



Screening and Production of Industrially milk Clotting by *Bacillus subtilis* carries a Potential Probiotic Character



Nouran A. Elattal¹, Eman A. Karam², Amany L. Kansoh², Mona A. Esawy^{1*}

¹Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Cairo, Egypt.

²Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt.

Abstract

The safety and potential of enzymes is the most significant trend in the microbial food sector. One excellent source of safe enzymes was the probiotic bacteria. The probiotic bacteria were a good source for safe enzyme. In this finding, among 23 honey isolates 13 isolates were detected as milk clotting producers and the most potent producer was the isolate No2 (2000 SU/ml). The most favorable conditions for enzyme production were 40 °C in the presence of maltose and soybean (2714 SU/ml). The microorganism safety was confirmed by the antimicrobial sensitivity and negative blood hemolysis. The isolate showed a promising antimicrobial activity against different pathogens such as *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*, *Candida albicans*, and *Asperigillus niger*. The isolate tolerated the temperature elevation until 80°C and up to 3M NaCl. The isolate could tolerate the severe acidic condition and remained 68.68% of its growth after incubation at pH2 for 6h. The isolate tolerated the 0.3% bile salt and remained 88% of survival cells and exceeding the survival cells of *L.casi* (81%). Our findings suggested that, compared with other types of probiotics, honey probiotics may be a better choice for producers and consumers.

Keywords: Honey isolates-probiotic-milk clotting-*Bacillus subtilis*

1. Introduction

The microorganism source had a significant role in enzyme potentiality and safety. Honey isolates were previously reported by our group as an active strain produced potential enzymes such as levansucrase, amylase and milk clotting [1,2,3]. With the increasing demand for the milk clotting, their production has continuously attracted great research interest. Newborn ruminants' abomasum, or fourth stomach, is a major source of Chymosin (EC 3.4.23.4), also known as renin, is used in the dairy industry to coagulate milk, which is essential for the manufacture of cheese. But there are many issues limited the use of animal Chymosin (EC 3.4.23.4) such as the scarification of new born ruminants. Unquestionably, scientists are having difficulty finding potential milk clotting agent that can entirely replace animal rennet. Accordingly, attention has been focused on the production of milk-clotting enzymes (MCEs) from microbial sources for use as rennin substitutes. *Bacillus* sp. was mentioned in different researches as a milk clotting producer. *Bacillus* species, *Bacillus amyloliquefaciens* [3], *Bacillus velezensis* [4] *Bacillus methanolicus* [5], *Bacillus subtilis* [6], *Paenibacillus* spp [7], *Bacillus licheniformis* [8]. The safety of bacteria is crucial to the processes of milk coagulation and cheese maturation, has a substantial impact on the safety of cheese. Probiotic bacteria are a good example for save and benefit bacteria which could produce beneficial and efficient bioproducts. Probiotic strains have been shown to be capable of producing fermented milk products with strong inhibitory and antioxidant properties. Certain strains of lactobacilli, which can be found in traditional foods, have significant health benefits because they help balance the intestinal microbiota and control the immune system [9]. Probiotics may be utilized as an adjuvant in the treatment of gastrointestinal disorders, cancer, high blood pressure, and high cholesterol because of their capacity to alter gut flora and weaken the immune system [10]. According to Guo et al. [11], using fermented dairy products that contain lactic acid bacteria (LAB) with probiotic properties is one practical strategy to reduce cow milk allergy (CMA). This study emphasized honey as an excellent source for highly productive milk-clotting bacteria. The isolate that produced the highest amount of milk-clotting enzyme was identified as *Bacillus subtilis*. Various physiological and environmental factors affecting enzyme production by the isolate were examined. The probiotic properties of the isolate were evaluated from multiple perspectives. The research presented *Bacillus subtilis* as a safe and efficient milk-clotting strain with potential applications in the dairy industry.

2. Results

2.1 Isolation, screening and identification

Twenty-three bacterial isolates were screened for their milk clotting enzyme production. Thirteen isolates showed milk clotting activity with a considerable activity ranged from (110-2000 SU/ml). The highest activity was obtained by *Bacillus subtilis* (2000SU/ml), and NO 19,11 (1200SU) and the lowest activity was obtained by NO14 (110 SU/ml). The most potent isolate was identified as *Bacillus subtilis* based in morphological shape and 16sRNA (Table 1).

*Corresponding author e-mail: mona_esawy@hotmail.com; (Mona A. Esawy).

Receive Date: 12 April 2025; Revise Date: 03 May 2025; Accept Date: 26 May 2025

DOI: <https://doi.org/10.21608/ejchem.2025.375021.11588>

©2026 National Information and Documentation Center (NIDOC)

2.2 Optimization of the MCE

The most proper conditions for enzyme production were investigated. The highest milk clotting activity was obtained at 24h above and below this hour the enzyme production decreased gradually. The maximum MCE was obtained at 40 °C, the decrease or increase apart from this degree had adverse effect on the MCE production. Maltose was the best carbon for enzyme production \ (2400 SU/ml U). Starch had a negative impact in enzyme production (1000 SU/ml), followed by fructose (1500 SU/ml) and mannose (1750 SU/ml U). Evaluation of different nitrogen sources on enzyme production was achieved. Soybean and peptone had the highest enzyme production (2714 SU/ml). KNO₃ had adverse effect on the MCE activity and led to about 60% decrease on enzyme production. Also, the urea and gelatin inhibited the enzyme production with degree of variations (**Table 2**).

Table 1. Screening for milk clotting enzyme from different bacterial isolates

Number of strain	Diameter of clear zone (mm)	Activity of enzyme (SU/ml)
1	-	-
2	25	2000
3	-	-
4	14	600
5	13	400
6	12	343
7	13	218
8	-	-
9	-	-
10	-	-
11	20	1200
12	12	240
13	-	-
14	11	110
15	17	750
16	12	480
17	15	800
18	13	600
19	18	1200
20	-	-
21	-	-
22	-	-
23	-	-

Table. 2. Optimization of *Bacillus subtilis* milk clotting by different factors

Incubation time (h)	6	12	24	36	48	50	62
Activity (SU/ml)	400	1200	2000	1750	1500	1000	400
Temperature °C	25	30	35	40	45	50	55
Activity (SU/ml)	400	1200	2000	2181	2000	1000	400
Carbon source	Lactose	Glucose	Fructose	Mannose	Maltose	Sucrose	Starch
Activity (SU/ml)	2200	2200	1500	1750	2400	1500	1000
Nitrogen source	peptone	Casein (control)	Gelatin	Na NO3	Urea	soyabean	KNO3
Activity (SU/ml)	2714	2000	1750	800	1500	2714	1000

2.3 Antibiotic susceptibility

The antibiotic susceptibility on different types of antibiotics were investigated for their ability to inhibit the *Bacillus subtilis*. The result showed that the isolate was sensitive to Streptomycin* (10µg) Gentamycin* (10µg) Chloramphenicol* (30µg) Tetracycline* (30µg) Vancomycin*(30µg) Ampicillin* (10µg) and Erythromycin* (15µg) (**Table 3**). All the tested antibiotic recorded clear inhibition zone. The range of inhibition was (14-26) depend in the type of the used antibiotic.

Table 3: Antibiotic susceptibility profiles of *Bacillus subtilis* to different antibiotics.

Isolate No.	Inhibition zone (mm)						
	Streptomycin * (10µg)	Gentamycin * (10µg)	Chloramphenicol * (30µg)	Tetracycline * (30µg)	Vancomycin * (30µg)	Ampicillin * (10µg)	Erythromycin * (15µg)
<i>Bacillus subtilis</i>	12± 1.41 (S)	15± 0.70 (S)	24 ± 2.82 (S)	21 ± 2.82 (S)	14 ± 0.70 (I)	26± 1.41(S)	19± 2.82 (S)

Experiment was done by disc diffusion method. Inhibition zone diameter measured in mm as (mean ± SD). S, susceptible; I, intermediate.

*antibiotic-impregnated discs with amount, in µg shown in brackets.

Table 4: Antimicrobial activity of *Bacillus subtilis* against certain pathogens

	Gram positive		Gram Negative		Fungi and Yeast	
	<i>Bacillus cereus</i> (a)	<i>Pseudomonas aeruginosa</i> (a)	<i>Salmonella typhi</i> (a)	<i>Escherichia coli</i> (a)	<i>Candida albicans</i> (b)	<i>Asperigillus niger</i> (b)
	ATCC10876	ATCC 27953	ATCC 6539	ATCC 25922	NRRL Y-477	NRRL 599
<i>Bacillus subtilis</i>	27 ±2.82	30 ±1.41	24 ±1.41	28 ± 1.41	23 ±1.41	21.5 ± 1.41
Streptomycin (10 µg)	29 ±1.41	33 ±1.41	27 ±2.82	25 ± 2.82	-	-
Clotrimazole (15 µg)	-	-	-	-	28 ± 1.41	27.5 ±1.41

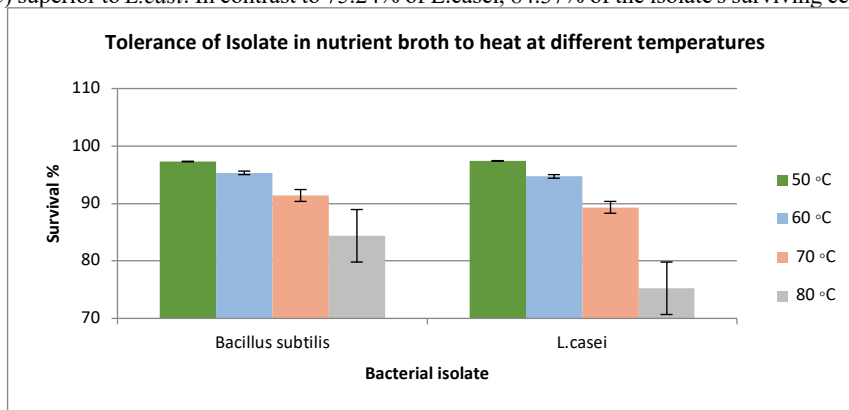
Antimicrobial activity of bacterial isolate by agar diffusion method. (a) grown on nutrient medium agar; (b) on potato dextrose agar; Diameter of inhibition zone measured in mm. each value is as mean±SD, pore size 8 mm.

2.4 Antimicrobial activity

The antimicrobial activities of the *Bacillus subtilis* against different types of microorganisms including (Fungi, bacteria and yeast). The results showed that the isolate had antimicrobial activity against all the tested microbial pathogen. The isolate had antimicrobial activity against *Escherichia coli* (28mm) which exceeded Streptomycin (23mm) (Table 4).

2.5 Tolerance of the isolate to heat

The effect of different temperature on the cell surviving was examined. *Bacillus subtilis* revealed high tolerance to the elevated temperature (89°C) superior to *L.casi*. In contrast to 75.24% of *L.casei*, 84.37% of the isolate's surviving cells were still present.

**Fig 1. Tolerance of *Bacillus subtilis* to heat at different temperatures in nutrient broth media.**

Results shown are average of two replicate experiments and error bars show standard deviation.

2.6 Tolerance of the isolate to NaCl

The tolerance of the vegetative cells of *Bacillus subtilis* to 3M NaCl was investigated after incubation of the cells at 3 and 6 h at 3M NaCl (Fig.2.). The result showed that the isolate *Bacillus subtilis* was high tolerant to NaCl in compared to *L.casie*. For instance, after 6h the surviving cells was 93.27%, 90.32% for the isolate and *L.casie* respectively.

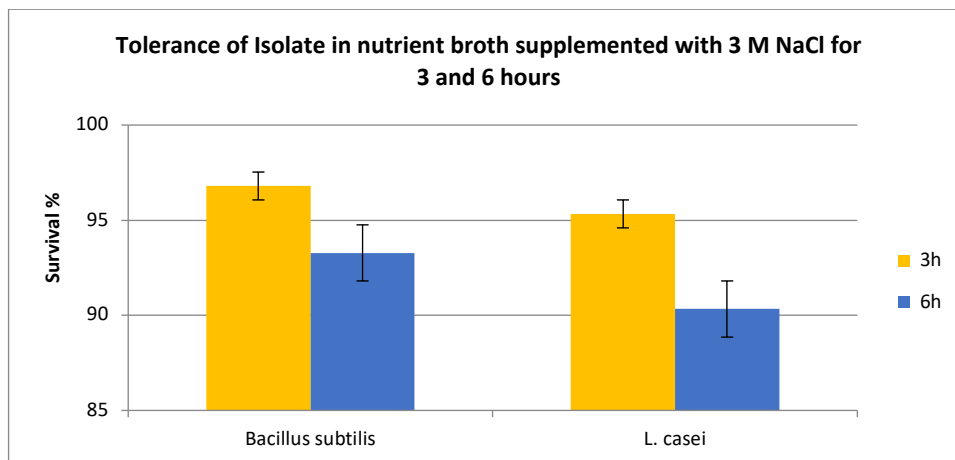
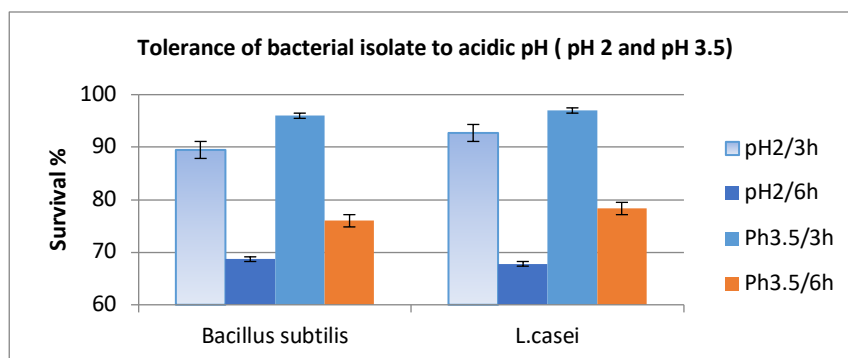


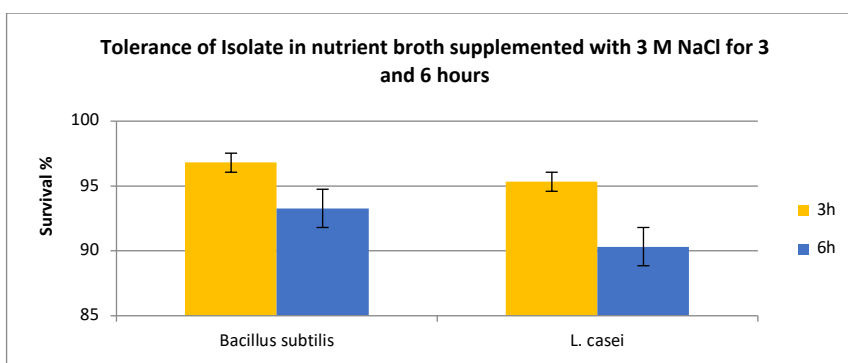
Fig 2. Tolerance of *Bacillus subtilis* in nutrient broth supplemented with 3 M NaCl for 3 and 6 h. Results shown are average of two replicate experiments and error bars show standard deviation.

2.7 Tolerance of the isolate to acidic and alkaline pHs

The incubation of the isolate vegetative cells for 3 and 6 h at acidic pHs and estimation of the remained surviving cells. The results showed that the *Bacillus subtilis* tolerance was in approach with the *L.casie*. For instance, at pH3.5 after 3 incubation, the surviving cells recorded 95.98%,96.82% for the isolate and *L.casie* respectively. After 6-hour incubation at pH3.5 *Bacillus subtilis* and *L.casie* showed (75.99%,78.27%) surviving cells. The results revealed clearly that *Bacillus subtilis* showed high surviving rate at pH2 after 3, 6h incubation (89.74%,68.68%) respectively. This result is near the result of *L.casie* after the same incubation h (92.7%,76.80%) respectively. Also, *Bacillus subtilis* showed a promising tolerance the pH9 at 3 and 6h incubation time. The isolate and *L.casie* recorded surviving cells of 72.87%, 75.70% respectively (Fig.3.).



(a)

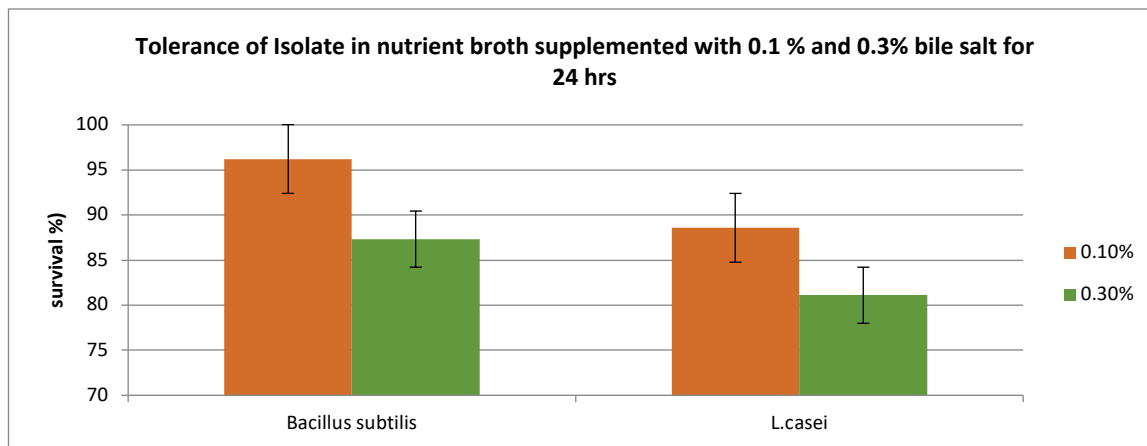


(b)

Fig 3. Tolerance of bacterial isolate to acidic pH (pH 2 and pH 3.5) (a) and alkaline (pH 9) for 3 and 6 h (b). Results shown are average of two replicate experiments and error bars show standard deviation.

2.8 Tolerance to bile salt

Tolerance of *Bacillus subtilis* in nutrient broth supplemented with 0.1 % and 0.3% bile salt for 24 h was tested. The results showed that at 0.1 % bile the surviving cells% of *Bacillus subtilis* (96.21%) was clearly higher than the *L.casie* (88.58%). Also, at 0.3 % bile salt *Bacillus subtilis* recorded (87.32%). (**Fig.4.a**). Tolerance of *Bacillus subtilis* in nutrient broth supplemented with 0.15% pancreatic enzyme for 24 h was examined. The results showed that the surviving cells of the isolate was (91.58% and of *L.casie* was (95.27%)



Tolerance *Bacillus subtilis* in nutrient broth supplemented with 0.15% pancreatic enzyme for 24 hours was examined. The results appeared that the surviving cells of the isolate was (91.58% and of *L.casie* was

(95.27%) (**Fig.4.b**).

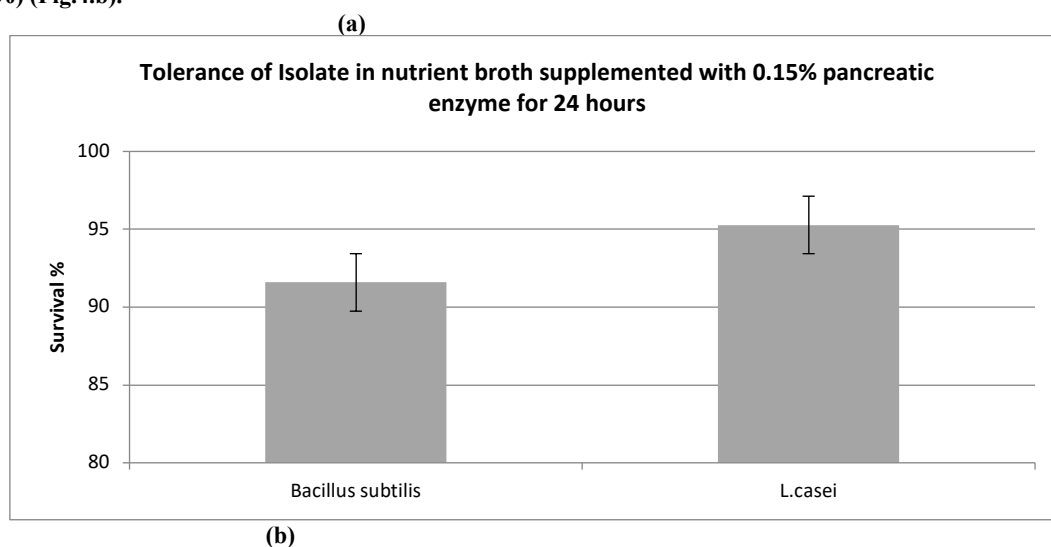


Fig 4. Tolerance of *Bacillus subtilis* in nutrient broth supplemented with 0.1 % and 0.3% bile salt for 24 h (a) and with 0.15% pancreatic enzyme for 24 h (b).

Results shown are average of two replicate experiments and error bars show standard deviation.

Tolerance to H₂O₂

The isolate showed 75.48% tolerance to H₂O₂ in comparison to 71.08% for the control.

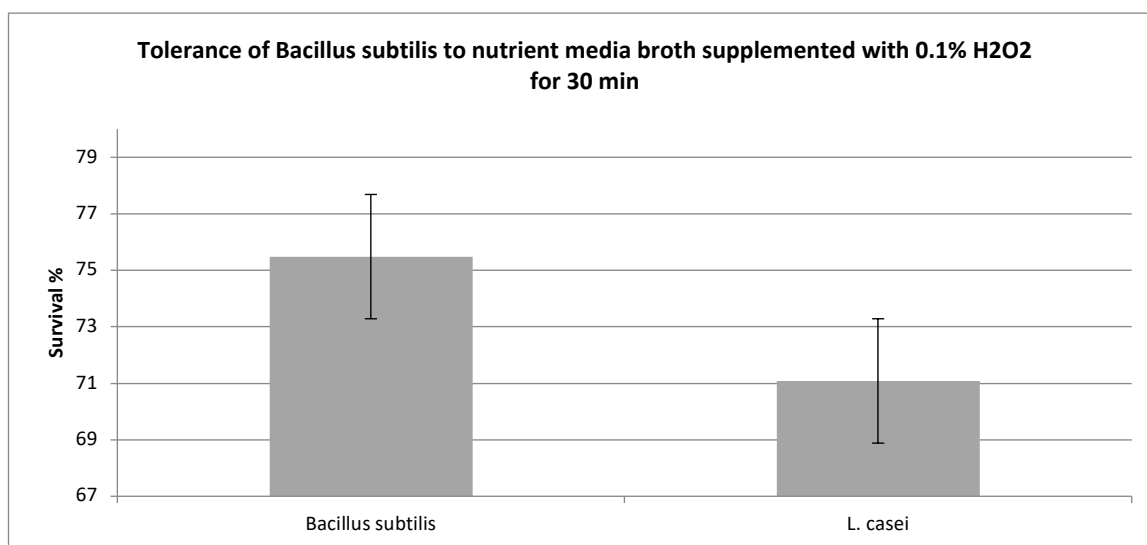


Fig 5. Tolerance *Bacillus subtilis* in nutrient broth supplemented with 0.1% H₂O₂ for 30 min. Results shown are average of two replicate experiments and error bars show standard deviation.

2.9 Antioxidant activity

One recognized method for evaluating antioxidant capacity is a DPPH free radical scavenging. The *Bacillus subtilis* culture filtrate is added to the violet DPPH solution in the DPPH assay in a concentration-dependent manner, reducing it to the yellow product, diphenylpicryl hydrazine. In this work the result of the antioxidant activity of the crude culture of *Bacillus subtilis* milk clotting showed that recorded about 75% which exceeded *L. Casi* by about 4%.

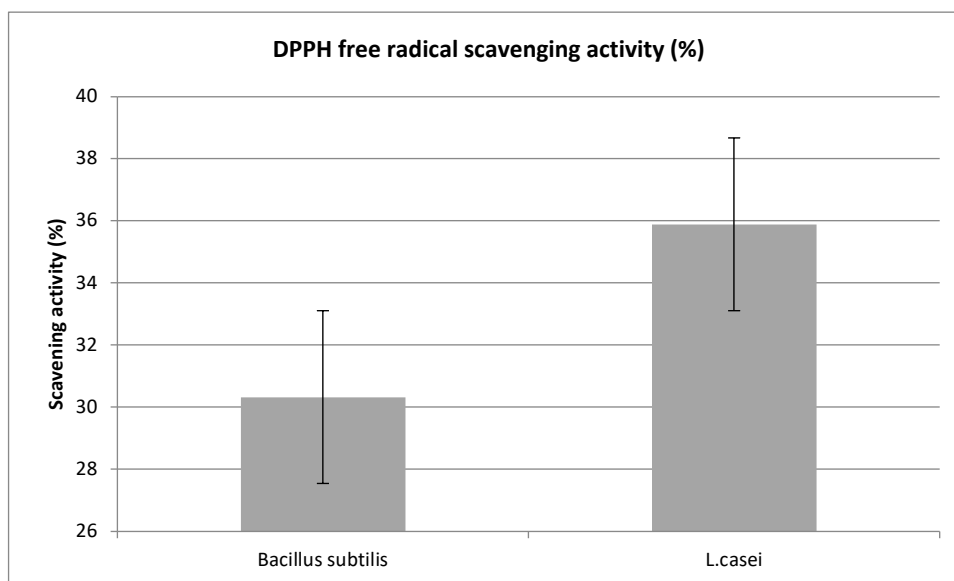


Fig 6. DPPH free radical scavenging activity (%).

Results shown are average of two replicate experiments and error bars show standard deviation.

4. Discussion

The findings demonstrated important developments in the microbial food sector, especially with relation to the safety, efficiency, and possible uses of enzymes. The results of the study showed that bacteria found in honey are prospective sources for the safe production of enzymes, which is essential for a number of applications in food processing [12]. Finding and separating possible probiotic species in honey could greatly increase their use in the food and pharmaceutical sectors, providing a host of health [13]. In this work, out of the 23 honey isolates that were analyzed, 13 were found to produce milk-clotting enzyme. The previous results recommended honey as a new reservoir for potential milk clotting bacteria. In the last few years many authors talk about the honey isolates as an efficient source of important enzymes such as levansucrase [1], amylase [2], invertase [14]. The result detected isolate No. 2 as the most efficient isolate, with an activity level of 2000 SU/ml. This amount of activity was superior to *Bacillus amyloliquefaciens* SP1 milk clotting activity [15] about 21-fold. In terms of production cost,

biodiversity, gene modification, material utilization, and by fermentation control, bacterial MCE outperforms fungal MCE [16]. Additionally, certain bacterial MCE exhibit significant milk-clotting productivity (MCA) and other traits that make them ideal for the production of cheese [17]. In this study, honey bee bacteria showed high productivity without the need to use expensive technologies to increase productivity, which reduces production costs. The isolate was identified based on 16 sRNA as *Bacillus subtilis*. Many authors talk about *Bacillus subtilis* milk clotting [18], [19], [20]. The optimum conditions for enzyme production are an important step to get the proper productivity. In this study, the optimal conditions for enzyme production were found to be 40°C with the presence of maltose and soybean or peptone. These conditions can be leveraged in industrial settings to enhance enzyme yield and efficiency. **Guleria et al. [15]** said the optimum conditions for *Bacillus amyloliquefaciens* SP1 milk clotting enzyme was 30°C in the presence of sucrose and soybean. Microbiological testing is necessary during manufacturing to guarantee the safety of products like polysaccharides and antibiotics. Hemolysis and antibiotic sensitivity caused a big problem to the human health. In this study, the microorganism showed sensitivity to the antibiotics and the absence of hemolysis. These results indicated that *Bacillus subtilis* is not only effective but also safe for consumption. **Hamdy et al. [21]**, recorded *Bacillus subtilis* HMNig-2 and *B. subtilis* MENO2 as non-hemolytic and sensitive to all the tested antibiotics. The antimicrobial activity of *Bacillus subtilis* against a range of pathogenic microorganisms including *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger*—further emphasizes its potential as a natural preservative. This attribute could benefit food producers by extending shelf life and ensuring food safety. **Sharma and lee [22]** said one important consideration when choosing possible probiotic strains is the evaluation of antibacterial activity against infections. Thus, preventing food spoiling and guaranteeing consumer safety, *Bacillus subtilis* resilience to extreme conditions, such as temperatures up to 80°C, high salt concentrations (3M NaCl), and low pH (surviving pH 2 for 6 hours), demonstrated its robustness, making it suitable for various food processing environments. The ability to maintain 88% cell viability in the presence of bile salts, exceeding that of *Lactococcus lactis* (81%), suggest that these honey probiotics could be effective in digestive health applications as well. Previously, honey isolates were detected as high NaCl tolerant and survived at high temperature [21], [23], [24]. The avoidance of free radicals' detrimental effects on the human body and the degradation of lipids and other food ingredients is known as antioxidant activity [25]. *Bacillus subtilis* milk clotting showed encouraging antioxidant properties, according to a recent study. This result pointed the importance of the bacterial isolates in protection against severe diseases such as cancer and peptic ulcer [21, 26]. **del Carmen et al. [27]** reported in the role of antioxidant activity of the lactic acid bacteria in colorectal cancer treatment.

Given that milk clotting enzyme is a crucial step in the making of cheese, this shows that probiotics produced from honey have a lot of potential for application in cheese products. **Fresno et al. [28]** mentioned the effect of feeding dairy calves with milk fermented with selected probiotic strains in curing Calf-diarrhoea. Also, it was reported in the incorporation of fermented dairy items that include lactic acid bacteria (LAB) known for their probiotic benefits has been proposed as an effective method to alleviate cow milk allergy (CMA) [11].

4. Experimental

4.1 Isolation of bacterial strains from mountain Nigerian honey

One hundred microliters of each honey samples (mountain Nigearian honey, Sedr honey and Citrus honey) were spread in petri dishes contained nutrient agar medium (NA). The plates were incubated at 37°C for 24 hours, or until the bacterial colonies were three to five millimeters in diameter. The bacterial isolates were streaked onto agar slants and then stored at 4°C. Microscopy was used to assess the purity of the isolates.

Screening for milk clotting

The twenty-three isolates were screened for milk clotting production. The Basel medium (BM) consisted of g/l: Sucrose 5g/l, casein 6, K₂HPO₄ 1 & MgSO₄ 0.2 was used

4.2. 16S rDNA-Based Molecular Identification

Genomic DNA was extracted from bacterial isolates grown in LB broth using a commercial purification kit. The 16S rRNA gene was amplified via PCR using universal primers 8F and 1492R. PCR products were visualized on agarose gel electrophoresis, purified, and sequenced. Sequence data were analyzed using BLAST for identification and aligned with ClustalW. A phylogenetic tree was constructed using the neighbor-joining method in MEGA X [29]. **The GenBank accession number(s) for isolate nucleotide sequence(s): Bacillus OP223462**

4.3. Medium optimization

Each experiment was done under the optimized conditions. The effect of different incubation temperatures (25–55°C), incubation periods (6–62h), different sucrose concentrations (20–180%), different sodium chloride concentrations (0.5–5%), different carbon sources (5g/L) such as (Starch, Sucrose, Maltose, Mannose, Fructose, Glucose and Lactose), different nitrogen sources were substituted at equivalent amounts of nitrogen such as (KNO₃, soyabean, Urea, Na NO₃, Gelatin, Casein (control) and peptone) The optimized medium contents (g/L): sucrose, 120; yeast extract, 1; MgSO₄, 0.2; K₂HPO₄; starch 5; NaCl 5; Baker yeast 12.5; and AlCl₃ (5µM)

Characterization studies

4.4. Heat resistance

Tolerance of strains to heat was determined according to **Chaiyawan et al. [30]** with different range of temperature and incubation time. Freshly prepared vegetative cells were heated for 30 min at a range of temperatures of 50, 60, 70 and 80 °C.

Culture cells were transferred to nutrient broth (NB) and incubated for 24 h at 37 °C. Control was prepared by cultivation of non-heated cells at 37 °C for 24 h and viable count was determined.

4.5. NaCl and H₂O₂ tolerance

Vegetative cells of isolates were incubated in nutrient liquid medium containing 3 M NaCl for 3 and 6 h. Cell cultures were transferred into freshly prepared NB medium and the same procedures were done as described above.

Tolerance of strains to H₂O₂ was assessed by the method of Li et al. [31] but with only 30 min incubation time. Overnight grown cultures of the isolates were inoculated (1% v/v) into NB medium (control) and NB medium containing 0.1% hydrogen peroxide and incubated at 37 °C for 30 min.

4.6. Probiotic criteria

4.6.1. Catalase and protease activities

Catalase activity was assessed according to Aebi [32] and detected by re-suspension of freshly prepared culture in 3% hydrogen peroxide solution. Also, protease activity was estimated qualitatively by the method of Harely and Prescott [33]. Freshly prepared bacterial cultures (100 µL) were streaked on milk agar plates for proteolysis activity examination, then incubated for 24 h at 30 °C, presence of clear zones indicated positive results.

4.7 Resistance to low and high pH

The method of Conway et al. [34] was employed to study the acid tolerance of bacterial isolates. Tolerance to pH 9 was also tested. Freshly prepared isolate cultures were transferred into NB medium (5%) adjusted to pH 2 and 3.5 with 2 M HCl and to pH 9 with 1 M NaOH. They were then incubated at 37 °C and culture samples were taken after 3 and 6 h. Medium neutralization was done by serial dilutions in phosphate buffer (0.1 M, pH 7.0) and re-culture on nutrient agar (NG) plates. NG plates were incubated at 37 °C for 24 h and survival % was determined by comparing the viable bacterial count after incubation at pH 2.0, 3.5, and 9.0 with the control bacterial count incubated at pH 7.

4.8. Pancreatic enzyme and bile tolerance

Overnight grown cultures of the isolates were inoculated into NB with 0.15% pancreatic enzyme and 0.1 or 0.3% bile salt (oxgall) at 37 °C for 24 h. Survival percentage was calculated [35].

4.9. Antibiotic susceptibility

Susceptibility of the isolates to antibiotics was determined using the disc diffusion method according to the guidelines outlined by Clinical and Laboratory Standards Institute (2014) (CLSI) (<http://clsi.org>). Since there is little official data for the sensitivity to antibiotics for the *Bacillus* genus, characterization of susceptible or resistant phenotype was made with reference to judgments based on CLSI interpretative standards for gram positive bacteria. Antibiotic disks (Oxoid, England) e.g., streptomycin (10 µg), ampicillin (10 µg), gentamycin (30 µg), tetracycline (30 µg), vancomycin (30 µg), erythromycin (15 µg) and chloramphenicol (30 µg) were tested. Inhibition zone was measured after 24 h of incubation at 37 °C. Results were recorded as sensitive (S), resistant (R) and intermediate (I) based on inhibition zone diameter.

4.10. Antimicrobial activity

Antimicrobial activity of the isolates was carried out by agar well diffusion method according to (Mishra and Prasad) [36] against six indicator microorganisms. Indicator strains were *Bacillus cereus* ATCC 10876, *Candida albicans* NRRL Y-477, *Aspergillus niger* NRRL 599, *Pseudomonas aeruginosa* ATCC 27953, *Salmonella typhi* ATCC 6539 and *Escherichia coli* ATCC 25922. Overnight culture of test organisms (106 cfu/mL) was inoculated in NA media for bacteria and potato dextrose agar for yeast and fungi, then poured immediately in sterile petri dishes. After centrifugation, supernatant samples of isolate cultures (100 µL) were poured directly into the 8 mm wells of agar plates. The inoculated plates were incubated for 24 h at their optimum growth temperatures, and the diameter of the inhibition zone was measured in mm. Streptomycin (10 µg) and clotrimazole (15 µg) were used as positive controls.

4.11. Antioxidant activity

This was done according to the method of Cuendet et al. [37]. Two mL of the bacterial isolates spore suspension were added to 50 mL NB medium. 1 mL of the culture filtrate was lyophilized and re-dissolved in 1 mL of methanol, then 50 µL of this solution was added to 5 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanolic solution (0.004%). After a 50-min incubation period in darkness, the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH percentage was as follows: where (A blank) is the absorbance of the control reaction (containing all reagents except the test compound), (A sample) is the absorbance of the test sample.

$$[(\text{control absorbance} - \text{sample absorbance}) / \text{control absorbance}] \times 100.$$

5. Conclusion

One of the most important problems facing the dairy industries when using microbial products is the instability of the milk clotting enzyme in difficult environmental conditions, as well as the safety of the bacterial source, in addition to the lack of high productivity of the degradation enzyme. Overall, the findings propose that honey-derived probiotics may offer superior benefits compared to traditional probiotics, presenting an exciting avenue for producers looking for safe, effective, and versatile

microbial solutions in food production. This could lead to innovations in probiotic formulations, potentially enhancing both consumer safety and product quality in the food industry.

Conflict of interest: The authors declare no competing interests.

Acknowledgments

This work was supported by National Research Centre, Chemistry of Natural and Microbial Products Department.

References

- [1] Salama, B.M., Helmy W.A., Ragab T.I.M., Ali M.M., Taie H.A.A., Esawy M.A., Characterization of a new efficient low molecular weight *Bacillus subtilis* NRC16 levansucrase and its levan. J. Basic Microbiol., 59: 1004-1015 (2019).
- [2] Karam E.A., Wahab W.A.A., Saleh S.A.A., Hassan M.E., Kansoh A.L., Esawy M.A., Production, immobilization and thermodynamic studies of free and immobilized *Aspergillus awamori* amylase. Int. J. Biol. Macromol, 102:694-703 (2017).
- [3] Karam E. A. , Hassan M. E. , Elattal N. A., Kansoh A. L., Esawy M. A., Cell immobilization for enhanced milk clotting enzyme production from *Bacillus amyloliquefacien* and cheese quality. MCFs, 23(1):283 (2024).
- [4] Wang Y., Aziz T. , Hu G., Liu J., MinZ., Zhennai Y., Alharbi M., A.Alshammari , Alasmari A. F., Production, purification, and characterization of a novel milk-clotting metalloproteinase produced by *Bacillus velezensis* YH-1 isolated from traditional Jiuqu. LWT., 184 (15): 115080 (2023)
- [5] Li L., Zheng Z., Zhao X., Wu F., Zhang J., Yang Z., Production, purification and characterization of a milk clotting enzyme from *Bacillus methanolicus* LB-1. Food Sci Biotechnol., 28(4):1107–1116 (2019)
- [6] Narwal R.K., Bhushan B., Pal A., Malhotra S.P., Optimization of upstream process parameters for enhanced production of thermostable milk clotting enzyme from *Bacillus subtilis* MTCC 10422. J. Food Process Eng., 40(2): 103055 (2017).
- [7] Hang F., Wang Q., Hong Q., Purification and characterization of a novel milk-clotting metalloproteinase from *Paenibacillus spp.* BD3526. Int. J. Biol. Macromolecules, 85 : 547-554 (2016).
- [8] Zhang Y., Xia Y., Ding Z., Lai P.F.H., Wang G., Xiong Z. , Liu X., Ai L., Purification and characteristics of a new milk-clotting enzyme from *Bacillus licheniformis* BL312. J. Dairy Sci., 106 (10): 6688-6700 (2023).
- [9] Rutella G.S., Tagliazucchi D., Solieri L., Survival and bio activities of selected probiotic lactobacilli in yogurt fermentation and cold storage: new insights for developing a bi-functional dairy food. Food Microbio., 60:54-61(2016).
- [10] Saleh S.A.A., Shawky H., Ezzat A., Taie H.A.A., Salama B., El-Bassyouni G.T., El Awdan S.A., Awad G.E.A., Hashem A. M., Esawy M.A., Abdel Wahab W. A. Prebiotic-mediated gastroprotective potentials of three bacterial levans through NF-κB-modulation and upregulation of systemic IL-17A. Int. J. Biol. Macromol., 250:126278 (2023).
- [11] Guo Z.H., Wang Q., Zhao J.H., Xu Y.P., Mu G.Q., Zhu X.M., Lactic acid bacteria with probiotic characteristics in fermented dairy products reduce cow milk allergy. Food Bioscience., 55:103055 (2023).
- [12] Katiku M. M, Matofari J. W., Nduko J. M., Preliminary evaluation of probiotic properties and safety profile of *Lactiplantibacillus plantarum* isolated from spontaneously fermented milk. Heliyon, 8(8) (2022).
- [13] Gamal A.A., Abbas H.Y., Abdelwahed N.A.M., Kashef M.T., Mahmoud K., Esawy M.A., Ramadan M.A., Optimization strategy of *Bacillus subtilis* MT453867 levansucrase and evaluation of levan role in pancreatic cancer treatment. Int. J. Biol. Macromol., 182: 1590-1601 (2021).
- [14] Esawy M.A., Kansoh A.L., Kheiralla Z.H., Ahmed H.A., Kahil T.A.K, Abd El-Hameed E.K., Production and Immobilization of Halophilic Invertase Produced from Honey Isolate *Aspergillus niger* EM77 (KF774181). Int. J. Biotech. Well., 3:36-45(2014).
- [15] Guleria S., Walia A., Chauhan A., Shirkot C. K., Optimization of milk-clotting enzyme production by *Bacillus amyloliquefaciens* SP1 isolated from apple rhizosphere. BIOB., 3(1):30 (2016).
- [16] Wehaidy H. R., Abdel-Naby M. A, Shousha W. G., Elmallah M. I.Y., Shawky M.M. Improving the catalytic, kinetic and thermodynamic properties of *Bacillus subtilis* KU710517 milk-clotting enzyme via conjugation with polyethylene glycol. Int. J. Biol. Macromol, 111: 296-301 (2018).
- [17] da Silva R.R., Duffeck C.E., Boscolo M., da Silva R., Gomes E., Milk clotting and storage-tolerant peptidase from *Aureobasidium leucospermi* LB86. Process Biochem., 85: 206-212 (2019).
- [18] Wehaidy H.R., Abdel-Naby M.A., Kholif M.M., Elaaser M., Bahgaat W.K., Abdel Wahab W.A., The catalytic and kinetic characterization of *Bacillus subtilis* MK775302 milk clotting enzyme: comparison with calf rennet as a coagulant in white soft cheese manufacture. Journal of Genetic Eng. & Biotechnol., 21(1):61(2023).
- [19] Wehaidy H.R., Abdel Wahab W.A., Kholif A.M.M. b, Elaaser M., Bahgaat W.K., Abdel-Naby M. A., Statistical optimization of *B. subtilis* MK775302 milk clotting enzyme production using agro-industrial residues enzyme characterization and application in cheese manufacture. Biocatalysis and Agricultural Biotechnolo, (25): 101589 (2020).
- [20] Meng F., Chen R., Zhu X., Lu Y., N. Ting, Lu F., Lu Z., Newly Effective Milk-Clotting Enzyme from *Bacillus subtilis* and Its Application in Cheese Making. J Agric Food Chem., (24):6162-6169 (2018).
- [21] Hamdy A.A., Elattal N.A., Amin M.A., Ali A.E., Mansour N.M., Awad G.E.A, Awad H.M., Esawy M.A., Possible Correlation Between Levansucrase production and Probiotic Activity of *Bacillus* sp. Isolated from Honey and Honey Bee. World J Microbiol Biotechnol, (33):69 (2017).
- [22] Sharma A., Lee H.G., Antimicrobial Activity of Probiotic Bacteria Isolated from Plants: A Review. Foods, 14(3):495 (2025).
- [23] Esawy M. A., Awad G. E.A., Abdel Wahab W. A., Ahmed El-diwanaya, Abdel-Fattah A. F., Easa S.M.H., El-beihb F.M., Immobilization of halophilic *Aspergillus awamori* EM66 exochitinase on Grafted k-carrageenan- alginate Beads. Biotech 3, 6 (1): 29(2016).

- [24] Esawy M.A., Mansour S. H., Ahmed E. F., Hassanein N. M., El Enshasy, H. A., Characterization of Extracellular Dextranase from a Novel Halophilic *Bacillus subtilis* NRC-B233b a Mutagenic Honey Isolate under Solid State Fermentation. E. J. Chem., (9):1494-1510 (2012).
- [25] Gülçin İ., Antioxidant activity of food constituents: an overview. Arch Toxicol., 86(3):345-91 (2012).
- [26] Ragab T. I. M., Shalaby A. S. G., El Awdan S., El-Bassyouni G. T., Salama B.M., Helmy W.A., Esawy M.A., Role of Levan extracted from Bacterial Honey Isolates in Curing Peptic Ulcer: In Vivo.Int. J. Biol. Macromol , (42): 564-573 (2020).
- [27] del Carmen, S. A., de Moreno de LeBlanc, A., Levit R., Azevedo V., Langella P., Bermúdez-Humarán L. G., Le Blanc J. G., Anti-cancer effect of lactic acid bacteria expressing antioxidant enzymes or IL-10 in a colorectal cancer mouse model. Int. Immunopharmacol., (42):122-129 (2017).
- [28] Fresno A. H., Alencar A. L. F., Liu G., Wridt M. W., Andersen F. B., Pedersen H. S., Henrik Nielsen L. M., Aabo S. S., S., Elmerdahl Olsen J., Jensen A. N., Effect of feeding dairy calves with milk fermented with selected probiotic strains on occurrence of diarrhoea, carriage of pathogenic and zoonotic microorganisms and growth performance Veterinary. Microbiol., 286, 109885 (2023).
- [29] Weisburg, W.G., et al. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*, 173(2): 697–703.
- [30] Chaiyawan N., Taveeteptaiku P., Wannissorn B., Itsaranuwat P., Characterization and probiotic properties of *Bacillus* strains isolated from broiler. Thai J. Vet. Med., 40:207-214 (2010).
- [31] Li S., Zhao Y., Zhang L., Zhang X., Huang L., Li D., Niu C.H., Yang Z., Wang Q., Antioxidant activity of *Lactobacillus plantarum* strains isolated from traditional Chinese fermented foods. Food Chem., 135:1914-1919 (2012) .
- [32] Aebi, H., Catalase in vitro Methods Enzymol 1984:105:121-6. doi: 10.1016/s0076-6879(84)05016-3. PMID: 6727660 DOI: 10.1016/s0076-6879(84)05016-3
- [33] Prescott, L.M., Harley, J.P. and Klein, D.A. (2002) Laboratory Exercises in Microbiology. 5th Edition, McGraw-Hill Corporation, Pennsylvania, 45-47, 170-175.
- [34] Conway P.L., Gorbach S.L., Goldin B.R., Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci., 70:1-12. (1987).
- [35] Gilliland S.E., Staley T.E., Bush L.J., Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. J. Dairy Sci., 67:3045-3051(1984) .
- [36] Mishra, V. and Prasad, D. (2005) Application of in Vitro Methods for Selection of *Lactobacillus Casei* Strains as Potential Probiotics. International Journal of Food Microbiology, 103, 109-115.
- [37] Cuendet, M., Hostettmann, K., Poterat, O. and Dyatmiko, W. (1997) Iridoid Glucosides with Free Radical Scavenging Properties from *Fagraea blumei*. Helvetica Chimica Acta, 80, 1144-1152.