the standard compound arbutin in 50 ml of distilled water. The concentrations were prepared as shown in the following table (2-1) from the solution The standard compound is stored by taking a volume of 1 ml of the stock solution, which has a concentration of 1000 micrograms, in test tubes and using the dilution law, adding appropriate volumes of distilled water to it so that the volume becomes 2 ml, Add 1 ml of 2.5 mM potassium periodate solution to the concentrations prepared from the stock solution of the standard compound and incubate the closed tubes at room temperature for 20-25 minutes. 0.5 ml of this reaction mixture is withdrawn, and 7 ml of biuret buffer solution is added to it. After that, 2 ml of potassium periodate solution is added to it. The absorbance is then measured at a wavelength of 351 nm.

Table (2-1) Concentrations used in preparing the Arbutin standard curve

Number	Absorbance at 351 nm	Concentration (µg/ml)
1	0.39	25
2	0.708	50
3	0.999	75
4	1.375	100
5	1.598	125
6	1.970	150

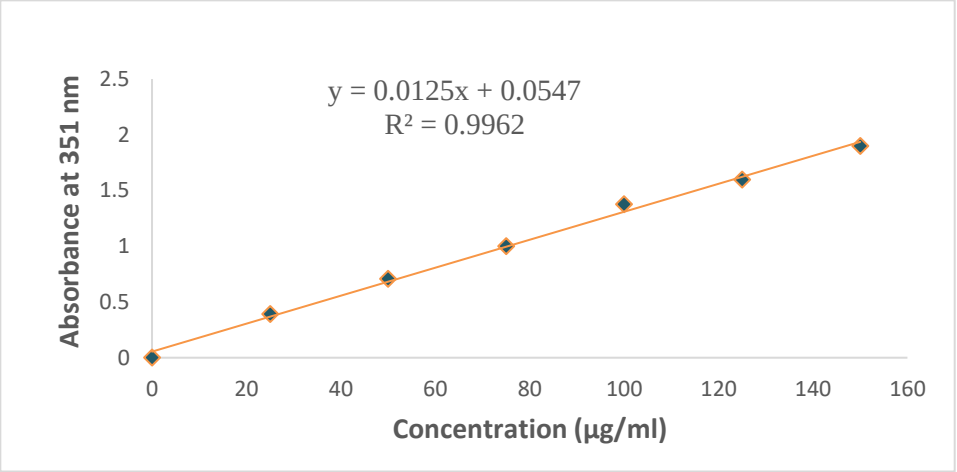

Figure (2-2) Standard curve of the active compound Arbutin

Isolation of bacteri

The process of isolating bacteria was conducted from three different soil sources. They were collected from wheat fields in Waist Governorate/Suwayra, fruit tree orchards in Waist, and agricultural fields belonging to the Department of Horticulture and Landscape Engineering of the College of Agriculture/University of Baghdad. The isolation was carried out by suspending 10 grams of soil samples. After cleaning and eliminating impurities in 90 ml of distilled water in dilution bottles, a series of decimal dilutions was performed by transferring 1 ml of the first dilution to 9 ml of sterile distilled water (second dilution), then transferring 1 ml of the second dilution to 9 ml of sterile distilled

water (dilution III). Implantation was performed by the plate casting method as described by Harrigan and Maccanse (1976); using the solid nutrient medium prepared and then incubating the dishes at a temperature of 37°C for 48 hours, the process of colony development was carried out by capturing the colonies, which are described as being irregular in shape, with an opaque cream-colored edge, and repeating the planting process individually using the Streaking method. on the solid nutrient medium (N.A) and using the metal Loop conveyor at a rate of two replicates for each colony. The process aimed to obtain the colonies in a morepure form. In addition, the pure isolates were preserved as salts containing the N.A at refrigerator temperature

Production of Arbutin

Liquid cultures were used to produce arbutin. 250 ml flasks containing 50 ml of production medium were inoculated with 1 x 10^6 cells/ml inoculum. The flasks were incubated at 37 °C in a shaking incubator at a speed of 150 rpm for 5 hours of incubation. An amount of Hydroquinone at a rate of 0.5% (v\w) and the same incubation conditions were repeated for 24 hours.

Isolating of isolates

The production medium was used to investigate the ability of bacterial isolates to produce arbutin by inoculating 250 ml flasks containing 50 ml of arbutin production medium with the previously purified colonies. Then hydroquinone was added as a precursor in the process of arbutin biosynthesis; then centrifugation was performed—central 400 rpm to remove the bacterial cells and obtain the filtrate. Arbutin was detected by qualitative detection of glycosides (Fehlenck detection A and B), and quantitative compound estimation was carried out using the standard curve prepared in paragraphs (3-11).

Qualitative detection:

Conduct the Fehlinck test for the specific detection of arbutin by mixing two equal parts of Fehlinck solutions (A) and (B) with an equal amount of the bacterial culture filtrate and leaving it to boil for 10 minutes in a water bath. The resulting compound is indicated by the red colored precipitate that forms.

Quantitative determination of Arbutin

The standard curve for arbutin, paragraph (3-11), was adopted in the quantitative estimation of the filtrate of bacterial cultures to determine which of them produces the highest compound, so that it is possible to choose the most efficient one.

Diagnosis of selected isolation

Initial diagnostic tests were conducted for the selected isolate, which was distinguished by its highest production of the active compound arbutin among other isolates. This included conducting cultural, microscopic, and biochemical properties tests using the Vitech 2 system, in addition to performing genetic diagnosis.

Identifications Media

1-The nature of growth on the solid medium: The properties of the colonies growing on the solid nutrient medium (N.A.), at a temperature of 37°C and for a period of 48 hours, were studied in terms of the colony's shape, color, edge, and height.

2-The nature of growth on the liquid medium: The liquid nutrient medium (N.B) was inoculated with the selected isolate, and the tubes were incubated for 48 hours at a temperature of 37°C, while monitoring the nature of growth on the aforementioned nutrient medium.

Biochemical diagnosis of the selected isolate using the Vitek 2 System

The isolate was diagnosed with the Vitek system using the Vitek2 compact system device, as shown

in Figure (3-5). The diagnostic kit for the Bacillaceae family, symbolized by the symbol BLC, was used, which contains 46 biochemical tests, followed the procedure according to the instructions of the manufacturer BioMerieux.

Bacillus Subtilis Identification Using 16S rRNA

Using the method of amplifying the 16S rRNA gene and studying its sequences for the purpose of diagnosing the isolate under study.and The method described was followed by (Alzobaidy *et al*.,2024) and 16SrRNA was used to identify it, The Presto Mini Gdna Bacteria kit, supplied by the Taiwanese company Geneaid, was used to extract DNA from bacteria Using PCR, the 16S rRNA gene was amplified. Polymerase chain reaction (PCR) technology was used to amplify the 16S rRNA gene to confirm the type of isolate selected.using the following primers:

Table (2-3) Primers used to amplify the 16S rRNA gene

Sequence	The number of bases that are nitrogenous	Primer
5⁻CGGGTGAGTAACACGTG 3⁻	17	Primer Forward 104F
5⁻CGGTGTGTACAAGGCCC 3⁻	17	Primer Reverse 1390R

anneal to specific gene areas, enabling gene amplification PreMix PCR was used to produce the amplification in 20 µ volumes an agarose gel was used in order to analyze the amplified PCR result. to determine the nitrogenous base sequences of the products of the 16SrRNA gene amplification reactions of the selected local isolate, the amplification products were sent to the Korean company macrogen to determine the nitrogenous base sequences and the nitrogenous base sequences were compared with the available information about the 16Sr RNA gene in the gene bank of the National Center for Biotechnology Information (NCBI). Using the website (lnt.1) and according to the BLAST Nucleotide program, the type of isolate selected is identified.

Genetic improvement of the selected bacterial isolate (physical mutagenesis):

The mutagenesis process was carried out using U.V. radiation, as stated by Liu et al. (2013), with some modifications by the study's researcher. Young cultures of the isolate selected for bioproduction were prepared on N. A medium at an angle and the physiological solution (Saline) were inoculated with the bacterial culture cells at a density of 1 x 10^6 cells/ml. Then, 5 ml of the bacterial suspension was transferred to a sterile dish. The dish was exposed to U.V. rays for different periods (50, 40, 30, 20, 10, 2, and 60) minutes. The wavelength of the rays used was 257 nanometers (20 w) and at a distance (20)cm from the plate; after the end of the treatment periods, 0.1 ml was transferred from each dish that had been treated with radiation. It was spread on the plates using a sterile glass diffuser on the prepared N.A. medium, with three replicates for each treatment. The plates were incubated at a temperature of 37°C for 24 hours. After the end of the incubation period, a smear of the growing colonies was taken using the carrier. The bacteria are transferred to other dishes under the same incubation conditions above, with three replicates for each treatment. The production media prepared for the active compound are inoculated and inoculated with 1 x 6 10 cells/ml of the isolates obtained after mutagenesis. Mutation lethality (death) due to UV radiation was calculated following the method described by Demirkan *et al*.,(2018). According to the following equation:

$$\text{Killing rate} = \left(\frac{T-U}{U}\right) \times 100\%$$

- **U**: Represents the number of colonies in the wild strain

- **T**: Represents the number of novae in the mutant strain

3. Result and Discussion

Isolation of bacteria that produce the active compound Arbutin

4.1.1- Initial Isolation

More than 30 bacterial isolates were obtained from different soils and grown on the solid nutrient medium N.A. They were given the symbols A, B, and C according to the location from which they were transferred. Then, they were grown on the same nutrient medium several times, arriving at a pure isolate in preparation for the initial isolation. Then, the bacteria were grown several times on the same nutrient medium—some pure colonies on the synthetic production medium for the active compound (3-5-3). The result of the Fehlinck test on the bacterial culture filtrate was used as a preliminary isolation to select the productive isolate indicative of the color change from blue to red or reddish-brown as a result of the presence of glycosides produced in the bacterial filtrate, and this was considered the case. This indicates the bacterial isolate's ability to produce glycosides (arbutin), so 12 isolates were chosen out of 30, as in Table (4-1).

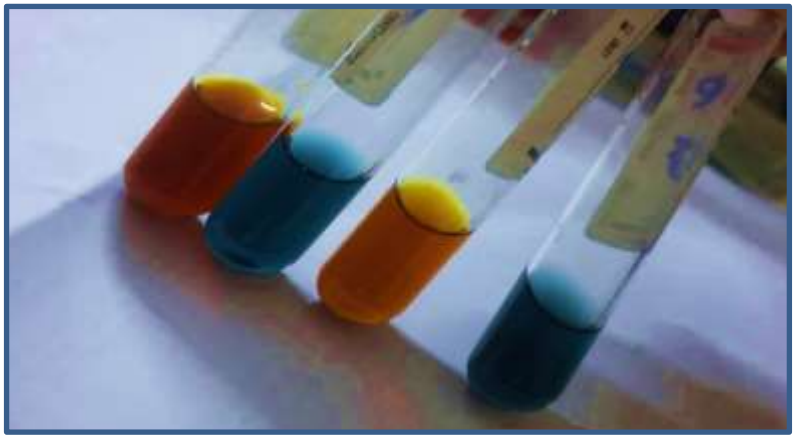

Figure (4-1) A bacterial isolate positive for the Fehling's test and another negative for the Fehling test

Table (4-1) Results of Fehling test on filtrate of bacterial isolates

Number	Fahling test result	Name and number of bacterial isolate
1	(-)	A1
2	(-)	A2
3	(-)	A3
4	(-)	A4
5	(-)	A5
6	(+)	A6
7	(+)	A7
8	(-)	A8
9	(-)	A9
10	(-)	A10
11	(+)	B1
12	(-)	B2
13	(-)	B3
14	(+)	B4
15	(-)	B5
16	(+)	B6

17	(-)	B7
18	(+)	B8
19	(-)	B9
20	(+)	B10
21	(-)	C1
22	(-)	C2
23	(+)	C3
24	(-)	C4
25	(+)	C5
26	(-)	C6
27	(+)	C7
28	(-)	C8
29	(+)	C9
30	(+)	C10

- Test positive (+)
- Negative test (-)

Secondary Isolation

A test was conducted for the twelve isolates that gave a positive result from the previous step (Fehlenk test). Then, a comparison was made between them to select the isolate with the highest productivity using a quantitative estimation of the active compound arbutin. It appears from Figure (4-1) that isolates A7 and C10 gave the best productivity. Isolate C10 outperformed isolate A7 in arbutin concentrations, reaching 8.5 microg/ml and 5.3 microg/ml, respectively. Therefore, isolate C10 was adopted in the subsequent experiments of the current study.

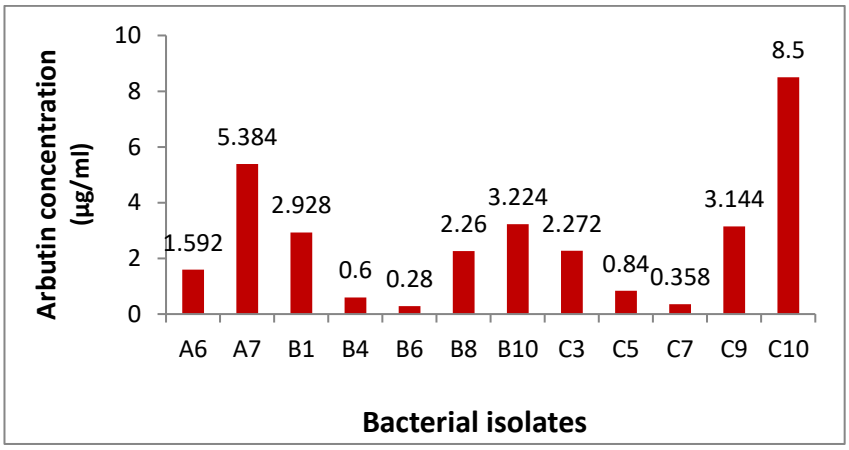

Figure (4-2) Efficiency of the isolates produced in the secondary screening stage in producing Arbutin when grown on the liquid production medium

Diagnosis of elected isolation

The most productive bacterial isolate, C10, was identified through cultural and microscopic examinations. The productive isolates were transferred to Nutrient Agar to incubate at a temperature of 37°C. The isolates were preserved in good condition.

Cultural and microscopic characteristics

The selected isolate C10 underwent a set of microscopic and cultural tests where the characteristics of the isolate were studied After growing it on a solid nutrient medium (Nutrient agar), colonies appeared in an irregular shape and cream color with irregular, dense, wavy edges (Figure 4-2). These results are consistent with what was reported by (Al-Attar), 2016 when he isolated Bacillus subtilis bacteria and

diagnosed them culturally. When grown on a liquid medium (Nutrient broth), the nature of growth was that the cells floated on the surface of the fluid medium with each other, forming a thin membrane (Pellicle). These results are consistent with what Ali (2019) described about the growth of Bacillus subtilis bacteria in the liquid medium. In addition, the microscopic characteristics of the selected bacteria were studied by staining them with gram stain and examining them under the microscope to appear as single or double rod cells or as gram-positive chains, forming endospores. Its location is in the central or subcentral center of the cell and is oval with a circular end, as in Table (4-2). These results agreed with what was mentioned by Collins and Lyne (1985). It is also consistent with what was reported by (Holt *et al*., 1994). Several other researchers also pointed out the general characteristics of the genus Bacillus, as it has a facultative, anaerobic nature of spread on the surface of the dish, and the colonies are cream-colored, with a wrinkled surface and irregular edges (Mandic-Mulec *et al*., 2016; Šimunovi´ *et al*., 2022). (Omar and Jabber,2017) pointed to the isolation of bacteria Twenty-five bacteria isolates (*Bacillus sp*.) were isolated from different local sources soil of college of Agriculture/university of Baghdad. The researcher (AL-Mosawi and AL-Maliki,2023) reported *Bacillus subtilis* are important in improving soil characteristics and phosphorus solubility at high levels, *Bacillus subtilis* have the ability to analyze cellulosic hydrolysis and semi-cellulosic (Hamza et al.,2022).Alzobaidy *et al*.,(2024) reported that *Bacillus subtilis* amenability to genetic manipulation and cultivation for its resilience to extreme heat.

Table (4-2) Cultural and microscopic characteristics of the selected isolate after the primary and secondary screening stage

Number	**Cultural characteristics**	
1	Form of growth on a liquid media	Together, the cells float on the liquid medium's surface to form a thin membrane Pellicle.
2	Form of growth on a solid medium	widespread and slightly flat
3	The colony's edges	Unequal
4	Colony color	Off-White
5	Colony surface	Creased
Number	**microscopic characteristics**	
1	reaction to the Gram	Gram-positive
2	Cell form	Long stick or medium length

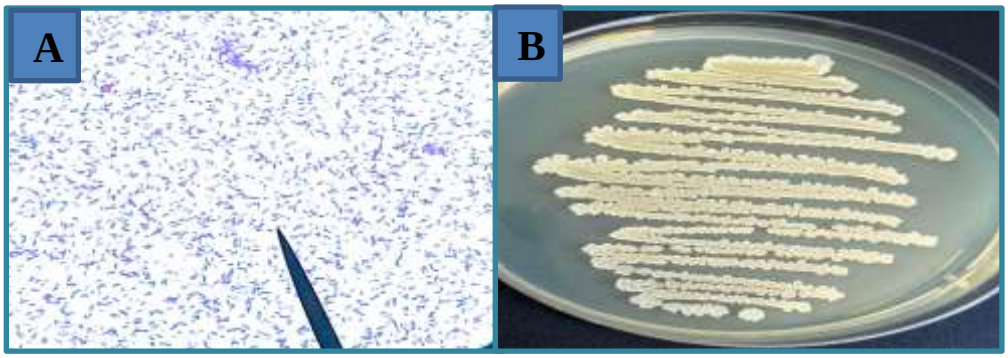

Figure (4-3) (A) Microscopic characteristics of bacterial cells after Gram staining under the microscope (B) Cultural characteristics of C10 bacteria colonies after growing them on N.A medium

Diagnosing isolation using the Vitek2 system

Diagnosing the selected C10 isolates using the Vitech 2 system includes biochemical tests in a

diagnostic kit containing 63 tests. This BCL diagnostic kit was used to diagnose 42 species belonging to the genus *Bacillus* (Halket *et al*., 2010). The Vitek-2 compact system is used in the diagnosis of bacteria using biochemical tests, Mursyidah *et al*., (2021) It has been indicated that it is used in the diagnosis of three types of the genus *Bacillus.* diagnostic tests for the selected isolate showed a 98% probability that the isolate was *Bacillus subtilis*, as shown in the figure (4-4).

bioMérieux Customer: **Microbiology Chart Report** Printed February 1, 2022 7:48:11 AM CST

Patient Name: Patient ID:
Location: Physician:
Lab ID: 2 Isolate Number: 1

Organism Quantity:
Selected Organism : Bacillus subtilis

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	14.12 hours	**Status:**	Final
Selected Organism	98% Probability	Bacillus subtilis		
	Bionumber:	0377001574346661		
ID Analysis Messages				

Biochemical Details

1	BXYL	-	3	LysA	-	4	AspA	-	5	LeuA	(-)	7	PheA	+	8	ProA	-
9	BGAL	-	10	PyrA	+	11	AGAL	-	12	AlaA	-	13	TyrA	+	14	BNAG	+
15	APPA	-	18	CDEX	-	19	dGAL	-	21	GLYG	+	22	INO	-	24	MdG	-
25	ELLM	(+)	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	-	31	dMAN	-
32	dMNE	-	34	dMLZ	-	36	NAG	+	37	PLE	-	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	-	45	PVATE	(+)	46	AGLU	(-)	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	-	61	ESC	+	62	TTZ	-	63	POLYB_I	+						

Figure (4-4) shows the results of the biochemical tests that were conducted on the selected isolate. The test results showed that isolate 10 C belongs to *Bacillus subtilis* with a probability of 98%.

Genetic diagnosis of the selected isolate C10

In this type of diagnosis, it is based on the *16S rRNA* gene. The genetic material of the selected isolate was initially extracted to diagnose the selected isolate. Its purity was confirmed by measuring the absorbance ratio at a wavelength of 260 to 280 nanometers, which reached 1.8. After that, the amplification process was performed, and the results showed that Obtained as a result of electrophoresis of the amplified gene was the presence of one band, as shown in Figure (4-3), which reflects the success of the process of binding the primers to the target gene (*16S rRNA*) without the rest of the other parts of the DNA extract of the selected bacterial isolate, The molecular size of the amplification product was also estimated, as the base pairs reached (1256) pairs. Lakshmi *et al*., (2014) stated that the *16S rRNA* gene is successfully used to distinguish between different types of bacteria and gives decisive results in diagnosis. It was also reported by Hasan *et al*., (2017) that the length of the fragment he amplified from Bacillus sp is 1082 base pairs. At the same time, Özdemir *et al*., (2011) indicated that the size of the *16S rRNA* gene fragment he amplified from B. subtilis amounted to 1031 base pairs. In addition, researchers Bukhari and Rehman (2015) stated that the length of the gene segment he amplified from the *B. subtilis* isolate is 500 base pairs (bp).

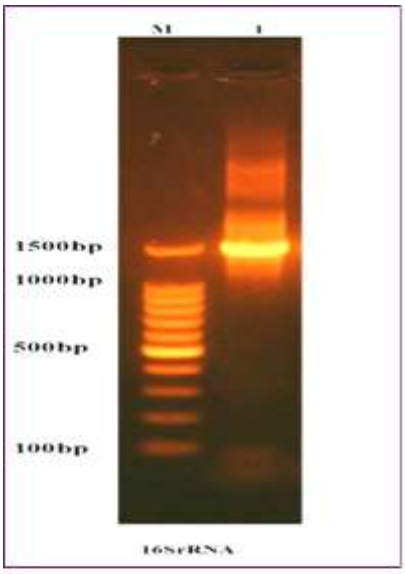

Figure (4-5) Electrophoresis of the selected isolate C10 on an agarose gel and the size index Ladder ranging from 1500-500 base pairs

Analysis of sequences of amplification products

The amplification products were sent to the Korean company Macrogen, which studied the sequences of the nitrogenous bases of the 16SrRNA gene of the selected isolate under study.

After analyzing the results using the BLAST program to find genetic homology or match with NCBI's Gene Bank at the National Center for Biotechnology Information (NCBI) and comparing it with the available information, the results showed that there was a match in the sequences of the nitrogenous bases of the 16SrRNA gene that was amplified for the selected local isolate C10 by (100%) and between the sequences of the nitrogenous bases of the 16SrRNA gene for the strains of Bacillus subtilis registered in NCBI, as shown in Table (4-4). Thus, the selected local isolate was considered to belong to Bacillus subtilis. The result of the genetic diagnosis is identical to the result of the phytic test. The genetic diagnosis is considered one of the most essential modern methods followed by several researchers (Wang *et al*., 2016).

Table (4-5) The percentage of identical nitrogenous base sequences of the local isolate C10 with 8 strains of *Bacillus subtilis* bacteria registered in the National Center for Biotechnology Information .(NCBI)

Number	Strain	Identity%	Accession
1	*Bacillus subtilis*Strain 3667	100	MT538531.1
2	*Bacillus subtilis* Strain 3617	100	MT538489.1
3	*Bacillus subtilis* Strain NB9	100	MT534552.1
4	*Bacillus subtilis* Strain YEBN5	100	MT372156.1
5	*Bacillus subtilis* Strain YEBBR3	100	MT372149.1
6	*Bacillus subtilis* Strain YEBFR5	100	MT332715.1
7	*Bacillus subtilis* Strain HSY21	100	MT513998.1
8	*Bacillus subtilis* Strain YP201707201	100	MT312783.1

Improving arbutin production by physical mutagenesis of Bacillus subtilis NN2M isolate

The effect of exposing the isolate *Bacillus subtilis* NN2M to the physical mutagenic factor, ultraviolet radiation, was studied at different periods (10-60) minutes. The results showed that the bacterial cells were killed by 94% after 60 minutes, and a mutagenic isolate was obtained after exposure for a period, thirty minutes of ultraviolet radiation was distinguished by its superior production over the wild isolate, as the maximum concentration reached 154.66 µg/ml for the resulting compound. In contrast, the non-mutagenic isolate produced 80 µg/ml. Our results regarding UV exposure time are consistent with what was reported by Muzzamal *et al*., (2015) when a 30-minute exposure to UV radiation on bacterial strains *Bacillus subtilis* and *B. amyloliquefaciens* improved polygalacturonase enzyme productivity. This strain showed more than seven times the production. Özalpar *et al*., (2024) also used ultraviolet radiation for periods ranging from (1-120) minutes to cause random mutations in the isolate Bacillus subtilis ATA38 to improve its protease enzyme production. They found that the mutant strain showed an 18.2-fold increase in production compared to the wild strain when mutated. with ultraviolet radiation for 24 hours. Wang *et al*., (2016) found a mutagenic isolate of the *Bacillus subtilis* S1-4 strain when exposed to radiation for 24 hours and improved its production of the protease enzyme by 2.5 times that of the wild strain. Ultraviolet (UV) mutagenesis is widely used to increase bacterial mutation rates in laboratory experiments. UV radiation can be applied to acquire adaptive traits in bacterial cells rapidly (Shibai *et al*., 2019). Ultraviolet rays work to polymerize pyrimidines by causing many chromosomal changes, and deletion, repair, and mutation processes lead to mutation (Ibrahim, 2019). Sirisha *et al*., (2017) studied the effect of the physical mutation using ultraviolet rays for periods ranging from (5 to 60) with an interval of five minutes between each treatment on *Bacillus sp*. bacteria and achieved an improvement in the strain's productivity for the enzyme Alkaline α - Amylase) They were, moreover, obtaining maximum productivity. When exposed to radiation for 40 minutes. Khatun *et al*., (2023) improved the productivity of *Bacillus licheniformis* for the alpha-amylase enzyme through induced physical mutations. The highest activity of the enzyme (705.23 units/ml) was observed after 6 minutes of exposure, which is more than twice as high as the enzyme produced from the wild isolate. (Jebril,2020) indicated the use of ultraviolet mutagenesis technology for E. coli K12 and B. subtilis 168 bacteria to improve and increase their tolerance to cadmium and mercury

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