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Molecular characterization of *Bacillus subtilis* isolated from carrot and evaluation of anticancer potential

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ABSTRACT

***Bacillus subtilis,* a probiotic, has been used in the medical, food, and feed sectors, among others. Nevertheless, the processes that explain its benefits for hosts are still not well understood. This work concentrating on the isolation, characterization, and assessment of the anticancer and hemolytic properties of extract derived from *B. subtilis* obtained from naturally fermented carrots. The isolate was validated as *B. subtilis* DSM 10 using biochemical and physiological tests, followed by PCR amplification and 16S rRNA gene sequence study. We investigated the antitumor efficacy of *B. subtilis* DSM 10 against MDAMB-231 breast cancer cells. Our results stated that the extract had potent dose-dependent anticancer activity against MDAMB-231 breast cancer cells. Safety studies revealed little acceptable toxicity on human RBCs, with just 1.7% hemolysis seen at 320 µg/mL, indicating its possible applicability. In conclusion, *B. subtilis* DSM 10, extracted from carrot, shows potential as an anticancer agent against the triple-negative breast cancer cells, MDAMB-231. Its prospective anticancer property and its safety profile underscore its forthcoming uses in medication.**

INTRODUCTION:

The global rise in chronic lifestyle diseases, largely driven by the consumption of processed convenience foods and increasingly sedentary lifestyles, presents a significant public health challenge. This issue is exacerbated by a growing imbalance between caloric intake and physical activity levels (Caprara, 2021). To address this health crisis, research suggests that incorporating probiotics into functional foods could enhance their nutritional profiles and provide preventive health benefits. Such dietary interventions may play a vital role in reducing the prevalence of communicable and noncommunicable diseases (Cencic and Chingwaru, 2010; El Sohaimy, 2012). Probiotics are defined by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as live microbes that enhance the health of the host when provided in sufficient quantities. This definition positions probiotics as suitable for use in dietary supplements or as effective food sources for both humans and animals, highlighting their part in helping overall health and well-being.

Probiotics are defined as specific microbial strains that exhibit particular characteristics, such as being natural, nonpathogenic, and classified as generally regarded as safe (GRAS) according to Sarao and Arora (2017). Essential attributes of probiotics include their ability to withstand the acidic environment of the stomach and bile, significant surface hydrophobicity, and beneficial aggregation properties, which are crucial for their successful colonization of the gastrointestinal tract (GIT), as highlighted by Tuomola *et al.* (2021). Probiotic strains show a vital part in improving the health and safety of host organisms. They are essential for their ability to adhere to the gut wall and stimulate the immune system without causing inflammatory reactions, as supported by studies (Tuomola *et al.,* 2001; Li *et al.,* 2019). Additionally, probiotics obstruct the adhesion and colonization of

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pathogenic bacteria by producing bactericidal metabolites (Turchi *et al.,* 2013). For a probiotic strain to be effective, it must exhibit desirable technical characteristics such as long-term stability, resistance to bacteriophages, and scalability for mass production, all while maintaining no negative impact on the sensory properties of food items (Tripathi and Giri, 2014). Moreover, while in vitro studies are instrumental for initial strain screening, they alone cannot definitively determine the appropriateness of a probiotic strain for use.

The antibacterial properties of probiotic microbial strains, as highlighted by Ibrahim *et al.* (2021), play a crucial role in their metabolic functions, particularly in glucose fermentation. Lactic acid bacteria are accountable for the conversion of fermentable sugars into lactic acid and other metabolites. This process not only helps lower the pH in the gut, creating an environment that is detrimental to pathogenic bacteria, but also enhances nutrient absorption by improving digestibility and the bioavailability of nutrients for the host organism, as noted by Lingga *et al.* (2022). Probiotic microbial strains produce a variety of metabolites including lactic acid, bacteriocins, hydrogen peroxide, and several organic acids. These metabolites play a essential role in preventing the growth of pathogenic microbes, as evidenced by studies conducted by Shivsharan *et al.* (2023), and Nadirova *et al.* (2023).

Breast cancer ranks as the most predominant malignancy in women across the globe, with its presence reported in 157 out of 185 nations. In 2022, the World Health Organization estimated a surprising 2.3 million new breast cancer cases and nearly 670,000 deaths attributable to the disease worldwide. This cancer type is recognized as a critical health concern, as it stands as the foremost reason for cancer-related deaths among women (Zhang *et al.,* 2025). Chemotherapy remains the primary treatment approach for breast cancer, associated with a range of side effects. These side effects can include immediate pain and may lead to long-term complications that persist years after treatment. Conventional therapies such as radiotherapy, chemotherapy, targeted immunotherapy, and surgery often inflict adverse effects and toxicity on healthy cells. However, using natural compounds derived from various natural sources presents a promising alternative. These natural compounds offer broad-spectrum therapeutic benefits while significantly minimizing or potentially eliminating associated side effects (Zhao *et al.,* 2025).
Approximately 13,000 natural chemicals, each with unique medicinal and pharmacological properties, have been sourced from bacteria, highlighting the rich diversity of beneficial secondary metabolites produced by these microorganisms (Mohan *et al.,* 2022). This appreciation for bacterial metabolites spans over a century, recognizing their importance in pharmaceutical applications. Several studies have demonstrated the effectiveness of these metabolites as anti-cancer agents, showing promising results in combatting various types of human malignancies (Mohan *et al.,* 2022), thereby underscoring the potential of bacteria in the development of novel therapeutic agents. Some notable bacterial-derived anti-cancer agents currently utilized in clinical settings include Bleomycin, Dactinomycin, Doxorubicin, and Carflzomib, each referenced through various studies. Bleomycin has been outlined in works by Chen *et al.* (2020) and Shaikh *et al.* (2023), while Dactinomycin by Liu *et al*. (2019).

The genus Bacillus is known for its ability to produce secondary metabolites that exhibit a range of biological activities, including antiviral, antibiotic, and anti-cancer properties (Abdel-Nasser *et al.,* 2024, Tran *et al.,* 2022; Aimaier *et al.,* 2023). Specifically, *Bacillus subtilis* synthesizes a variety of bioactive compounds with both linear and cyclic structures, which are mainly notable for their anti-cancer property (Kaspar *et al.,* 2019; Dan *et al.,* 2021). Research indicates that several of these compounds from Bacillus subtilis have demonstrated lethal effects on breast cancer cells. These compounds have been further investigated through molecular docking experiments targeting the HER2 protein, revealing significant binding energies that suggest strong interactions with the cancer-associated receptor (Kumari and Ram, 2021). The findings highlight the possible therapeutic applications of *B. subtilis* in cancer treatment, underscoring the importance of this genus in pharmaceutical research and development.

The study aimed to isolate, identify, and characterize a strain of *B. subtilis* from carrot known for its anticancer potential. In addition to the main objective, the research included a hemolysis study to evaluate the safety profile of the isolated strain, ensuring that its anticancer properties do not compromise safety.

MATERIAL AND METHODS:

Sample collection, isolation of bacteria and characterization

Strains of *Bacillus* spp. have been successfully isolated from carrot samples which were obtained from the Warangal, Telangana, India, where a systematic process was employed to culture these bacteria. Methodology was followed as described by the Koilybayeva *et al.* (2023) with slight modifications. A 10 g of carrot material was

homogenization in 90 ml of a saline solution, specifically 0.85% sodium chloride (NaCl), which was agitated for 20 min. at a rotational speed of 150 rpm. Following this initial preparation, the mixture underwent a series of incremental dilutions. This was then subjected to a water bath treatment, where the samples were heated to 90°C for a duration of 10 minutes, a critical step for eliminating competing flora and enhancing the recovery of *Bacillus* spp. After the heat treatment, the samples were allowed to cool to room temperature. Subsequently, 0.1 ml aliquots of the cooled sample were carefully transferred onto nutritional agar medium (5.0 g/L peptone, 5.0 g/L NaCl, 1.5 g/L beef extract, 1.5 g/L yeast extract, 15 g/L agar). Plates were incubated at 37°C till the emergence of bacterial colonies. Uncontaminated bacterial cultures were acquired using the streak plate technique and incubated at 37 °C. Each bacterial pure culture's distinct colony was added to 5 mL of Nutrient Broth and incubated for 24 hours at 37 °C and 120 rpm in a shaking incubator. The inoculum was increased by moving it into 20 mL of fresh medium whenever it reached an appropriate level of turbidity, which is a sign of bacterial multiplication. After reaching maximum growth, the culture broth was centrifuged using a low-speed centrifuge for 15 minutes at 3000 rpm in order to get a clear supernatant. This process is known as differential centrifugation.

The characteristics of the separated bacterial colonies were assessed using a range of biochemical tests, such as the mannitol fermentation test, Voges-Proskauer (VP) test, and catalase test, in addition to their color, shape, and Gram staining.

Extract preparation:
The extract preparation used the liquid–liquid extraction technique, with minor changes as outlined by Burianek and Yousef (2000). In order to accomplish phase separation, equal amounts of supernatant and a freshly prepared chloroform–methanol combination (2:1 v/v) are vortexed in a sterile Eppendorf, and then the mixture is centrifuged at 15,000 rpm for 15 minutes at 4 °C. Without affecting the remaining contents, the separated polar phase (top layer) was moved to a new, sterile falcon. A revolving vacuum evaporator operating at reduced pressure was used to concentrate the extracts. A hot air oven was then used to dry the concentrated extracts at 55 °C. For later usage, the final dehydrated crude extract was stored at -20 °C.

Molecular identification of strains:
Bacterial isolates were subcultured in nutritional broth and incubated at 37°C for 24 hours. Subsequent to incubation, genomic DNA was extracted using the SPINeasy DNA Kit. The quality and amount of the extracted DNA were assessed by a NanoDrop spectrophotometer, with measurements at 260/280 recorded.

Polymerase chain reaction (PCR) of 16S rRNA of Bacterial Isolates:
Molecular identification of bacterial strains was conducted using 16S rRNA gene sequencing with specified primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R: 5'-GGTTACCTTGTTACGACTT-3'). PCR amplification was performed in a 50 µl reaction mixture comprising 5 µl of genomic DNA, 25 µl of GoTaq (Green Master Mix), and 1.25 µl of each primer. The first step in the amplification process was to denaturate the DNA at 94°C for 3 minutes. This was followed by 30 rounds of denaturation at 94°C for 46 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 min. The last step was to extend the DNA at 72°C for 10 min. After PCR amplification, gel electrophoresis on a 1% agarose gel was used to separate the amplicons. The targeted PCR products were excised, concentrated, and purified with the SPINeasy PCR purification kit. The purified amplicons were sequenced and evaluated for species-level identification.

Phylogenetic Analysis:
The acquired sequences were submitted to the NCBI nucleotide BLAST database, and those exhibiting 98–100% similarity were chosen for phylogenetic analysis. A phylogenetic tree was generated using Mega 11 software, based on the top BLAST hits, with 500 bootstrap repetitions used to evaluate the robustness of the grouping.

Hemolysis Activity of B. subtilis:
The hemolytic capacity of the *B. subtilis* extract on human erythrocytes was evaluated. The human red blood cells (RBC) were derived from healthy persons, and stored with EDTA to inhibit coagulation. The blood cells were separated from the whole blood by spinning them at 2000 rpm for 15 min. at 27°C. The red blood cells were then washed three times with 150 mM NaCl phosphate-buffered saline (PBS) containing 10 mM sodium phosphate buffer (pH 7.0). The silt that was left over was mixed with PBS to make a 5% (v/v) RBC/PBS suspension. Then, different amounts of the extract (20 to 320 g/ml) were added to the same buffer. The mixture was then left to sit at 37 °C for one hour. After being left to sit for a while, the liquids were centrifuged at room temperature for 10 minutes at 2500 rpm. We checked the optical density (OD) of the supernatant at 540 nm. SDS and phosphate-buffered saline (PBS) were used to

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set up positive and negative standards, respectively.

The hemolysis % was determined using the formula given below: Hemolysis percentage (%) = OD(crude extract treated sample)−OD(buffer treated sample) /OD(SDS treated sample)−OD(buffer treated sample) × 100

Anti-cancer activity:
The study evaluated the cytotoxic effects of bacterial extract specifically on the MDA-MB231 human breast cancer cell line. The assessment was carried out using the MTT (3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide) test, which is a widely recognized method for measuring cell viability and toxicity based on mitochondrial activity. Cells were inoculated at a density of 1 × 104 cells per well on a flat-bottom 96-well plate, using a cell culture medium composed of 88% Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 2% Penicillin/Streptomycin. The cells were incubated at 37 °C in a humidified 5% CO2 environment for 24 hours. Subsequent to the incubation time, the culture medium was substituted, and cancer cells were administered 20 µL of bacterial extracts (40, 80, 160 and 320 µg/ml dissolved in DMSO) for an additional 24 hours at 37 °C. Subsequent to treatment, 10 µL of MTT solution (5 mg/mL) was introduced to each well, and the plate was incubated for a further 4 hours at 37 °C. Upon completion of incubation, the medium underwent careful extraction. The formed formazan crystals were then solubilized using 50 µL of DMSO for each well. To evaluate cytotoxicity, absorbance measurements were taken at 570 nm using an ELISA plate reader. The study's findings indicated that cell viability was assessed as a percentage of viable cells when compared to control groups. This determination of viability followed the methodology outlined by Tippani *et al.* (2013).

RESULT AND DISCUSSION:

Isolation and Identification of Bacterial Isolates

Twelve distinct bacterial colonies were successfully isolated from the Petri plates, leading to the establishment of pure cultures for further analysis and experimentation. The preliminary characterisation included microscopic inspection of Gram-stained isolates (Figure 1), which indicated that 5 of them were classified within the Bacillus genus, namely S1, S2, S3, S4, and S5. The isolates were identified as Gram-positive and had a rod-shaped morphology (Figure 1). Then, a secondary screening included biochemical assays, counting the catalase test, Voges-Proskauer (VP) test, mannitol fermentation assessment. The findings obtained are enumerated in Table 1.

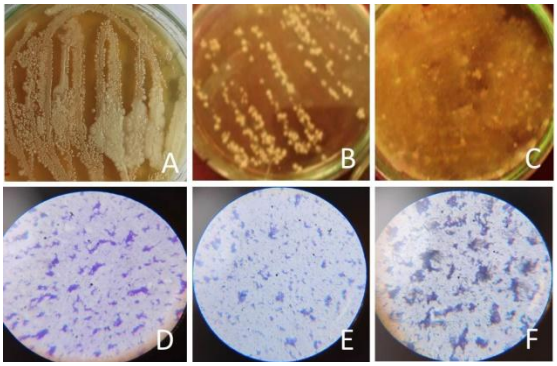

Figure. 1 Colonies and gram staining of probiotic bacillus species A,D) S4; B,E) S5; C,F) S2

Table 1. Biochemical features of samples of Bacillus species.

SNo	Tests	S1	S2	S3	S4	S5
1	Gram staining	+	+	+	+	+
2	Mannitol fermentation	-	+	-	+	+
3	VP test	+	+	+	+	+
4	Catalase test	+	+	+	+	+

Molecular Identification and Characterization of Bacterial Isolates:

Genomic DNA was extracted with the SPINeasy DNA Kit. PCR amplification was performed with universal 16S rRNA primers, resulting in a unique amplicon of about 1.5 kb, as seen on a 1% agarose gel (Figure 2), corroborating previous findings (Cheng *et al.*, 2014). The amplified product was excised, scaled, and purified using the SPINeasy PCR purification kit. Sequencing was performed with the forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), and the quality of the purified product was verified by sequence analysis. The resultant sequences were examined via NCBI BLAST, and those exhibiting 98–100% similarity were selected for further phylogenetic analysis. A neighbour-joining phylogenetic tree was generated using MEGA 11, using 500 bootstrap replications to guarantee statistical reliability.

The isolates S2, S4, and S5 detected in this investigation were classified as *Bacillus subtilis*, while isolates S1 and S2 were categorized as *B. cereus*. Therefore, S4, designated as *B. subtilis*, was chosen for further *in vitro* investigation. Through a comparison study of the genomes of the newly discovered strain with those in the GenBank database, the bacterial strain exhibiting 91% similarity was identified as *B. subtilis* DSM 10 for the S4 isolate(Figure 3), previously acknowledged as the BSL CDC strain (El-Helow, 2001).

B. subtilis DSM 10 is an extensively studied type strain often used as a reference organism in biotechnology and microbiological research. It is recognized for generating elevated concentrations of surfactin and secreting diverse proteases,

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rendering it significant for research pertaining to biosurfactants, enzymology, and industrial bioprocesses. This strain is preserved by the German Collection of Microorganisms and Cell Cultures (DSMZ) and has a publicly accessible genome sequence under the GenBank accession number JAEPVU000000000. Due to its well documented genome and strong physiological characteristics, *B. subtilis* DSM 10 is an exemplary model for several research and industrial applications.

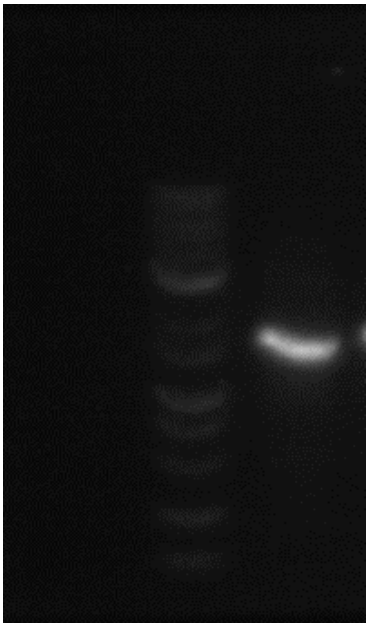

Figure. 2. PCR amplification of 16S rRNA gene amplified at 1.5kb resolved onto agarose gel electrophoresis (1%).

The obtained sequences listed below BSLCDC.

Sequence of culture

>0424-082_001_PCR_BSLCDC_27F_D12.ab1

agagtttga tcctggctca ggacgaacgc tggcggcgtg cctaatacat gcaagtcgag
cggacagatg ggagcttgct ccctgatgtt agcggcggac gggtgagtaa cacgtgggta
acctgcctgt aagactggga taactccggg aaaccggggc taataccgga tggttgtttg
aaccgcatgg ttcaaacata aaaggtggct tcggctacca cttacagatg gacccgcggc
gcattagcta gttggtgagg taacggctca ccaaggcaac gatgcgtagc cgacctgaga
gggtgatcgg ccacactggg actgagacac ggcccagact cctacgggag gcagcagtag
ggaatcttcc gcaatggacg aaagtctgac ggagcaacgc cgcgtgagtg atgaaggttt
tcggatcgta aagctctgtt gttagggaag aacaagtacc gttcgaatag ggcggtacct
tgacggtacc taaccagaaa gccacggcta actacgtgcc agcagccgcg gtaatacgta
ggtggcaagc gttgtccgga attattgggc gtaaagggct cgcaggcggt ttcttaagtc
tgatgtgaaa gcccccggct caaccgggga gggtcattgg aaactgggga acttgagtgc
agaagaggag agtggaattc cacgtgtagc ggtgaaatgc gtagagatgt ggaggaacac
cagtggcgaa ggcgactctc tggtctgtaa ctgacgctga ggagcgaaag cgtggggagc
gaacaggatt agataccctg gtagtccacg ccgtaaacga tgagtgctaa gtgttagggg
gtttccgccc cttagtgctg cagctaacgc attaagcact ccgcctgggg agtacggtcg
caagactgaa actcaaagga attgacgggg gcccgcacaa gcggtggagc atgtggttta
attcgaagca acgcgaagaa ccttaccagg tcttgacatc ctctgacaat cctagagata
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gagatgttgg gttaagtccc gcaacgagcg caacccttga tcttagttgc cagcattcag
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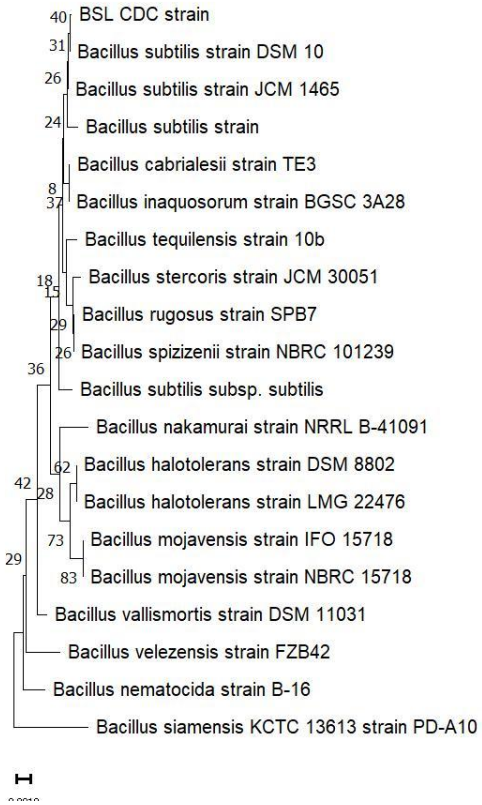

Figure 3. Phylogenetic tree of the culture strain Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, viewing relations between different strains of the genera *Bacillus*.

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Hemolysis activity of *B. subtilis* DSM 10:

The hemolytic activity of the *B. subtilis* DSM 10 extract was evaluated at different concentrations. The findings, as seen in the figure, demonstrated no significant hemolytic effects on human erythrocytes. At the maximum dose examined (320 µg/mL), the *B. subtilis* DSM 10 extract exhibited acceptable hemolytic activity with no discernible toxic effects seen at the first dosages. Although hemolytic activity increased to 1.7% at the highest dose, this is significantly lower than the 100% hemolytic activity showed by the TritonX100, positive control, indicating minimal erythrocyte damage even at higher concentrations (Figure 4). Based on the ASTM E2524-08 standard, a hemolysis score exceeding 5% is deemed harmful to erythrocytes (Choi *et al.,* 2011). Consequently, the extract of *B. subtilis* DSM 10 is shown to be biocompatible at the concentrations tested in this study. Recent research has utilized the hemolysis method as a preliminary approach for toxicity assessment and biocompatibility evaluation (Porika *et al.,* 2025; Dharavath *et al.,* 2025).

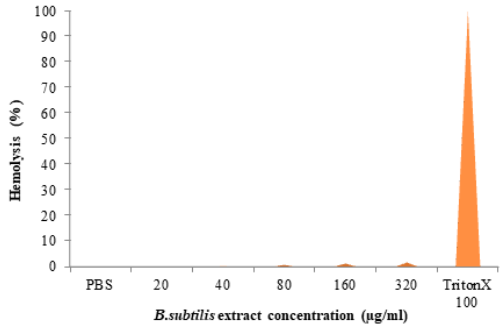

Figure 4. Hemolytic activity of the extract produced by *B. subtilis* DSM 10

Growth suppression effect on MDAMB-231 cells caused by *B. subtilis* DSM 10 extract.

Recent advancements in cancer therapy have heightened the significance of cancer drug development, which plays a crucial role in overall cancer treatment strategies. The diverse functional properties of microbial metabolites present a viable avenue for drug development, particularly through the use of microorganisms, with probiotics being paramount in this context. In a recent study, the effects of the extract from *B. subtilis* DSM 10 were investigated on the triple negative breast cancer cell line MDAMB-231 using an in vitro approach. Specifically, the study aimed to quantify the inhibition percentage of cells through the MTT assay, which is a common method employed to evaluate cell viability in cancer research. The study examines the effects of *B. subtilis* DSM 10 extract on the growth inhibition of MDAMB-231 breast cancer cells across diverse concentrations (40–320 µg/mL) and incubation times (24, 48, and 72 hours) (Figure 5). Results indicate that the extract effectively inhibits cell proliferation in a dose and time-dependent manner. Specifically, at the highest concentration of 320 µg/mL, the observed inhibition rates were approximately 49.06%, 70.79%, and 74.14% for 24, 48, and 72 hours, respectively (Figure 5). These findings suggest significant cytotoxic properties of *B. subtilis* DSM 10 extract, marking it as a potential adjunctive therapeutic agent for breast cancer treatment. The research aligns with recent studies highlighting the cytotoxic effects of bacteria and their metabolites on various cancer types, as noted by Mohan *et al.* (2022), Laliani *et al.* (2020).

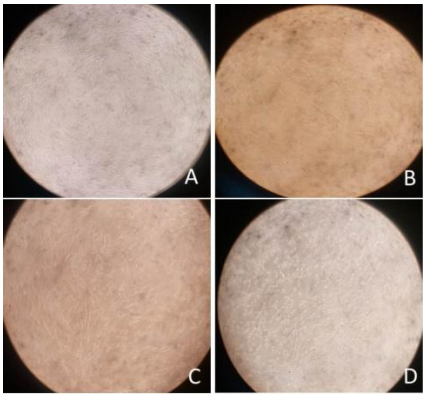

Figure 5. Representative phase contrast pictures of MDAMB-231 breast cancer cells cultured with *B. subtilis* DSM 10 extract for 72 hours- A) Control; B) 80; C) 160; D) 320µg/ml.

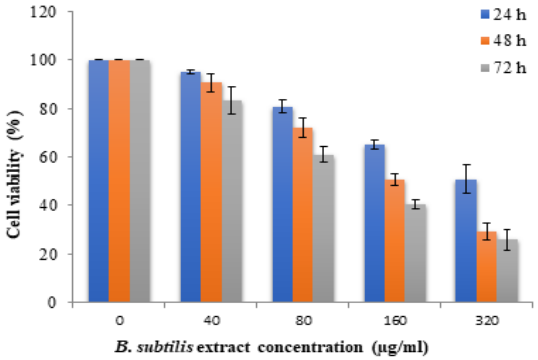

Figure 6. The growth inhibitory effects of *B. subtilis* DSM 10 extract on triple negative breast cancer cell line, MDAMB-231 for 24, 48 and 72 h. Data are expressed as the mean ± SD.

CONCLUSIONS:

This study's results demonstrated that the extract from *B. subtilis* DSM 10, derived from fermented carrot, had potent anticancer activity against the triple-negative breast cancer cells, MDAMB-231. The findings demonstrated a dose-dependent suppression of breast cancer cells. The extract's safety is confirmed, since it demonstrated no hemolytic action on human red blood cells, confirming its appropriateness for usage. Consequently, it may function as an eco-friendly

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and promising bacterial strain proficient in antibiotic production for disease management across several domains. Subsequent study need to concentrate on elucidating the chemical structure of the active compounds or metabolites produced by this strain, as well as examining its enzymatic activity to fully harness its potential as an effective anticancer agent.

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