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Isolation and Identification of Bacillus subtilis for usage as probiotic bacteria

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Abstract

Background and Objective: Bacteria known as probiotics can help the host's health when given in sufficient doses. For many years, Bacillus species have been utilized as probiotics. In the present study, one bacterial isolate of bacteria from Human milk was isolated and identified by morphological, biochemical tests and then confirmed by 16S rRNA sequencing. The strain was positive for Gram stain, Spore formation, Motility, Catalase, Nitrate reduction, Acidity production from glucose, Starch and Casein hydrolysis, Gelatin liquefied, Citrate utilization and Voges-Proskauer reaction. While this strain was negative for KOH, Egg-yolk lecithinase, and Toxin crystal produced. After polymerase chain reaction (PCR) amplification, DNA sample fragments were obtained. These fragments were sequenced and submitted to GenBank at the National Center for Biotechnology Information (NCBI) under the accession number "OP522284". Isolate Bacillus was identified as Bacillus subtilis strain AZ1EGY through 16S rRNA sequencing. In addition, the outcomes also revealed that the Bacillus subtilis strain AZ1EGY presented probiotic characteristics such as tolerance of acidic and bile salt, and as well tolerance of gastric and intestinal juice.

Key words: Human milk; Bacillus Probiotics; characteristics; GenBank; 16S rRNA

INTRODUCTION

Since the field of helpful bacteria was first developed by Russian Nobel winner Elie Metchnikoff, interest in it has increased exponentially. Over time, the term "probiotics," used as an undisputed synonym for "useful bacteria," has acquired acceptance and found use in a variety of clinical and general health scenarios [1]. The World Health Organization defines probiotics as live microorganisms that, when given to a host in sufficient proportions, impart a health benefit by mean of contributing to the balance of the host's gut microbes [2,3,4].

Recently, researchers' focus has turned to the function that probiotics play in human health and disease. This may be partly attributed to the probiotics' good results in preventing and treating diseases, as well as the public's growing interest in alternative treatments. Probiotics such as Lactobacillus spp.; Bifidobacterium spp.; Propionibacterium spp.; Streptococcus spp. and several Saccharomyces species are some of the classic probiotics that have been the subject of studies mostly on the treatment of gastrointestinal (GI) diseases at this time. [5,6]. Yet, the above-mentioned microorganisms scarcely have a chance of surviving in harsh environments [7]. For instance, while classic probiotics like Lactobacillus and Bifidobacterium species have excellent probiotic properties, their survival rates are often low, ranging between 1:15 %, with certain strains being even more ineffective [8,7].

Bacillus spores ingested orally can grow for a certain time, in the intestine, according to several investigations [5,9]. Spore-forming probiotics are gaining popularity because of their ability to survive in unfavorable environmental conditions [10,11]. These attributes enable researchers to create unique functional food products that consumers are needed [12,13].

Commercial Bacillus probiotics (Bacillus subtilis, Bacillus clausii, and Bacillus pumilus) might survive in the mouse GI tract for up to 16 days, according to fecal analyses [5]. Short-chain fatty acids, organic acids, and bacteriocin are among the antimicrobial compounds found in probiotics, which have an antimicrobial and antiadhesion effect on pathogen strains and modify GI diseases [14]. As a result, probiotics of bacillus have pulled the attention of scientists. Certain non-pathogenic Bacillus species are employed as probiotics, albeit they are less well-known than yeasts and lactic acid bacteria (LAB) [15]. Due to their capacity to create spores, these bacteria have significantly higher survival and stability compared to other probiotics. Hence, creating spore-forming probiotics for functional foods is the best option [16].

Bacillus subtilis was examined and revealed no signs of toxicity, according to the European Scientific Committee on Animal Nutrition. Animal investigations on acute and chronic toxicity also supported the safety of these strains. The Food and Drug Administration (FDA) has typically recognized Bacillus subtilis as safe, indicating that it is not dangerous to humans

[17]. The European Food Safety Authority (EFSA) has deemed Bacillus subtilis species as safe. As a result, the Bacillus subtilis strain is safe for eating by humans and is not harmful [18]. The ideal multipurpose probiotic bacterium for humans could be Bacillus subtilis [17].

The goal of this study was to identify a new Bacillus subtilis strain that may be used as a probiotic in the food industry. As a result, different strains of Bacillus were isolated from human breast milk, identified using morphological and biochemical tests, and then verified through 16S rRNA sequencing. Further, their probiotic properties were characterized such as tolerance to gastric and intestinal juices as well as resistance to low pH and high bile salt concentrations.

Methods

Samples:

Twenty samples were collected from different healthy mother breast milk all samples were collected from Gharbia and Beheira City, Egypt. The samples were collected in sterile glass containers. All samples were transferred to a laboratory in the ice box and analyzed on the same day.

Preparation of samples to isolate some of Bacillus spore:

A portion of 1 ml of each mother's breast milk sample was added to sterilized tubes with 9 ml of sterilized physiology solution. And then heated at 80ºC for 10 min and cooled in melting ice prior to plating, and the serial dilution technique was applied.

Isolation of some species of Bacillus genus:

To isolate Bacillus spore-forming bacteria after preparation of samples were determined using the pour plate method by using trypticase soya agar (TSA) or Nutrient Agar (NA), followed by incubation for 24 hr. at 37 ºC under aerobic conditions. After that the typical colonies isolated on TSA or NA were transferred to slant nutrient agar which were incubated for 24 hr. at 37 ºC under aerobic condition. A maximum of four typical colonies from each sample was retained for complementary tests, after four successive purifications on the same medium. A stock culture of each isolated culture was maintained through bimonthly transfers on trypticase soya agar and stored at 4 ºC. All isolates were transferred before use from stock culture into trypticase soya broth and incubated at 37 ºC for 24 hr. under aerobic condition. Subsequently, to insure, an active culture, two transfers of each culture to new tubes of TSB were made, followed by incubation as described by [19].

Morphological and biochemical tests of isolated aerobic spore-forming bacteria:

Isolated typical colonies from TSA or NA were tested for Gram-staining according to [20], KOH [21], catalase activity [22], Gelatin Liquefaction [23]. All isolates were also examined for additional tests such as motility, catalase, nitrate reduction, Utilization of citrate, acidity production from glucose, Starch Hydrolysis, Utilization of Citrate, Voges-Proskauer reaction, Egg-yolk lecithinase and Toxin crystal produced according to [19]. To evaluate the Hydrolysis of casein, the strains were plated on milk agar. The milk agar was prepared with nutrient agar (NA) supplemented with 1% skimmed milk powder according to [24]. The plates were incubated at 30 ºC for 48 hours and 10 ºC for 7 days and considered positive the colonies with transparent halo.

Molecular Identification:

DNA Extraction:

DNA was extracted from the bacteria and actinomycetes according to [25], this described method is an improved method of the standard phenol/chloroform method described by [26].

16S analysis:

a) PCR Reactions:

The PCR amplification was performed in a total volume of 50 µl, containing 1X reaction buffer, 1.5 mM MgCl2, 1U Taq DNA polymerase (Promega), 2.5mM dNTPs, 30 picomole (pmol; 1 picomole = 10^-12 mole) of each primer and 30 ng genomic DNA.

Primer Code	Sequence	Product Size
27F	5′- AGAGTTTGATCCTGGCTAG -3′	1500bp
1492R	5′- GGTTACCTTGTTACGACTT -3′	

b) Thermo-cycling PCR program:

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 30 sec., an annealing step at 54ºC for 30 sec. and an elongation step at 72ºC for 1 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

c) Detection of the PCR Products:

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. A 100bp DNA ladder was used as a molecular size

standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000).

Purification of PCR Products:

Amplified products for all PCR were purified using EZ-10 spin column PCR products purification PCR reaction mixture was transferred to 1.5 ml microfuge tube and three volumes was added of binding buffer 1 after that the mixture solution was transferred to the EZ-10 column and let it stand at room temperature for 2 minutes after that centrifuge, 750 µl of wash solution was added to the column and centrifuge at 10.000rpm for two minutes, repeated washing, 10.000 rpm was spine for an additional minute to remove any residual wash solution. The column was transferred into a clean 1.5 ml microfuge tube and adds 50 µl of elution buffer, incubated at room temperature for 2 minutes and when store purified DNA at -20 °C.

16S sequencing analysis:

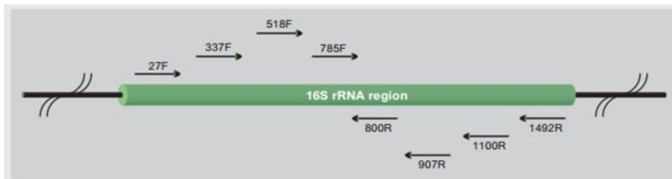
The sequencing of the product PCR was carried through in an automatic sequencer ABI PRISM 3730XL Analyzer using Big Dye TM Terminator Cycle Sequencing Kits following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using Rbcl Forward primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Microgen Company).

Computational analysis (BLASTn) ITS:

The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) Sequences were aligned using Align Sequences Nucleotide BLAST.

Identification by 16S rRNA:

All bacteria contain 16S ribosomal RNA (rRNA) genes of approximately 1500bp in length. RNA genes contain regions of variable DNA sequence that are unique to the species carrying the gene. The species identity of an unknown bacterium may therefore be deduced from its unique rRNA gene sequence rRNA genes are first amplified using PCR technology. After amplification, PCR cycle sequencing is performed, and the rRNA sequence determined using a capillary sequence analyzer. The resulting sequence is then matched to known rRNA sequences in Gen-Bank® and validated using a rigorous review process.



Probiotic properties of isolated strains:

Acid tolerance:

All strains were evaluated for their ability to grow in low pH values (1.5, 2 and 3) the method which described by [27].

Bile tolerance:

Bile tolerance was estimated as described by [27].

Tolerance of artificial gastric juice (AGJ) and artificial intestinal juice (AIJ):

Gastric and pancreatic juices were prepared fresh by dissolving pepsin (Sigma- 3050 Spruce Street Saint Louis, Missouri 6310- USA) from porcine stomach mucosa (3g/ L) and pancreatin (Sigma) from porcine pancreas (1g/ L) in sterile saline (5g/ L) (Charteris et al., 1998). Subsequently, the pHs of the gastric and pancreatic preparations were adjusted to 2.0 and 8.0 with 5 M/L HCl or 1M/L NaOH, respectively.

RESULTS AND DISCUSSION

Identification and characterization of bacterial isolates by using Morphological and Biochemical tests:

Twenty breast milk samples were obtained from various healthy mothers and used to isolate Bacillus spp. strains by using TSA and NA media. All isolated colonies (43 isolates) grown upon TSA and NA media after being heated at 80ºC for 10 minutes as previously reported were identified at the gender level by mean of several traditional methods according to Berge's Manual 2009 [28] such as Gram stain, Spore formation, Motility, Catalase, Nitrate reduction, Acidity production from glucose, Starch hydrolysis, Casein hydrolysis, Gelatin liquefied, Citrate utilization, Voges-Proskauer reaction, Egg-yolk lecithinase, and Toxin crystals produced.

The characteristics of isolated strains are generally presented in Table (1). The data obtained in this table showed that 14 (32.6 %) of the 43 isolates were gram positive, rod shape, spore forming, and motility. In contrast, the remaining 29 (67.4 %) strains that gave opposite results from the previous results were excluded. Also, the results in Table (1) clearly indicate that 14 (100 %) out of 14 isolates were able to Catalase production, Utilization of Citrate, glucose fermentation, and were

positive for the Voges-Proskauer test. While these strains gave negative results with KOH, Gas formation from glucose and Toxin crystals production. In addition, from these strains, 12 (85.7%), 5 (35.7%), 8 (57%), 10 (71.4%), 9 (64.3%), 10 (71.4%), and 9 (64.3%) gave positive results with Hydrolysis of starch, Egg-yolk lecithinase, Hydrolysis of casein, Gelatin liquefied, Nitrate reduction, Growth in NaCl 7% and Growth at 50 °C, respectively.

Table (1): Morphological and Biochemical tests used for the identification of isolates:

Source of samples	No. of samples	No. of isolates	Morphological tests by used Microscope							
			Gram staining		Morphology		Spore-forming		Motility	
			+	-	Rods	Cocci	+	-	+	-
Mother breast milk	20	43	28	15	17	11	14	3	14	-

Biochemical tests used for identification of isolates																	
No. of isolates	KOH		Catalase production		Starch Hydrolysis		Egg-yolk lecithinase		Hydrolysis of casein		Voges Proskauer		Gelatin liquefied		Nitrate reduction	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
14	-	14	14	-	12	2	5	9	8	6	14	-	10	4	9	5

Biochemical tests used for identification of isolates												
No. of isolates	Utilization of Citrate		Toxin crystal produced		Acid from glucose		Gas from glucose		Growth in NaCl 7%		Growth at 50 °C	
	+	-	+	-	+	-	+	-	+	-	+	-
14	14	-	-	14	14	-	-	14	10	4	9	5

Molecular characterization of the tested isolate:

One strain was chosen from the 14 isolates after initial identification by morphological and biochemical tests because it matched the characteristics of Bacillus subtilis as described in Systematic Bacteriology in Bergey's Manual for the year 2009 [28]. Subsequently, this strain was subjected to molecular analysis.

For one strain of Bacillus studied, the results of the PCR technique amplification of fragments and electrophoresis fragments using the 16S ribosomal RNA (rRNA) gene in a 1.5% agarose gel are presented in Fig (1). Based on the partial sequence of the 16S ribosomal RNA (rRNA) gene, strain identification was carried out. The results shown in Fig (1) indicate the amplified fragment of 1380 bp from the mother's breast milk obtained in lane (1) was Bacillus subtilis.

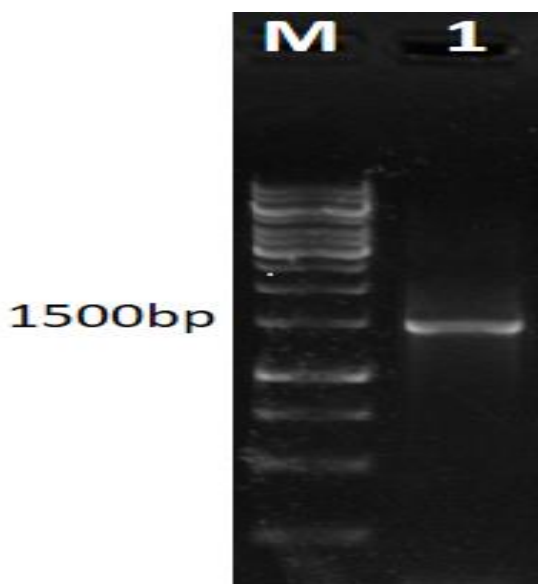


Figure (1): PCR amplified fragments and electrophoresis analysis using 16S ribosomal RNA (rRNA) gene in a 1.5 % agarose gel, for (1) Bacillus subtilis AZ1EGY (Lane M: DNA ladder), amplified fragment 1380 bp from Mother breast milk.

BLASTN-NCBI alignments of Bacillus subtilis sequences:

BLASTN analysis of Bacillus subtilis AZ1EGY sequence is shown in Figure (2) using the 16S ribosomal RNA (rRNA) gene. Through using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) against the nucleotide database, this resulting sequence exhibited considerable alignment with the other accessions of NCBI databases, showing strong similarity to nearly 100 strains of Bacillus subtilis as shown in Figure (2). The accession number "OP522284" had the highest similarity score, with 100% Query Cover and 99.42% similarity.

Phylogenetic relationships among the species of the genus Bacillus:

According to the information in Figure (3), 16S ribosomal RNA (rRNA) gene sequences allow researchers to determine the relationships between closely related species. Phylogenetic evidence reveals that strains of the same species are more closely related than those of other species. We have shown that the rates of divergence of the 16S ribosomal RNA (rRNA) gene sequences can discriminate across subspecies of the same species. Hence, the phylogenetic tree was created using the BLAST tree construct in https://www.ncbi.nlm.nih.gov/blast/treeview based on Fast Minimum Evolution, which was based on 16S ribosomal RNA sequence comparisons, length polymorphism of the PCR-amplified, and sequences from the database.

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all *100 sequences selected* — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus subtilis strain AZ1EGY 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2549	2549	100%	0.0	100.00%	1380	OP522284.1
Bacillus subtilis strain K1 chromosome, complete genome	Bacillus subtilis	2505	25040	100%	0.0	99.42%	4060823	CP113256.1
Bacillus subtilis strain HBUAS74019 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2505	2505	100%	0.0	99.42%	1514	OP904234.1
Bacillus stercoris strain D7XPN1 16S ribosomal RNA, partial sequence	Bacillus stercoris	2505	2505	100%	0.0	99.42%	1455	NR_181952.1
Bacillus subtilis strain O4 chromosome, complete genome	Bacillus subtilis	2505	25046	100%	0.0	99.42%	4217848	CP112873.1
Bacillus subtilis strain K24 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2505	2505	100%	0.0	99.42%	1436	OP836620.1
Bacillus subtilis strain MG-1 chromosome, complete genome	Bacillus subtilis	2505	25001	100%	0.0	99.42%	4356867	CP110634.1
Bacillus sp. (in: Bacteria) strain KO3 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bact…	2505	2505	100%	0.0	99.42%	1524	OP788114.1
Bacillus subtilis strain CHD1 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2505	2505	100%	0.0	99.42%	1483	OP787953.1
Bacillus subtilis strain MX50 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2505	2505	100%	0.0	99.42%	1451	OP782434.1
Bacillus sp. (in: Bacteria) strain LMR1031 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bact…	2505	2505	100%	0.0	99.42%	1462	OP750285.1
Bacillus subtilis strain XP chromosome, complete genome	Bacillus subtilis	2505	24946	100%	0.0	99.42%	4049856	CP072845.1

Figure (2): BLASTN-NCBI alignments of Bacillus subtilis AZ1EGY sequences.

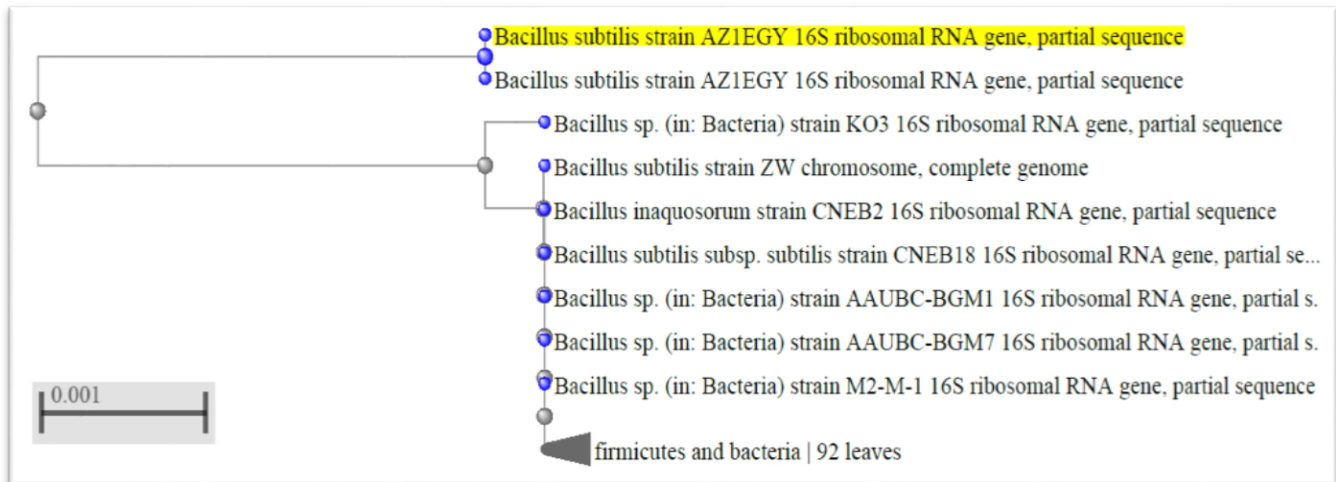


Figure (3): The relationships between individuals belonging to the Bacillus subtilis AZ1EGY are presented in a phylogenetic tree based on 16S ribosomal RNA sequences.

Database submissions and accession numbers:

The flat file in Figure (4) shows that the first report describing the identification of a new Bacillus subtilis strain by isolating from the mother's breast milk in Egypt using molecular identification. Then, the NCBI database's Genebank presented its sequence analysis findings. It has been confirmed to be entered into Genbank and made available to the public under the new accession number "OP522284" under the name Bacillus subtilis AZ1EGY. Probiotic criteria for this strain were studied as will be seen later.

Probiotic criteria of Bacillus spp. Isolate:

Live microorganisms that, when taken in sufficient quantities and supplied to the gastrointestinal tract, boost the consumer's health are known as probiotics. Yet, isolated bacteria must integrate into the regular microbial flora in the intestine, endure the digestive process, and be able to stick to and colonize the intestinal tract in order to be considered probiotics [29]. A healthy person's gastrointestinal tract is a tough environment since it contains bile acids, digesting enzymes, and gastric juices. Probiotic strains are seriously threatened by these conditions.

To achieve consistent and beneficial probiotic effects, strict selection criteria for the identification of probiotic strains are necessary. Collins et al [30] recently produced a list of 12 crucial factors to consider when choosing a possible probiotic strain. These requirements basically said that the chosen strains had to be able to withstand low pH, high bile concentration, and survive gastric and intestinal juices.

Tolerance of acidic:

Bacteria that are added to foods as probiotic adjuncts are frequently ingested, so they start their journey to the lower intestine tract by the mouth. From the moment of entry to the time of release from the stomach, according to [31], is 90 minutes. Although the stomach has a pH as low as 1.5, where cellular stress first manifests itself [32], pH 3.0 has typically been employed in vitro tests [33]. This might be because strain viability is frequently seen to decline significantly at pH 2 or lower [34].

As a result, the tolerance of one identified Bacillus spp. to low pH (1.5, 2 and 3) in TS broth was tested. Table (2), which summarizes the results, showed that the tested strains appeared to be more harmed by pH 1.5, which was used in the current investigation. According to [35], the bactericidal effect of acid is visible at pH levels lower than 2.5.

After 180 minutes of incubation at pH 1.5, it was also evident that B. subtilis AZ1EGY was acid-tolerant and had a viable cell count of 11.83 log CFU/ml. It is intriguing to note that following 180 minutes of exposure at pH 1.5.

Interestingly, the obtained results strongly suggested that B. subtilis AZ1EGY successfully survived in low pH (1.5, 2.0, and 3.0) and recorded variable increases in their populations. However, the percentage of increases as viable cell counts at pH 1.5 throughout incubation time (180 minutes), was 2.60%, while at pH 2.0 was 2.97 %, whereas high figures were detected at pH 3.0 was 6.59 %.

Bacillus subtilis strain AZ1EGY 16S ribosomal RNA gene, partial sequence

GenBank: OP522284.1

FASTA Graphics

Go to:

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LOCUS       OP522284                1380 bp    DNA     linear   BCT 30-SEP-2022
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            sequence.
ACCESSION   OP522284
VERSION     OP522284.1
KEYWORDS    .
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  ORGANISM  Bacillus subtilis
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1380)
  AUTHORS   Nour,M.A., Ali,H.M., Soliman,S.A. and Elkholy,A.I.
  TITLE     Isolation and identification of bacillus subtilis and their use as
            probiotic bacteria
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1380)
  AUTHORS   Nour,M.A., Ali,H.M., Soliman,S.A. and Elkholy,A.I.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-SEP-2022) DAIRY - FACULTY OF AGRICULTURE, AL-AZHAR
            UNIVERSITY, El_Mokhayam El_Daem, Nasr City, 11651 11651, Egypt
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//
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Accession No. OP522284

Figure (4): NCBI Flat file for Egyptian *Bacillus subtilis AZ1EGY* 16S ribosomal RNA gene, partial sequences.

Tolerance of Bile salt:

The ability of a bacteria to survive and grow in the conditions present in the intestine is the most important factor to consider when choosing it to use as a probiotic. It has been proven that the entry of bile salt into the duodenal region of the small intestine reduces the survival of bacteria once it enters the intestine. The catabolism of cholesterol in the liver produces surface-active compounds known as bile salts [36]. Probiotics must be able to tolerate bile [37].

Although bile salt concentration fluctuates, the human GI tract's average intestinal bile salt concentration is thought to be 0.3% w/v [33,38]. So, most studies utilize this quantity of bile salt to check for bile-resistant bacteria [39,40]. To simulate approximate levels in the digestive tract, three distinct concentrations of bile salts 0.3, 0.5, and 1.0% (w/v) ox gall were utilized in the current investigation. Data collected are displayed in Table (3). B. subtilis AZ1EGY strain generally demonstrated its resistance to bile salt. After 12 hours of incubation, improved growth was shown in media with 0.3% bile salts and the count of viable cells was 14.25 log cfu/ml. Notably, after 12 hours of incubation, the Bacillus subtilis AZ1EGY strain exhibited increased growth in media containing 0.3% bile salts, with a growth increase of 45.55%. Also, after 12 hours of incubation, it was capable to grow positively in the existence of 0.5 and 1% bile salts.

Table (2): Impact of low pH on the survival of Bacillus spp.:

Strains	pH	Time of incubation						
		Zero	60 min.		120 min.		180 min.	
		log cfu/ml.	log cfu/ml.	% increase	log cfu/ml.	% increase	log cfu/ml.	% increase
Bacillus subtilis AZ1EGY	1.5	11.53	11.97	3.82	12.02	4.25	11.83	2.60
	2	12.13	12.72	5.11	13.21	8.90	12.49	2.97
	3	11.38	12.69	11.51	13.53	18.89	12.13	6.59

Increase% in viable count = ((log cfu/ml at 1,2 and 3 h - log cfu/ml at 0 h) / log cfu/ml at 0 h) × 100

cfu = colony forming unit

Table (3): Impact of bile salts concentration on viability of tested Bacillus spp.:

Strains	Bile salts Concentration %	Time of incubation		
		Zero	12 hr.	
		log cfu/ml.	log cfu/ml.	% increase
Bacillus subtilis AZ1EGY	0.3	9.79	14.25	45.55
	0.5	10.28	14.01	36.28
	1	8.81	11.91	35.19

Increase% in viable count = ((log cfu/ml at 1,2 and 3 h - log cfu/ml at 0 h) / log cfu/ml at 0 h) × 100

cfu = colony forming unit

Bacillus spp. were tested for survival in simulated gastric and small intestine fluids:

Each day, roughly 2.5 L. of gastric juice with a pH of 2.0 and a salt concentration of at least 0.5% w/v are secreted [41]. In contrast, roughly 0.7 L. of pancreatic juice, with a pH of about 8.0 and a salt concentration of at least 0.5% w/v, is secreted into the proximal intestine each day [42].

These secretions work in conjunction with bile salt and peristalsis to prevent the resting small intestine from becoming highly colonized except under situations of stasis. They also operate as a pH and enzymatic barrier to the survival of ingested bacteria during digestion. Thus, it was discovered that surviving gastrointestinal transit was a crucial functional characteristic of the investigated probiotic bacteria [43,44].

The vitality of the B. subtilis AZ1EGY strain tested in this part of the study was examined in relation to the impact of simulated stomach and small intestine fluids. In brief, 1.0 ml of either gastric juice (pH 2.0, 0.3% pepsin), or intestinal juice (pH 8.0, 0.1% pancreatin), and 0.2 ml of B. subtilis AZ1EGY strain were mixed. Then, the mixtures were incubated at 37°C and the sampling intervals for determining the total viable count were after 60, 120, and 180 minutes and also, after 120, 240, and 360 minutes, respectively, while testing intestinal or stomach tolerance. Two times the experiment was repeated.

Survival in gastric juice simulator:

Table (4) displays the outcomes of gastric juice tolerance. In general, the Bacillus subtilis AZ1EGY strain displayed gastric juice resistance. After 60 minutes of incubation, where demonstrated growth of the Bacillus subtilis AZ1EGY strain in gastric juice and had a value for viable cell counts of 14.84 log cfu/ml. and a ratio of growth increase was 8.56%. However, within 180 minutes, the viable cell counts had increased to 14.32 log cfu/ml, and the growth rate had increased by 4.75 %. Last but not least, our findings demonstrated that Bacillus subtilis AZ1EGY was thought to be intrinsically tolerant of gastric juice and could pass through the human stomach, reach the intestinal tract, and function there.

Survival in intestinal juice simulator:

Table (5) presents the impact of simulated intestinal juice (SIJ) on the viability of the Bacillus subtilis AZ1EGY strain. According to the results, the B. subtilis AZ1EGY strain is intrinsically tolerant to intestinal transit because it maintained viability during growth in simulated small intestinal juice. In this regard, according to [45], the majority of probiotic strains were naturally resistant to simulated pancreatic juice and did not exhibit any decreased viability up to 4 hours. Whilst different levels of intestinal juice tolerance have been noted before [46,47,48].

Also, a number of authors have previously come to the conclusion that strains may affect variances and the degree of responsiveness to intestinal juice [49, 46, 50, 47].

The Bacillus subtilis AZ1EGY strain was able to retain vitality, according to the results shown in table 5. After 360 minutes of exposure to simulated intestinal juice (SIJ), the growth rate increased by 13.37%, as a result, this strain is deemed to be intrinsically tolerant of intestinal juice.

Table (4): Impact of simulated gastric juice on the survival of examined Bacillus spp.:

Strains	Time of incubation						
	Zero	60 min.		120 min.		180 min.	
	log cfu/ml.	log cfu/ml.	% increase	log cfu/ml.	% increase	log cfu/ml.	% increase
Bacillus subtilis AZ1EGY	13.67	14.84	8.56	15.25	11.56	14.32	4.75

Increase% in viable count = ((log cfu/ml at 1,2 and 3 h - log cfu/ml at 0 h) / log cfu/ml at 0 h) × 100

cfu = colony forming unit

Table (5): Impact of simulated intestinal juice on the survival of examined *Bacillus spp.*:

strains	Time of incubation						
	Zero	120 min.		240 min.		360 min.	
	log cfu/ml.	log cfu/ml.	% increase	log cfu/ml.	% increase	log cfu/ml.	% increase
Bacillus subtilis AZ1EGY	11.67	12.32	5.57	12.98	11.23	13.23	13.37

Increase% in viable count = ((log cfu/ml at 1,2 and 3 h - log cfu/ml at 0 h) / log cfu/ml at 0 h) × 100

cfu = colony forming unit

SIGNIFICANCE STATEMENT

It can be concluded that Bacillus subtilis AZ1EGY strain of healthy mother breast milk was isolated, and identified by morphological, biochemical tests and then confirmed by 16S rRNA sequencing. This strain was negative for the Egg-yolk lecithinase test and did not produce a Toxin crystal. After polymerase chain reaction (PCR) amplification, DNA sample fragments were obtained. These fragments were sequenced and submitted to GenBank (NCBI) under the accession number "OP522284". The probiotic properties of the B. subtilis AZ1EGY strain included acidic tolerance, bile salt tolerance, and gastric and intestinal juice tolerance. The B. subtilis AZ1EGY strain may be employed as probiotic bacteria, according to these findings. So, B. subtilis AZ1EGY is a promising probiotic strain and needs many medical studies for its use in functional and therapeutic foods.

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