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# Production of Exopolysaccharides by Diverse Bacterial Strains Isolated from Cheese Whey

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**Background and objectives**
Microorganisms's ability to produce exopolysaccharides (EPS) has drawn special attention from the food sector. EPS enhance the rheological characteristics of fermented foods and are considered natural thickeners. The objective of this study was to evaluate the ability of some bacterial strains isolated from cheese whey to produce EPS under different environmental conditions and to select the most potent EXP producing candidates.

**Materials and methods**
Based on their physical characteristics and 16S rRNA gene sequencing, the selected bacterial isolates *Priestia aryabhattai* (PC) and *Bacillus pumilus* (MC), were identified as non-similar strains. The one-factor-at-a-time approach was adopted to customize cultivation environments to maximize the EPS production for the isolated strains.

**Results and conclusion**
The EPS maximum yield of 12.09 and 8.91 g/l were achieved by the isolates *Priestia aryabhattai* and *Bacillus pumilus*, respectively. Both extracts' infrared (FT-IR) spectra showed the usual patterns of polysaccharide absorption. Furthermore, HPLC analysis showed that EPSs are heteropolysaccharides composed of glucose, galactose, rhamnose, and arabinose. The produced polysaccharide was composed of high ratios of carbon, oxygen, phosphorus, calcium, and chloride, as confirmed by Scanning electron microscope (SEM) and Energy dispersive X-ray (EDX) analysis. The EPS showed antioxidant activity as well as a strong antibacterial effect against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella typhimurium* which are hazardous human pathogens, suggesting their application in the food industry. Also, the produced EPS had a nontoxic effect on Brine shrimp toxicity *(Artemia salina)*.

**Keywords:** Exopolysaccharides, characterization, biological activity, cheese whey

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## Introduction

The escalation of global expansion has led to heightened utilization and requisition of natural resources, necessitating strategies to address the substantial influx of industrial waste and residual outputs originating from a multitude of commercial sectors, notably the food industry. Noteworthy among the prominent waste streams generated are residues derived from fruit processing, grain refinement within processing facilities, vegetables, and by-products from the animal production sector [1].

Microorganisms exhibit remarkable biosynthetic capabilities, enabling them to synthesize a wide array of bioactive substances characterized by distinctive chemical scaffolds and functionalities. These compounds demonstrate significant pharmaceutical potential, exemplified by their abilities to inhibit the proliferation of pathogenic bacteria and fungi, impede the growth of tumor cells, scavenge free radicals and mitigate inflammation. The diversity of these bioactive metabolites encompasses peptides, polypeptides, lactones, fatty acids, polyketides, terpenoids, isocoumarins and exopolysaccharides [2, 3]

Exopolysaccharides (EPSs) are high molecular weight carbohydrate polymers characterized by long chains. Many diverse types of organisms naturally produce these substances, including prokaryotic producers like eubacteria and archaebacteria and eukaryotic producers like plants, phytoplankton, algae, and fungi [4].

Microbial Exopolysaccharides (EPSs) represent significant extracellular macromolecules that are

synthesized and released as capsules or slime layers. Extensive researches were conducted on diverse microorganisms, encompassing bacteria, yeasts, fungi, and algae, to investigate their capacity for EPS production [5]. Microbial Exopolysaccharides (EPSs) encompass both homopolysaccharides and heteropolysaccharides. Their cost-effectiveness renders them as viable substitutes for polysaccharides derived from plants and animals. The substantial production of EPSs by microbial cells through efficient biotechnological processes, utilizing economically favorable substrates like industrial wastes, allows for rapid generation within a shortened time frame [6]. Cheese whey is a dairy by-product since every kg of cheese produces around 9–10 L of whey [7]. Due to the dairy industry's explosive growth, cheese whey output has increased [8]. According to a previous study [9], the worldwide production of cheese whey in 2023 was estimated to be approximately 200 million tons. Over the last 45 years, half of the whey produced ended up as waste, being repurposed for uses like animal feed, biofertilizers in irrigation, or discarded directly into the environment [10-11]. Numerous factors, such as the type of cheese being produced, the properties of the milk, the animal's nutrition, its lactation stage, and general management procedures, affect the composition of whey [12]. About 90% of cheese whey is made up of water, with the remaining 4-5% being lactose, 0.6-0.8% soluble proteins, 0.4-0.5% lipids, and 0.5-0.7% minerals [13]. Additionally, it has a high chemical and biological oxygen demand (BOD and COD), which suggests that improper management could have a major negative influence on the ecosystem. Because it lowers the amount of dissolved oxygen in the water, this presents significant hazards to aquatic life, the environment, and public health when disposed of in water sources [14]. About half of the production is processed and used in food for humans or animals; while the other 50% is either sent for effluent treatment, which comes with extra expenses, or comes from inappropriate sewage disposal, which contaminates water or soil and poses environmental risks [15]. Due to its high nutritional value and daily production, cheese whey is of great interest to the industry and has the potential to be used as a food ingredient [16]. Sweet whey is utilized as an ingredient in bread, meat products, beverages, and baby food. Acid whey is used to make fermented milk, fruit drinks, and spices for salads [17]. Besides, [18, 19] separated whey fat by centrifugation and explored its potential for use in the production of certain dairy products. This syudy aimed to to isolate bacteria that can utilize cheese whey as a growth medium and produce EPS.

## Materials and methods

### Cheese whey

Cheese whey obtained from DANON, Meknes, Morocco, was used for isolation and screening for EPS producing bacteria as well as a culture medium for the bacterial cultivation. After about 20 minutes of boiling, the whey was chilled and filtered through muslin fabric to produce a clear filtrate. The resulting whey was autoclaved for 15 minutes at 121°C [20]. Various chemical analyses were performed, including assessments of moisture content (determined via drying at 105 °C), total solids, ash content, total nitrogen (utilizing the micro Kjeldahl method), and protein content. These parameters were determined and computed by the methods outlined by the Association of Official Analytical Chemists [21]. Lactose content was assessed using the phenol-sulfuric acid assay technique as described in [22]. The determination of fat content was carried out following the methodology outlined in [23]. Measuring The samples' pH was measured utilizing a pH-meter (Orion SA720, U.S.A.).

### Isolation and screening of exopolysaccharide-producing bacteria

For isolation of the EPS producing isolates, an aliquot of 10 ml of whey was inoculated into 100 ml of nutrient broth in a 250 ml conical flask and incubated for 24 hours at 30-37 $^{o}$C. A loopfull of the culture was streaked on nutrient agar plates and incubated for 48 hours at 30-37 °C where the visually promising colonies were verified as EPS producers. The mycoid isolates were sub-cultured and repeatedly streaked on nutrient agar plates for purification; therefore, they were further screened for EPSs production. Purity of the isolates was microscopically confirmed and the pure ones were morphologically and genetically characterized.

### Molecular identification of the isolates

The genomic DNA was extracted from two selected bacterial isolates [24]. The 16S rRNA gene was amplified using the universal primers FD-1 (5′-AGAGTTGATCCTGGCTCA-3′) and RP2 (5′-ACGGCTACCTTGTTAGGACTT-3′) after DNA from 48-hr-old bacterial cultures was extracted using the automated kit of the "MagPurix Bacterial DNA Extraction Kit" platform. A 16S rRNA bacterial identification PCR (ABI "Verity" heat cycler) was used to perform polymerase chain reaction (PCR) and create reaction mixtures. The following conditions were used for conducting PCR on a Gene Amplification PCR System: Using a PCR machine (ABI "Verity" thermal cycler), 35 cycles were performed at 95°C for 2 minutes (first

denaturation cycle), 95°C for 30 seconds (denaturation), 52°C for 30 seconds (annealing), 72°C for 30 seconds (elongation), and 72°C for 3 minutes (final elongation). Agarose gel electrophoresis was used to verify the PCR results. After the electrophoresis was completed in 1% agarose, the bands were examined and captured on camera using the BoxG documentation system. Every operation was carried out as directed by the manufacturer. After the data was sequenced, BLAST (http://www.ncbi.nlm.nih.gov/blast) was used to analyze the nucleotide sequences for sequence similarity. After obtaining the accession numbers, the sequences were added to the GenBank database. The phylogenetic tree was built with MEGA 5.05 (http://megasoftware.net/) [25], which aligned the sequences using the maximum composite likelihood technique and the neighbor-joining method [26].

**Production of exopolysaccharides by the selected strains**

**Preparation of inoculum**

Twenty-four-hr old Nutrient Agar (NA) slant culture of the examined strain was used to inoculate 100 mL of nutrient broth in conical flasks and incubated at 32°C for a full day.

**Optimization of growth conditions for production by the isolated strains**

**The impact of incubation period on exopolysaccharides (EPS) production**

For the EPS optimization, one variable was changed at a time while keeping the other constants using the single factor at a time method. An aliquot of 100 mL plain cheese whey was transferred to 250 ml conical flasks for each run. An inoculum of 3 % (v/v) of the examined bacterial strain broth culture was applied to each flask, and incubated for 6 days at 30 $^{o}$C on a rotary shaker incubator at 100 rpm. Every 24 hours, samples were taken to measure the production of EPS. Triplicate whey flasks were examined in each run. Effect of incubation temperature on exopolysaccharide production by the bacterial strains. Cheese whey inoculated with the examined bacterial strain was incubated at different temperatures *i.e*. 25, 32, 37, 45, 50, 55 and 60 ºC on a rotary shaking incubator at 100 rpm for 6 days. Samples were daily withdrawn for EPS production analysis.

**Effect of initial pH on exopolysaccharide production by the bacterial strains**

The pH of the growth medium was adjusted to various values (4, 5, 6, 7, and 8) using a pH meter (Jenway, 3510, UK). Subsequently, the medium was inoculated and incubated at an optimal temperature of 32ºC in a shaking incubator at 100 rpm for 6 days. The media were tested for producing EPS every 24 hours.

**Effect of agitation speed on exopolysaccharide production by the bacterial strains**

The effect of agitation speed on growth and EPS production in cheese whey was studied on a shaker incubator at different agitation speeds *i.e.*, 0, 50, 100 and 150 rpm.

**Effect of inoculum size on exopolysaccharide (EPS) production by the bacterial strains**

Growth of the isolated bacterial strains and their EPS production were examined in triplicate flasks containing 100 ml of cheese whey at pH 7 inoculated with different inoculum sizes *i.e.*, 3, 7, 10, 12 or 15 %, (v/v) and incubated at 32 °C on a rotary shaking incubator at 100 rpm for 6 days. Production of EPS in the examined flasks was daily analyzed.

**Bacterial exopolysaccharide (EPS) extraction**

Bacterial cells were eliminated by centrifugation at 4000 rpm for 20 minutes at 4 °C. Trichloroacetic acid (10% (w/v)) was added to the supernatant, and the precipitated proteins were separated by centrifugation at 4000 rpm, 4 °C, 20 min. The EPS was precipitated and kept overnight at 4 °C. The clear supernatant was collected, neutralized with NaOH 1N, and precipitated. Centrifugation was used to recover the precipitate for 20 minutes at 4000 rpm at 4 °C. The precipitate was dialyzed for two days at 4 °C against the same solution (changed twice daily) after being dissolved in deionized water and it was then dried in the oven at 40 °C. The dry weight (g /l) was taken as the amount of EPS produced [27].

**Exopolysaccharides characterizations**

**Fourier transform infrared spectroscopy (FTIR) analysis**

The spectroscopic analysis was performed using a Bruker Alpha II FTIR (Fourier Transform Infrared) spectrometer, equipped with an ATR (Attenuated Total Reflection) accessory featuring a diamond crystal. This accessory allows for direct analysis of both solid and liquid samples without extensive preparation. The spectrometer operated over a spectral range of 4000 to 400 $cm^{-1}$ with a resolution of 4 $cm^{-1}$. For each sample, 16 scans were performed to enhance the signal-to-noise ratio. The infrared light source used was a silicon carbide heated source, and the built-in detector was a DTGS (Deuterated Triglycine Sulfate) detector [28].

**High-performance liquid chromatography (HPLC) examination of exopolysaccharides**

A C-18 column (4.6 mm × 260 mm, 5 μm particle

size) and two LC-pumps were employed in the HPLC-(Agilent 1100) analysis after 20 mg of partially purified EPS had been hydrolyzed for 8 hours at 120°C using 3 ml of 2 M trifluoroacetic acid (TFA). Chromatograms were obtained and analyzed using the Agilent ChemStation Mobile phase separated by employing a mobile phase of:
100% acetonitrile was used in mobile phase A, and an acetonitrile and distilled water mixture (90:10, v/v) with 0.045% KH2 PO4 and 0.05% triethylamine buffer (pH 7.5) was used in mobile phase B. Gradient elution was carried out at 94-94-88-88% B with linear declines at 04-5-20 minutes. The volume injected was 20 µl. A Refractive Index (RI) detector operating at 35°C was employed [29].

**Scanning electron microscopy (SEM)**
Samples were carried out using a JEOL IT500 HR Scanning Electron Microscope. The instrument operates with a high-resolution secondary electron detector, enabling detailed surface imaging. Samples were mounted on aluminum stubs using conductive carbon tape and sputter-coated with a thin layer of gold (approximately 10 nm thick) to enhance conductivity and prevent charging under the electron beam. With a working distance of about 10 mm, the SEM was run at an accelerating voltage of 15 kV. Magnifications ranging from 500x to 20,000x were used depending on the required level of detail [30].

**Energy dispersive X-ray (SEM-EDX)**
Using an energy-dispersive X-ray (EDX) apparatus in conjunction with a scanning electron microscope (SEM), the chemical composition of the EPS was examined. The elements' X-ray spectra were obtained at a 15 keV accelerating voltage. Following the steps outlined by [30]. The EDX technique was used to perform elemental analysis and ion distribution mapping of the samples.

**Biological activities of the polysaccharides The antibacterial impact**
**Pathogenic bacterial strains and culture**
The antibacterial activity of was evaluated against both Gram-negative (*Salmonella typhimurium*, *Escherichia coli*) and Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*) obtained from the culture of the Department of Microbiology, Moulay Ismail University. The bacterial strains were seeded on Tryptone Soy Yeast Extract Agar medium (TSYEA; Biolife, Milan, Italy) and then incubated for 24 hours at 37 °C.

**The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the exopolysaccharide**
The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined using the microdilution technique [31]. One hundred microliters of sterile distilled water was added to each well of 96-well microplates with a flat bottom. To ascertain successive dilutions, 50 µL of dried EPS were added to the first well plate and combined with 500 mg/mL of sterile distilled water. Furthermore, 50 µL of bacterial suspensions ($10^8$ cfu/ mL) and 50 µL of Tryptone Soy Yeast Extract Broth were added to each well. The well containing the bacterial suspension with Tryptone Soy Yeast Extract Broth was used as the positive control, whereas EPS and sterilized distilled water served as the negative control. Moreover, the Clinical and Laboratory Standards Institute's methodology was followed while using ciprofloxacin as a reference medication. 40 µL of TTC (2, 3, 5-triphenyl tetrazolium chloride) was added to each well of the microplate after it had been incubated for 24 hr. at 37°C. The wells were then reinsulated for 30 minutes at 37°C. The lowest EPS concentration at which no bacterial growth was observed was used to calculate the MIC. However, the lowest EPS concentration was used to compute MBC, which prevented the growth of a bacterial colony. This was accomplished by plating two microliters of samples from the wells that showed no growth on Tryptone Soy Yeast Extract Agar plates and then incubating them at 37 °C for twenty-four hours. The bacteriostatic and bactericidal properties of EPS were confirmed by the MBC/MIC ratio. When the ratio of MBC/MIC is greater than 4, bactericidal effects take place, and when the ratio is less than 4, bacteriostatic effects take place [31, 32]

**Antioxidant activity of the bacterial exopolysaccharide using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay**
The assay was carried out as described before [33] where appropriate blanks (distilled water) and standards (TROLOX and ascorbic acid solutions in DW 5, 10, 50 and 100 µg/ml) were run simultaneously. Several concentrations of extracts (50, 250, 100 and 500 µg /ml) were prepared. DPPH's % inhibition was determined using the following equation.

$$\% \text{ inhibition} = \{(A_{blank} - A_{sample})/A_{blank}\} \times 100$$

Where A blank is the absorbance of DPPH solution without extract at 490 nm using ELISA reader and A sample is the absorbance of the sample with DPPH solution.

### Cytotoxicity assay with brine shrimp larvae (*Artemia salina*)

The brine shrimp eggs (*Anemia sauna*) were hatched in artificial seawater containing 40g/l sea salt, oxygenated by an aquarium pump, and illuminated. With some adjustments, nauphi were harvested using a Pasteur pipette after an incubation period of 48 hours at 22–29°C [34]. In the sample test, ten Artemia salina larvae were added at concentrations of 50, 100, 250, 500, and 1000 µg/ml, along with a control. In the test tubes, serial dilutions were prepared in triplicate using 100 µ1 seawater. DW-containing control wells were used in every experiment. Each tube was filled with a suspension of nauphi with 10–15 organisms (l00µ1) extract, and the tubes were then cultured for 24 hours at 22–29°C. A binocular microscope (x12.5) was then used to inspect the tubes, and the number of dead (non- motile) nauplli in each well was counted.

The larvae mortality percentage was calculated by using equation [35]:

% Mortality = (number of dead larva/number of tested larva) x100 Abbot's formula was used if there was a dead larva at Control:

%Mortality = {(% Mortality at treatment-% Mortality at control) / (100-% Mortality at control)} x100

### Statistical analysis

All statistics are expressed as the mean ± standard error (SE) of three independent determinations. The significance levels for comparing differences were determined using a one-way analysis of variance (ANOVA) at $P < 0.05$. This analysis was performed with the Minitab® 19.2020.1 software

## Results and discussion

### Cheese whey chemical composition

The cheese whey analysis indicated that, the total whey solid content was 11%; lactose 4.5%, protein 0.32%, fat 0.01% and ash 0.4%. Whey mineral content was < 30 K, 0.08 Fe, Cu 0.15 and < 0.05 Zn mg/L.

### Isolation and identification of exopolysaccharides producing bacteria

Cheese whey sample was screened for EPS synthesizing bacteria. Among the obtained isolates, an isolate coded PC and another coded MC were selected for further studies depending on their mucoid formation. Colonies of the isolates appeared slimy and mycoid on total plate count agar plates due to EPS synthesis. Microscopic examination revealed that both are Gram positive spore-forming non-motile rod-shaped bacterial isolates. A BLAST search for molecular identification showed that the PC isolate was similar to *Priestia aryabhattai*, whereas the MC isolate was comparable to *Bacillus pumilus*. The isolates partial 16S rRNA gene sequences were submitted to Gen Bank with Accession Numbers PQ778804 and PQ778811, respectively. The estimated 1500 bp of the 16S rRNA gene was amplified and sequenced. The neighbor-joining approach was used to create a phylogenetic tree (fig. 1) that shows the relative positions of these isolated strains and other Bacterial species.

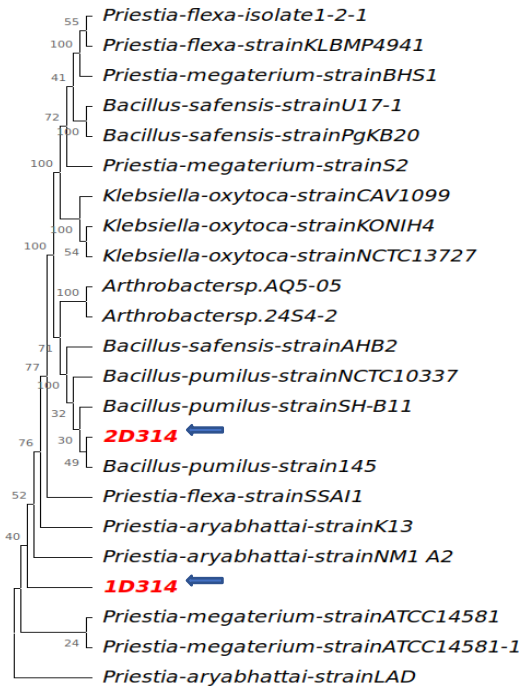


**Fig. 1** The phylogenetic tree of two isolates (1D314 and 2D314). A tree with one hundred replicas was bootstrapped. MEGA 11 was used to perform phylogenetic analysis

**Effect of incubation period**

Data illustrated by figure (2) show that *Bacillus pumilus* produced 8.26 g/l of EPS after 120 hours, while *Priestia aryabattai's* produced 12.09 g/l then sharply decreased after 96 hours and after 24 hours. EPS synthesis by *Priestia aryabhattai* started after the end of logarithmic growth phase and during the stationary phase, reaching its maximum after 24 hours (figure 2). This was in conformity with the results reported in a previous study [36] using lactic acid bacteria. On the other hand, *Lactococcus lactis*was found to produce the most EPS after 12 hours [37] and 48 hours [38].

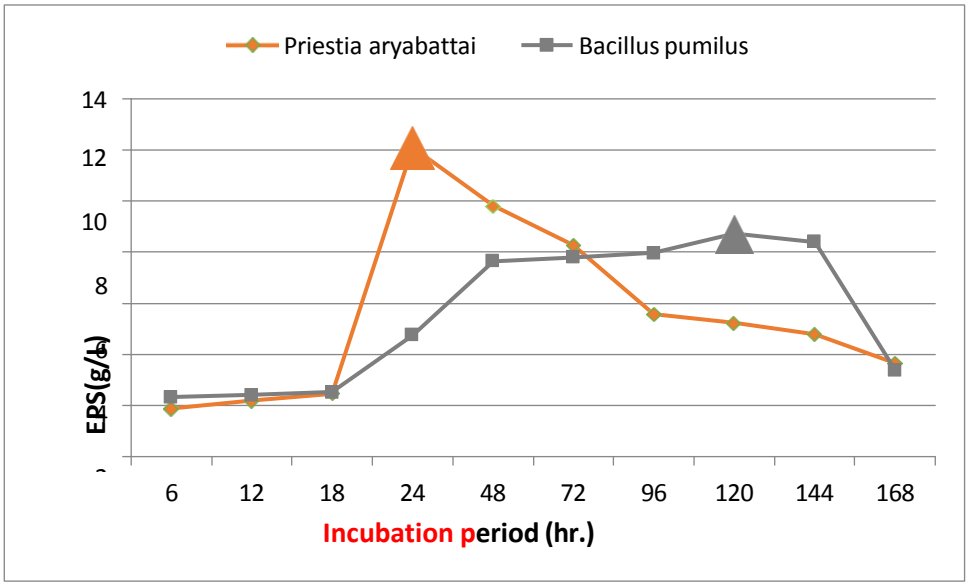


**Fig. 2** Effect of incubation period (initial pH 6 for *priestia aryabhattai* and 7 for *Bacillus pumilus*, incubation temperature (32 and 37 ºC), inoculum size 3% and shaking speed 100 rpm).

**Effect of initial pH**

The initial pH may also be regarded as a significant determinant of the yield of polysaccharides production by bacteria since it influences the biosynthesis of polymers, the general morphology and structure of the cell, and the cell's ability to absorb nutrients. The impact of pH on EPS production was examined at optimal temperatures (32, 37 °C) using a range of pH values from 2.0 to 8.0. *Priestia aryabhattai* and *Bacillus pumilus* produced the highest EPS yields at pH 6.0 and 7.0, respectively, according to the results in Fig. 3. These findings contrast significantly with those of *B. lentus* EPS production [39], where the maximum EPS yield was obtained at a starting pH of 6.5. Nonetheless, the results are similar to those obtained using *Brachybacterium phenoliresistens*, which had an optimal pH of 7 [40].

**Effect of incubation temperature on exopolysaccharide production by *Bacillus pumilus* and *P. aryhpatti***

An additional significant factor that is thought to impact the microbial growth and enzymatic activity is the incubation temperature. *P. aryhpatti* and *Bacillus pumilus* could grow and produce EPS in the temperature range of 25 to 37°C. On the other hand, Fig. 4 shows a considerable decrease at 37 °C, probably because of the denaturation of microbial enzyme system at higher temperatures. Better EPS production was observed in [41] within this temperature range. Other studies approved these results regardless of the microbial type; lactic acid bacteria [42], and *Bacillus sp.* [43].

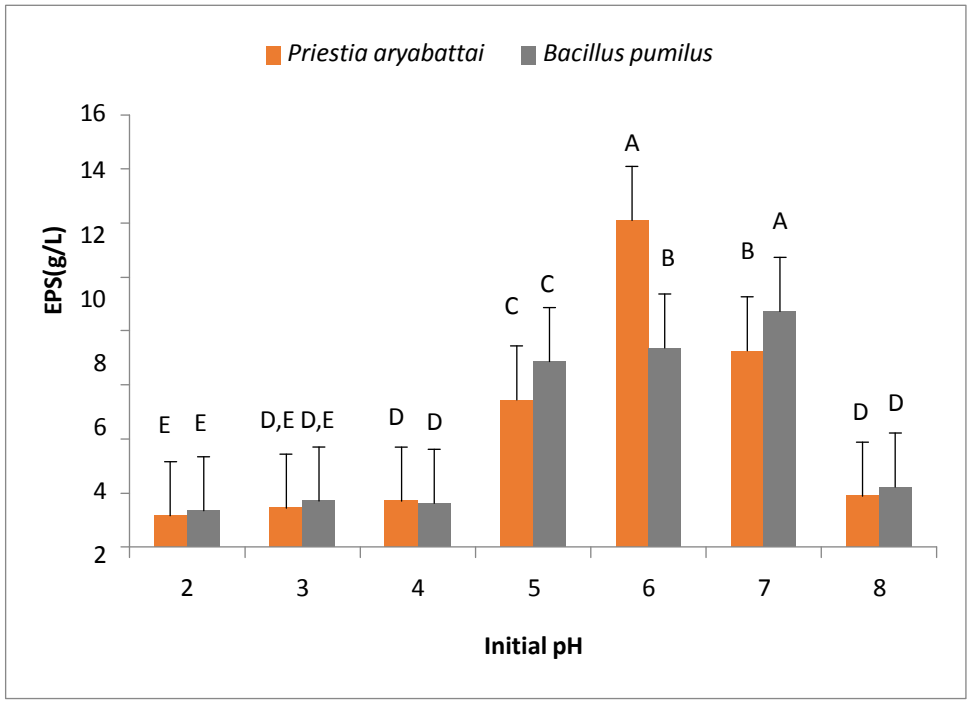


**Fig. 3** Effect of initial pH on exopolysaccharide production (incubation period for *priestia aryabhattai* 24 and 120 hr. for *Bacillus pumilus*, incubation temperature (32 ,37 ºC) inoculum size 3% and shaking speed 100 rpm.)

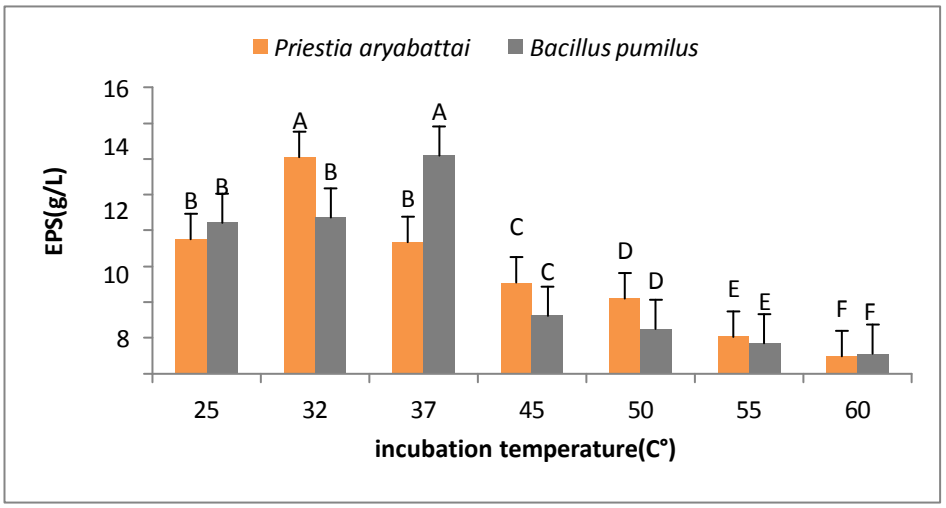


**Fig. 4** Effect of incubation temperature on exopolysaccharide production (incubation period for *priestia aryabhattai* 24 and 120 hr. for *Bacillus pumilus*, initial pH (6, 7), inoculum size 3% and shaking speed 100 rpm.)

**Effect of inoculum size on ESP production by by *Bacillus pumilus* and *P. aryhpatti***

As shown in Fig. 5, The two stains had the same appropriate inoculum size for the highest EPS production. Both strains achieved their maximum EPS production (12.09 and 8.72 g/l) at 3% inoculum size. The production of EPS was considerably decreased by inoculum sizes of more than 12% or less than 3%. According to the current findings, earlier studies have shown that the inoculum size (1–5%) significantly impacted the production of EPS [44, 45]. In another investigation [46], an inoculum between 10 and 11% was suggested for maximum EPS generation. Aeration conditions

The strain *priestia aryabattai* yielded 12.09 g/L of EPS within shaking conditions at 100 rpm, compared with 4.09 g/L in static culture (Fig. 6), and the EPS yield by *Bacillus pumilus* was 9.14 g/L at 100 rpm compared with as low yield as 3.17 g/L in static culture. This might be explained by the fact that the growth of these isolated strains substantially thrives by aerobic conditions because of their aerobic nature. In a previous study [47], using static or shaking conditions was considered while increasing the EPS production process for commercialization. According to this investigation, an optimal shaking speed of 150 rpm was recommended for maximum EPS production by lactic acid bacteria isolated from silage.

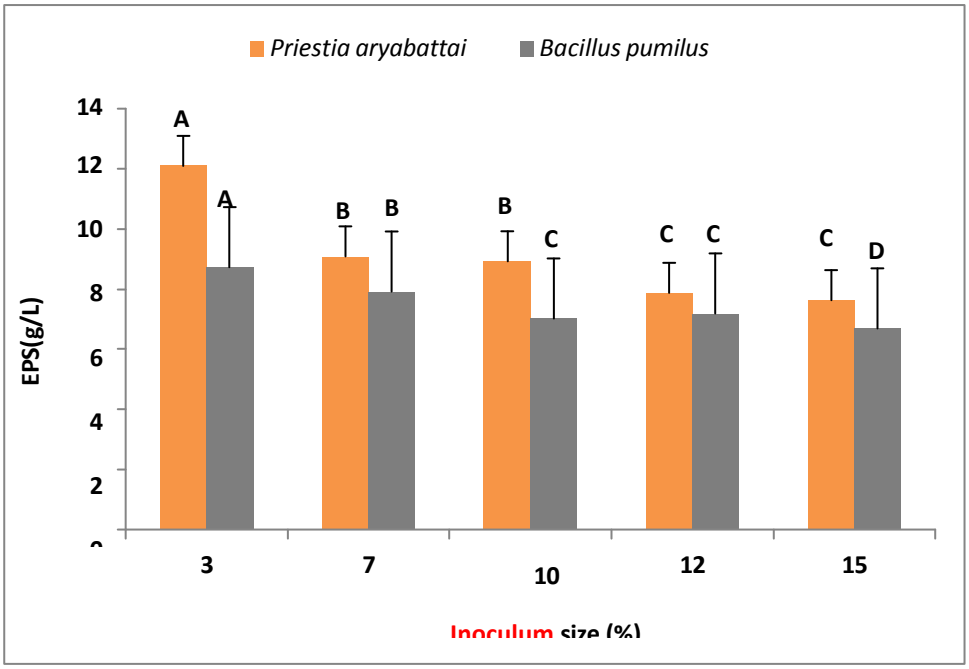


**Fig. 5** Effect of inoculum size on exopolysaccharide production (incubation period for *priestia aryabhattai* 24 and 120 hr. for *Bacillus pumilus*, initial pH (6 , 7) Incubation temperature (32 ,37) ºC inoculum and speed 100 rpm.)

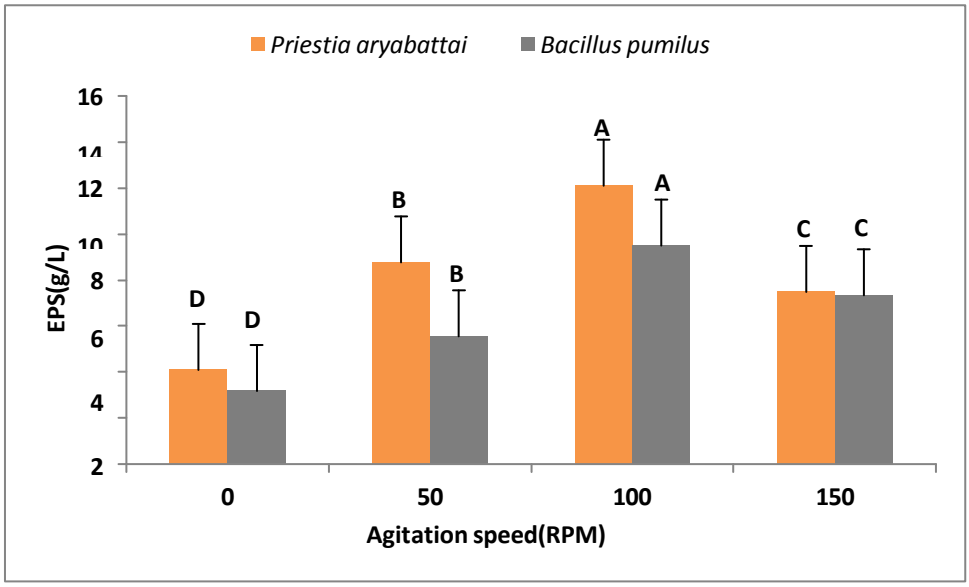


**Fig. 6** Effect of agitation speed (incubation period for *priestia aryabhattai* (24, 120) hr. for *Bacillus pumilus*, initial pH (6, 7) Incubation temperature (32, 37) ºC and inoculum size 3%).

**Exopolysaccharides characterization**

**Fourier-transform infrared (FT-IR) characterization of the exopolysaccharides**

The polymers' IR spectra revealed a wide, strong band with a resolution of 4 $cm^{-1}$ between 4000 and 400 $cm^{-1}$. The functional groups in the extracts of the two strains are not significantly different. The obtained spectra were compared against a spectral library integrated into OPUS, containing reference spectra for a wide range of organic and inorganic compounds Fig.7 (a), (b). Characteristic peaks were identified and associated with corresponding functional groups, including typical absorption bands for O-H, C=O, and N-H bonds. Two primary sections are frequently analyzed to describe the structure of polysaccharides: the sugar region, also known as the fingerprint area, which occurs between 1200-950 $cm^{-1}$, and the numeric region, which is found between 950 and 750 $cm^{-1}$. Saccharide-related peaks are usually seen in the 1000–1200 $cm^{-1}$ range, while bands in the 900–1150 $cm^{-1}$ region are thought to be caused by C–

O–C and C–O bond vibrations. Furthermore, the C-H bond exhibits a distinct peak at 619 $cm^{-1}$ [48].

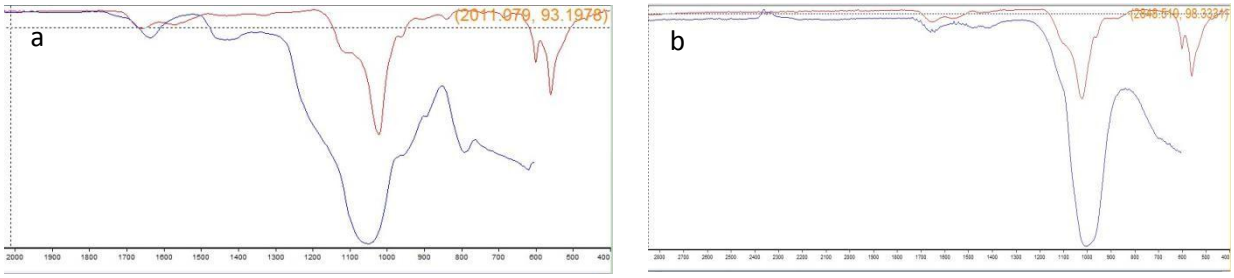


**Fig. 7** Fourier-transform infrared (FT-IR) analysis of exopolysaccharides produced by (a) *priestia aryabattai,* (b) *Bacillus pumilus)*

## HPLC analysis of the exopolysaccharides

The EPS structural features were characterized using the HPLC technique. Following partial hydrolysis, the refractive index chromatogram of EPS generated by *Priestia aryabattai* revealed five separate peaks at the identical retention periods of the standard sugars (12.30 µg/gm arabinose at 10.01, 10.98 µg/gm raminose at 8.1 minutes 9.14 µg/gm galactose at 6.8 minutes, 6.02 µg/gm glucose at 5 minutes and 5.74 µg/gm mannose at 9 minutes ).While the composition of EPS produced by *Bacillus pumilus* was (13.36 µg/gm galactose at 7 minutes, 5.85 µg/gm glucose at 5.2 minutes,5.29 µg/gm mannose at 9, 4.29 µg/gm xylose 12.01 minutes, 2.46 µg/gm raminose at 8.1 minutes, 2.36 µg/gm arabinose at 10.01 minutes and 2.36 µg/gm fructose at 14 minutes ) Fig.8 (a),(b). The structure and monomer composition of EPS are considered because its application is dependent on its structure and it is characteristic for each strain [49]. According to this result the EPSs produced may have antioxidant, antimicrobial and anti-inflammatory effect regarding its content of monosaccharaides and this is in agreement with [50].

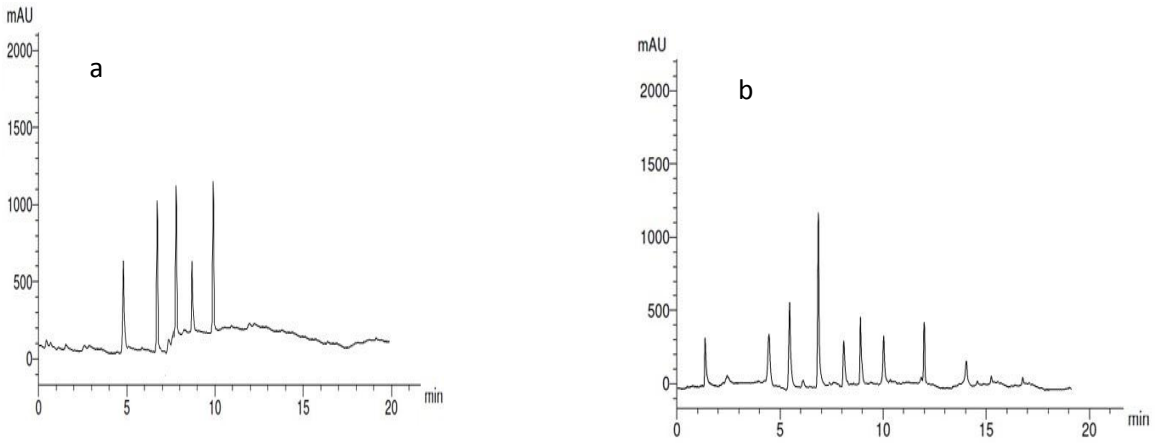


**Fig. 8** High-performance liquid chromatography analyses of exopolysaccharides produced by isolated strains (a) *priestia aryabattai* (b) *Bacillus pumilus*

## Scanning electron microscope (SEM) of exopolysaccharides

SEM is applied to investigate the extracted EPS's three-dimensional structure and surface morphology. According to previous studies, the EPS biopolymer has a very compact, non-porous, flake-like compact structure [51]. As shown by SEM analysis at 500 to 20.000 magnifications, respectively. Amorphous surface roughness made up of uniformly spaced macromolecular lumps was visible Fig. 9 [a, b].

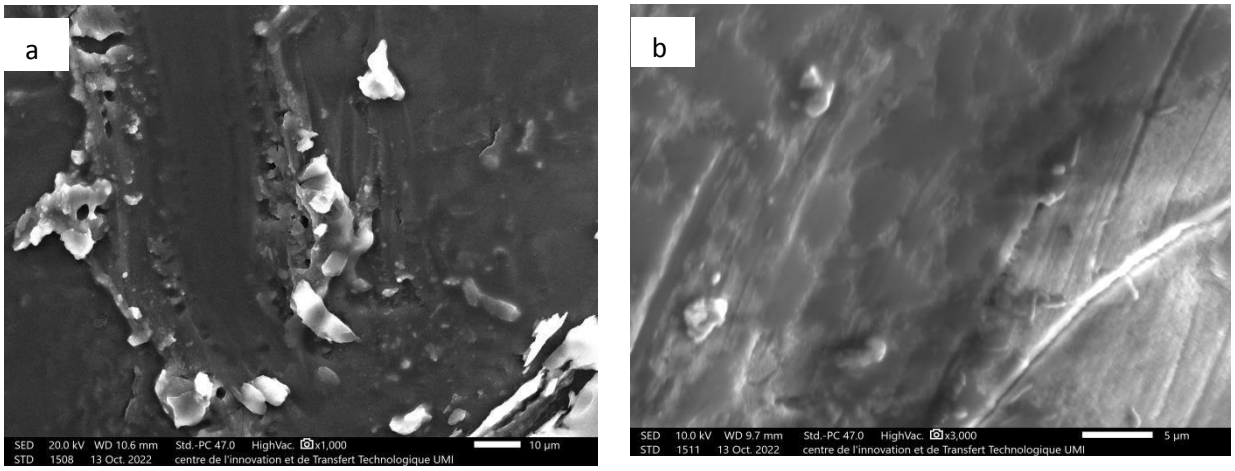


**Fig. 9** scanning electron microscope analyses of exopolysaccharides. (a) *priestia aryabattai*, (b) *Bacillus pumilus.*

## Energy dispersive x-ray (EDX)

The EPS from *priestia aryabattai* composed of some elements such as C, O, P, Na, Cl and Ca, in high ratios of its weight which reached 39.01, 39.19, 5.87, 3, 4.9, and 8% while the ratios of elements for EPS from *Bacillus pumilus* were 45.95, 33.91, 3.37, 3.49, 8.47, and 4.81% respectively (Table 1). Our results were in harmony with [52], who identified oxygen and carbon as the main constituents of EPS. Interestingly, it was discovered that the structure and function of any EPS were influenced by the elemental composition and the percentage diversity [53].

**Table 1** energy dispersive x-ray analysis of exopolysaccharides (*priestia aryabattai*, *Bacillus pumilus*)

| Elment | Mass% | | Atom% | |
|---|---|---|---|---|
| *priestia aryabhattai* | | *Bacillus pumilus* | *Priestia aryabhattai* | *Bacillus pumilus* |
| C K | 25.45± 0.13 | 31.12± 0.13 | 39.01± 0.19 | 45.95± 0.19 |
| O K | 34.05± 0.23 | 30.62± 0.23 | 39.19± 0.26 | 33.91± 0.26 |
| Na K | 3.76± 0.05 | 4.53± 0.05 | 3.01 0±.04 | 3.49 0±.04 |
| P K | 9.87 ± 0.05 | 5.89 ± 0.05 | 5.87 ± 0.03 | 3.37 ± 0.03 |
| Cl K | 9.46± 0.05 | 16.95± 0.05 | 4.91 ± 0.03 | 8.47 ± 0.03 |
| Ca K | 17.41± 0.08 | 10.87± 0.08 | 8.00 ± 0.04 | 4.81 ± 0.04 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 |

Values are expressed as the mean± standard error (SE) (n=3).

## Biological activities of exopolysaccharides produced by isolated strains Antioxidant activity by DPPH radical-scavenging method

Figure 10 displays the results of DPPH's determination of EPS's antioxidant activity. Both EPS exhibited antioxidant activity (44.5%–63%) that varied according to the sample concentration (50–500 µg/ml). For *priestia aryabhattai* and *Bacillus pumilus* EPS, the IC50 values were 170.8 and 174.9 µg/ml, respectively. It was found that [54] levan produced by *B. subtilis* had a strong ability to scavenge free radicals. In general, antioxidants have the potential to significantly reduce the risk of several types of diseases, including inflammation, cancer, heart disease, and immune system decline [55]. Therefore, the

produced EPS may include unique qualities, specifically anticancer, antioxidant, and immune-stimulating properties that can be used as efficient and secure medications [56].

**Antibacterial Activity**

The current study evaluated the antibacterial properties of EPS against the Gram-positive Staphylococcus aureus and Listeria monocytogenes, as well the Gram-negative Salmonella typhimurium and Escherichia coli. All tested bacteria demonstrated MIC to ciprofloxacin ranging from 4 to 8 µg/ mL, using the broth microdilution assay, as indicated in Table 2. The EPS extracts produced from the two isolated strains had MIC values ranging from 66.66 ±0.010 to 133.33 ±0.015 mg/mL against all tested microorganisms. Thus, these extracts showed bactericidal efficacy against Salmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes, and Escherichia coli. Additionally, [57] discovered that the EPS produced by Lactobacillus plantarum YW32 demonstrated a concentration-dependent inhibitory effect on the development of biofilms by a variety of pathogenic bacteria, which include Salmonella typhimurium S50333, Shigella flexneri CMCC (B), Escherichia coli O157, and Staphylococcus aureus AC1.

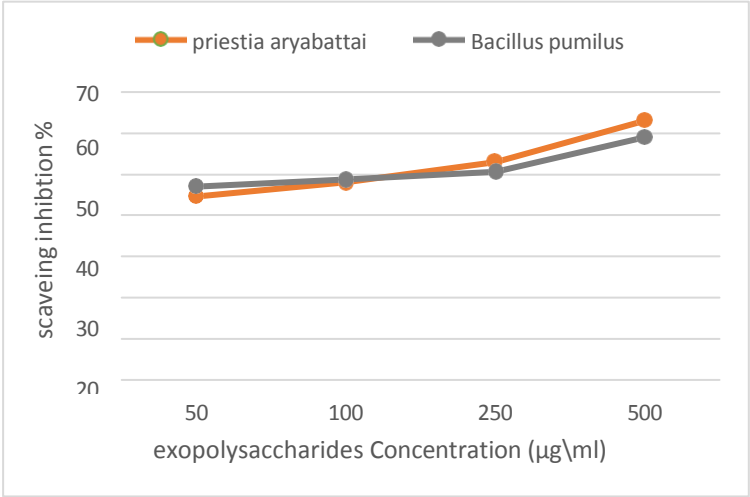


**Fig. 10** Antioxidant scavenging activity of exopolysaccharides produced by *isolated* strains (*priestia aryabhattai* and *Bacillus pumilus*).

**Table 2** MIC and MBC (mg/mL) exhibited by EPS extracts obtained from *priestia aryabattai* and *Bacillus pumilus*

| | *priestia aryabattai* | | | *Bacillus pumilus* | | |
|---|---|---|---|---|---|---|
| | MIC | MBC | MBC/MIC | MIC | MBC | MBC/MIC |
| ***E. coli*** | 66.66 ±0.015 | 133.33±0.011 | 2 | 66.66±0.015 | 133.33±0.01 | 2 |
| ***S. typhimurium*** | 133.33±0.017 | 133.33±0.010 | 1 | 66.66±0.0150 | 133.33±0.011 | 2 |
| ***S. aureus*** | 133.33±0.015 | 133.33±0.010 | 1 | 66.66±0.015 | 133.33±0.01 | 2 |
| ***L. monocytogenes*** | 66.66±0.015 | 66.66±0.015 | 1 | 133.33±0.011 | 133.33±0.014 | 1 |

Values are expressed as the mean± standard error (SE) (n=3).

**Toxicity activity by *Artemia salina***

LC50 values, which indicate the toxicity of EPS extract, were assessed by comparing them with either Meyer's or Clarkson's toxicity index. Meyer's toxicity index classifies extracts as non-toxic if their LC50 is larger than 1000 µg/mL and as hazardous if it is less than 1000 µg/mL. [58]. According to Clarkson's toxicity criterion, EPS extracts are categorized in the following order:

Low-toxic extracts have an LC50 of 500–1000 µg/mL, while non-toxic extracts have an LC50 greater than 1000 µg/mL. Extracts with an LC50 of 0-100 µg/mL are very hazardous, while those with an LC50 of 100-500 µg/mL are medium-toxic [59].

Our results indicated that the EPS extracts produced by two isolates have a non-toxic effect (Fig. 11), with IC50 (1004.198 and 1562.451 ppm), respectively. This result is similar to [60] who found that the EPS produced by *Bacillus licheniformis* is non-toxic to brain shrimp. Also [61] supports our result by finding the IC50 produced polysaccaride was 15,815.849 ppm which is non-toxic according to [62].

Several studies found that the $LC_{50}$ of brine shrimp and anticancer activity in the creation of novel medications from EPS were strongly positively correlated [63-65]. This relationship has been recognized as a successful prescreening method for antitumor and cytotoxicity tests [66]. These preliminary results could recommend forthcoming biotechnological applications of EPS1-T14, especially for the improvement of anti-cytotoxic drugs [65].

## Conclusion

Based on their potential to produce EPS, two bacterial strains (PC and MC) were selected for additional research in this study. Genetic and biochemical analysis revealed that the two strains (*Priestia aryabhattai* and *Bacillus pumilus*) were not similar. The one-factor-at-a-time approach was used to optimize how environmental factors affected the synthesis of EPS by both strains. Cheese whey may be an affordable substitute for synthetic carbon and nitrogen sources in the manufacture of more EPS. Both extracts displayed comparable main profiles that characterized the EPS, based on the functional groups analysis. According to chromatographic analysis, EPSs were heteropolysaccharides made up of glucose, galactose, and arabinose. When tested against both Gram-positive and Gram-negative microorganisms, the generated EPS showed a modest level of antioxidant and antibacterial activity. Additionally, a nontoxic impact was noted.

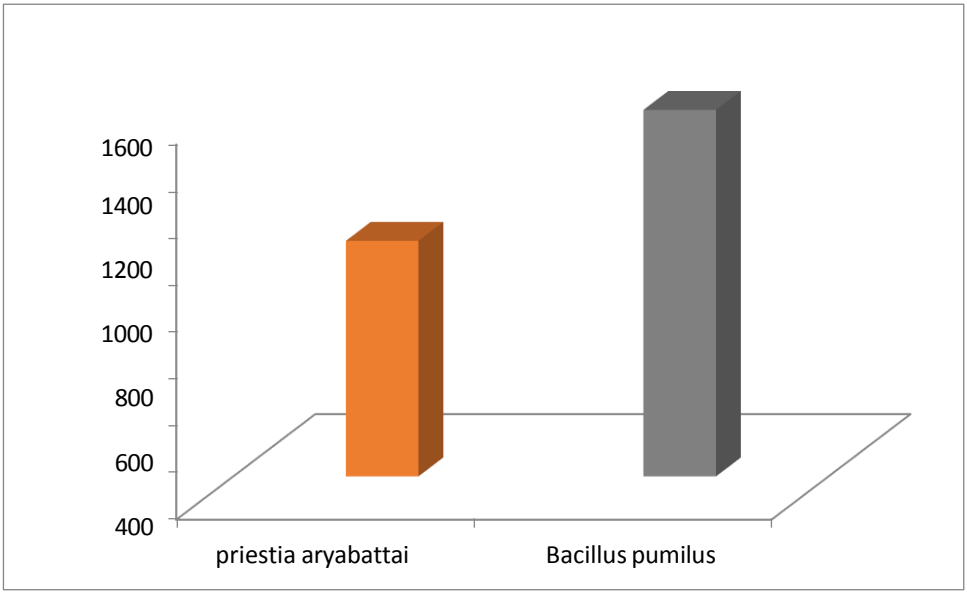


**Fig. 11** Brine shrimp toxicity ($IC_{50}$) of different exopolysaccharides extracts

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