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Bioactive Potential of Exopolysaccharides from *Bacillus strain CDB-1*; Isolated from Cow Dung and Evaluation of Antioxidant Activities

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KEYWORDS
Exopolysaccharides, Antioxidant, Bioactive polymers, Free Radical Scavenging, Green polymer

ABSTRACT:
Introduction: Exopolysaccharides (EPS) produced by microorganisms have attracted significant attention due to their diverse bioactive properties and potential applications in food, pharmaceutical, and biomedical fields. In the present study, a novel EPS-producing bacterial strain, *Bacillus strain CDB-1*, was isolated from cow dung and investigated for its EPS production, with yield 120 mg/ L. The physicochemical characteristics, and antioxidant potential was analyzed and found that EPS exhibiting. The EPS was extracted from the culture supernatant, purified, and subjected to detailed characterization. Fourier transform infrared (FTIR) spectroscopy revealed the presence of characteristic functional groups corresponding to polysaccharides, confirming the chemical nature of the EPS. Morphological analysis further supported its polymeric structure. The antioxidant activity of the purified EPS was evaluated using standard in vitro assays, including DPPH radical scavenging, ABTS radical cation decolorization, ferric reducing antioxidant power (FRAP), and reducing power assays.

Objectives: The major objective of the study was to explore CDB to isolate potential microorganism and to identify potential bioactive compound and apply these compounds for various field and green bioactive product.

Methods: A fresh CDB sample was collected for bacterial isolation, screening, and identification of exopolysaccharide (EPS)-producing strains using serial dilution. EPS producers were positively selected via Congo red dye and fermentation methods, with DH5α *E. coli* as the positive control, followed by biochemical identification per Bergey's Manual. EPS production occurred in LB medium supplemented with 3% sucrose, optimized for yield and biomass by varying pH, carbon, and nitrogen sources. EPS was extracted via chilled absolute ethanol precipitation, deproteinized with TCA, and lyophilized for further use.

Results: The isolated bacterium was Gram-positive and produced high EPS yields, with LB medium optimal at 120 mg/L. Sucrose proved the best carbon source, yeast extract the ideal nitrogen source, and pH 7.0 maximized production. Purified EPS showed key functional groups consistent with exopolysaccharides in prior studies; XRD confirmed its amorphous, semi-crystalline structure. Bioactivity tests revealed 97% antioxidant activity via DPPH assay and strong emulsification (79% index with groundnut oil).

Conclusions: A novel EPS-producing bacterium, *Bacillus* strain CDB-1, was isolated from cow dung and produced 120 mg/L of exopolysaccharides (EPS). The extracted and purified EPS was confirmed as a polysaccharide through FTIR analysis and morphological characterization. Antioxidant activity assessed through DPPH, ABTS, FRAP, and reducing power assays showed strong, dose-dependent free-radical scavenging and reducing abilities. These results highlight cow

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dung as a valuable source of EPS-producing microbes and demonstrate that EPS from strain CDB-1 has significant antioxidant potential, making it promising for food, pharmaceutical, and biotechnological applications.

1. Introduction

Extracellular polymeric substances (EPSs) are the biopolymers that are found in the surroundings of the outer microbial cell wall [1]. They may be attached loosely to the cell wall and completely dispersed to the environment for protective function under extreme environmental conditions [2]. The EPS are high molecular weight, ranging from 100 to 2000 kDa [3,4]. It is composed of carbohydrates like proteins, nucleic acids, uronic acids, fats, sulfates, lactates, and phospholipids, [5] which are the backbone chain of polysaccharides that are excreted by the cell walls of various eukaryotic (*Algae, fungi* and *phytoplankton*) organisms [5,2]. These polysaccharides are grouped into two: Natural polysaccharides and Semi-synthetic polysaccharides. Natural polysaccharides are obtained from various organisms such as algae, fungi, microorganisms, and plants. Semi-synthetic polysaccharides are produced by chemical or enzymatic modification of the macromolecules [6]. Based on their monomeric composition, EPS are classified into two: i) Homopolysaccharides (HoPS) and ii) Heteropolysaccharides (HePS). HoPS are composed of a single monosaccharide unit linked by a glycosidic bond, and HePS are composed of linear or branched monosaccharide units [7,8]. Also, EPS are structural monomeric units, such as pentoses like D-arabinose, D-ribose, D-xylose, hexoses (i.e., D-fructose, D-glucose, D-mannose, and D-galactose), deoxy-hexoses (L-rhamnose and L-fucose), uronic acids (D-glucuronic acid, and D-galacturonic acid), D-glucosamine, and D-galactosamine [2,4]. Microbial exopolysaccharides (EPSs) play an important role in water retention, the absorption of exogenous organic compounds for the accumulation of nutrients from the environment, and enhanced biofilm resistance to environmental stresses [9]. EPS are produced by bacteria, algae, fungi, and in very smaller amount by yeast and molds to protect the cell from toxic conditions. Thereby, wide varieties of EPSs are known by different genera such as *Bacillus cereus, Bacillus polymyxa*, *Bacillus thuringiesis*, *Bacillus lentus*, *Bacillus mycoides* [10], *Leuconostoc, Lactobacillus, Sphingomonas, Aureomonas, Xanthomonas, Pseudomonas, Azotobacter, Rhizobium, Streptococcus, Schizophylum, Zymomonas, Aureobasidium, Acetobacter, Mucorales, Achromobacter, Agrobacterium, Rhizobium, and Sclerotium* [11]. Furthermore, EPS can protect the microbial communities against extreme temperature, pH, salinity, and alkaline and also lack of nutrient accessibility by forming a barrier between the microbe and its environment [12]. Currently, many EPS are produced through biotechnology techniques. Each EPS has its own biosynthetic pathway for an extensive range of industrial applications, such as food, pharmaceutical, cosmetics [13], agricultural, textile, oil drilling, paper manufacturing, and medical industries, as well as in the chemical industries. EPS has high-value applications and is also useful to human life, and they are safe, nontoxic, with no side effects on the environment as focusing on greener production [11,14]. They are cost-effective alternatives to the environment because the microbial cells produced them in large quantities by biotechnological processes using low-cost, in a short time [11].In this study, EPS producing bacteria *Bacillus strain-CBD-1* were isolated from the cow-dung and confirmed by molecular 16sRDNA sequencing and biochemical assays. The EPS production was optimized for pH, temperature and purified by ethanol precipitation method. The EPS yield was also optimized using several factors such as carbon and nitrogen sources. Finally, the purified EPS was characterized by Fourier transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD) to identify the chemical nature and amorphous structural. Furthermore, antioxidant activity and emulsifying activity were analyzed to highlight the potential of EPS as bioactive ingredient for cosmetic industry as active biopolymer.

2. Material and Methods

2.1. Materials

Nutrient Agar Media (NAM) (HIMEDIA, Nashik), NaCl (Loba Chemicals, Mumbai), Gram Stains - Kit (Loba

Chemicals, Mumbai), Agar Powder (HIMEDIA, Nashik), MRS agar media (M641, HIMEDIA, Nashik), Glucose (HIMEDIA, Nashik), Sucrose (Loba Chemical Mumbai), Lactose (Loba Chemical Mumbai), Maltose (Loba Chemical Mumbai), Fructose (Loba Chemical Mumbai), Congo red agar (HIMEDIA, Nashik), Luria-Bertani Agar Media (HIMEDIA, Nashik), Yeast Extract (HIMEDIA, Nashik), Beef Extract (Loba Chemicals, Mumbai), Tryptone (Loba Chemical Mumbai) and other fine chemicals were purchased from HIMEDIA (INDIA).

2.2. Sample Collection

The cow dung sample was collected from the Kharora district area of Raipur, Chhattisgarh, India. Which is situated at 21°16’ North latitude and 81°36’ East longitude with an altitude of 298.58 meters above mean sea level. The isolated bacterial strain was further screened and characterized for the EPS production [10,15].

2.3. Isolation and screening of Exopolysaccharide producing microbial consortia

We collected a cow dung sample from Kharora, a district area of Raipur, Chhattisgarh, India. The sample was collected in a sterile plastic bottle under aseptic conditions and were transport to the laboratory. We added one gram (1 g) of cow dung sample in 10 ml sterile saline solution (0.9% NaCl w/v) in a test tube [16]. The serial dilution techniques were performed as precisely as possible to isolate bacterial colonies from the CD (cow dung) sample. Luria-Bertani (LB) agar medium (Peptone 10 g/L, NaCl 10g/L, Yeast extract 5g/L, Agar 15 g/L, pH 6.8±) is used [9] for plating. The dilution factors selected are 10^{-5}, 10^{-6}, 10^{-7}, and $10^{-8.}$ aseptically 100 µl from each dilution was transferred and spread on solidified LB plates, incubated at 37˚C for 24 h to determine the colonies. The isolated slimy colonies were purified and preserved in glycerol stock at – 80 °C freezer for further use [17,18] Aseptically.

3. Screening, Identification And Confirmation Of Isolated Bacterial For Exopolysaccharide Production

The gram strain was performed for each isolated bacterial colony. Gram straining (Himedia Gram stain kit) technique is useful for assessing the bacterial strain indicate (Gram positive or Gram negative). This typical method is for characteristic of micromorphology, identification and taxonomic division [19,20]. It is the pre confirming of the EPS producing by bacterial strain. Gram strain followed the standard protocol as per the kit, HIMEDIA [21].

3.1. Congo red screening assay

The Congo red agar test is used to identify a particular bacterial strain that differentiate between pigment or nonpigmented colonies. In this method, Congo red dye is used with sucrose on selected agar medium [22]. The Congo red test was used to analyze the conformation of purified EPS fractions. The bacterial strain form dark colour strain that indicates the EPS production by the strain [23]. The preparation of Congo red agar solution was done by taking 0.04 g of dye and dissolved in 50ml of DDW, and this solution was further used to make Congo red LB agar by incorporating LB agar media (HIMEDIA, Nashik) and autoclaved at 121˚C for 15 minutes. Additionally, a 10% sucrose solution was prepared separately and sterilized via autoclaved at 121˚C for 15 mins. Once all component was sterilized, it was allowed to cool down slightly, and 3 % sucrose solution was mixed aseptically, followed by plating and streaking of selected bacterial strain for confirmation and grown at 30°C for 24 hr [24].

3.2. Biochemical Characterization of isolates

The Biochemical test was examined by many tests for identification of bacterial strain as described by Bergey’s manual [25]. Different biochemical tests were conducted, such as Catalase Test, Amylase Test, Indole Test, Methyl Red Test, Voges-Proskauer Test, and Phosphate solubilization for carbohydrates of various sugars [26].

4.Growth Culture Media Optimization For Biomass And Eps Yield

The selection of different media to analyse the bacterial strain growth rate and the EPS yield by selected media, temperature, pH, carbon source, and nitrogen source. The inoculum was grown overnight in 250 mL flasks containing 50 mL of LB broth and grown at 30 °C for 48 hours in a shaker incubator at 150 rpm. The OD 600nm was recorded of EPS production from the cultured medium [27]. Here, four different media were selected for evaluation and media selection including; Nutrient medium (NM) broth (peptone 10g/L, NaCl 10g/L, yeast extract 5g/L, pH 6.8±0.2), Luria-Bertani (LB) broth (Peptone 10 g/L, NaCl 10g/L, Yeast extract 5g/L, pH

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6.8±0.2), and P medium broth (peptone 5g/L, glucose 1g/L, $K_2HPO_4.3H_2O$ 0.1g/L , $FeSO_4.7H_2O$ 0.01 g/L, (at 25℃) pH 7) [28,29]. All these media were taken in a flask and add 100ml of distilled water, and glucose stock solution was prepared and put it in the autoclave at 121˚C for 15 minutes 15 psi. The overnight inoculum was inoculated for the growth of culture medium to find the maximum growth in 5 days vs EPS production [9]. The studies on the selection of pH for growth rate and EPS production.

4.1. Effect of pH

This pH was analyzed for growth rate and EPS production for good results. Here few pH influences, the range of pH was 6,7,8, and 9, while the temperature conditions were included from 28℃, 30℃, and 37℃, respectively. The growth rate and EPS production were analysed of the selected parameter for enhancement of growth rate and EPS production, which are preferred for the further analysis of EPS [30].

4.1. Effect of carbon and nitrogen sources

The studies of carbon source to identify the enhancement of growth rate and EPS production [31]. Here different carbon source was use with 3% (w/v) of glucose, sucrose, maltose, lactose, and fructose were used in the bacterial culture medium to growth the EPS production. To obtain the suitable carbon source the effect on bacterial growth rate and EPS yield were observed [32]. In this study, nitrogen source different nitrogen source on bacterial growth and EPS production, to determine the growth rate and EPS production [33]. The cultures were inoculated with bacterial strain and incubated at 37˚C with agitation at 150 rpm for 5 days. At the conclusion of the growth period, the fermentation broth was centrifuged, and total sugar content was assessed. After analysis, that the nitrogen source concentrations of 0.5% (w/v) includes yeast extract, beef extract, tryptone that were identified for EPS production. Optimal culture volume for maximal EPS production was then investigated [34].

5.Extractionand Purification Of Exopolysaccharide

The pure CDB-1 strain was collected further by inoculated in 10 mL of LB broth medium for 24 hr, and 1% inoculum was then transferred into 500 mL of MSR broth in a 1 L flask and allowed to grow at 37°C for 72 h at 150 rpm [35]. Then the extraction of crude EPS was further proceeded in MRS medium on that inoculate CDB-1 strain and supplemented with 3% (w/v) sucrose solution and 0.5 % (w/v) yeast extract at 30℃ for 72 h. After incubation at 37°C for 72 h, then the culture was separated by added 70 % (w/v) trichloroacetic acid (TCA) to the culture to achieve a final concentration of 15 % (w/v). The mixture was precipitate to remove the proteins from the supernatant was kept at 4℃ for 24 hr, centrifugation 12,000 x g for 25 min at 4˚C. The supernatant was precipitated with three volumes of chilled absolute 95 % ethanol and incubated at 4°C for 24 h. The precipitated crude EPS was collected by centrifugation at same condition. The pellet was dissolved in deionized water and dialyzed against distilled water at 4°C for 24 h and then lyophilized. The EPS yield was collected [39].

6. Emulsification Activity

The emulsification activity of EPS was reported according [36]. Emulsion formation was assessed by 3 ml of different oils including kerosine oil, groundnut oil, mustard oil, sunflower oil, and coconut oil were added to 2 ml of the EPS solutions at a concentration of 1 mg/mL [37]. Then, each sample was vortex for 5-10 mins and left it for 1-5 days overnight. The emulsification index effects of EPS reading were taken to assess the emulsifications [38].

The emulsification index was calculated by formula: $EI = \frac{He}{Ht} X100\%$

Where, H*e* is the height of the emulsion layer and H*t* is the overall height of the mixture.

6.1. Oil holding capacity (OHC)

The experiment was conducted by the dried powder EPS (0.05 g) in 10 mL of selected oil. Then the mixture was vortex vigorously to disperse the sample [40]. This sample mixture was left undisturbed for at least 30 min and between that time period vortexing for 5 s every 10 min of intermediate, followed by centrifugation at 3500 rpm for 10 min. After discarding the supernatant, the falcon tube was weighed with EPS and without EPS [41].

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The OHC was calculated by the following equation:

$$\text{Oil Holding Capacity (OHC)}\% = \frac{\text{Oil bound weight (g)}}{\text{Initial sample weight (g)}}$$

6.2. Water Holding Capacity (WHC)

The measurement of the water holding capacity (WHC) of EPSs was performed by [42]. The sample of dried EPS was weighed at 0.5g in 10ml deionized water and vortex the mixer to form a solution. Then, dispersed material was centrifuged at 10000 rpm for 25 mins. The unbound excess water was discarded, and the weight of EPS was recorded [43].

$$WHC(\%) = \frac{\text{Total EPS weight after absorption}}{\text{Total dry EPS weight}} \times 100$$

6.3. Hydrocarbon

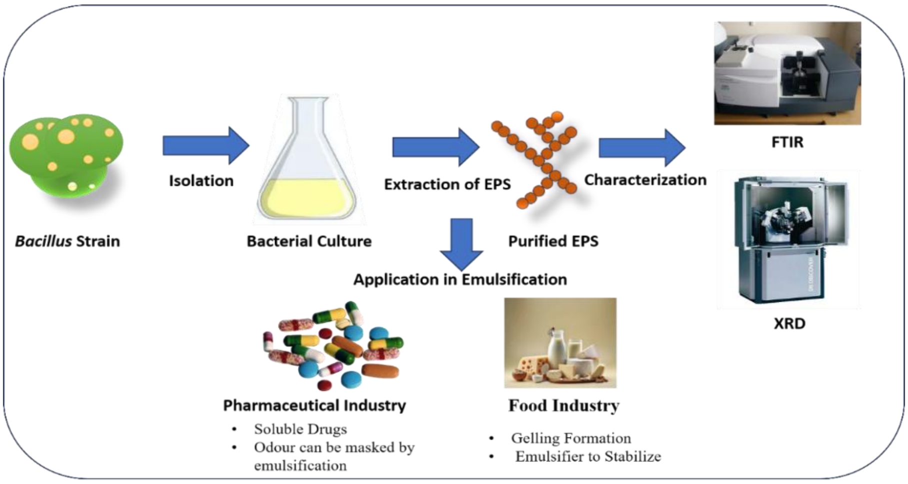

Figure 1.Graphical abstract: the process begins with the identification of a microbial source, followed by cultivation in a liquid growth medium to promote the secretion of exopolysaccharides.

In this hydrocarbons activity of EPS was analysed using the method by [44]. Briefly, the experiment was conducted by taken 2ml of the EPS cultured solution in a test tube. After that, in that test tube added 3ml of hydrocarbon compounds (Xylene, benzene, and chloroform) was added to the test tube and vortexed vigorously for 2 minutes.Then emulsion indexes of different hydrocarbons were determined after 24 hours, 168 hours, and 360 hours, as recorded [45].

The calculation Formula:

$$E24 = \text{Hight of the emulsionlayer(he)} / \text{Overall hightofmixture(ht)} \times 100$$

7. ANTIOXIDENT ACTIVITY

Table 1. Biochemical and several enzyme activities of CDB-1 isolates.

Biochemical Tests	CDB-1 Bacterial Strain	References
Catalase	Positive	(Zhang J. et al.; 2020)
Amylase	Positive	(Niyomukiza S. et al.; 2022)
Cellulase	Positive	(Niyomukiza S. et al.; 2022)
Oxidase	Positive	(Al-Dhabaan F. A.; 2019)
Urease	Positive	(Saleh F. et al.; 2014)
Gelatine	Positive	(Zhang J. et al.; 2020)
Citrate	Positive	(Zhang J. et al.; 2020)
Indole test	Negative	(Lu Z. et al.; 2018)
Methyl Red Test	Negative	(Lu Z. et al.; 2018)
Voges-Proskauer	Positive	(Zhang J. et al.; 2020)

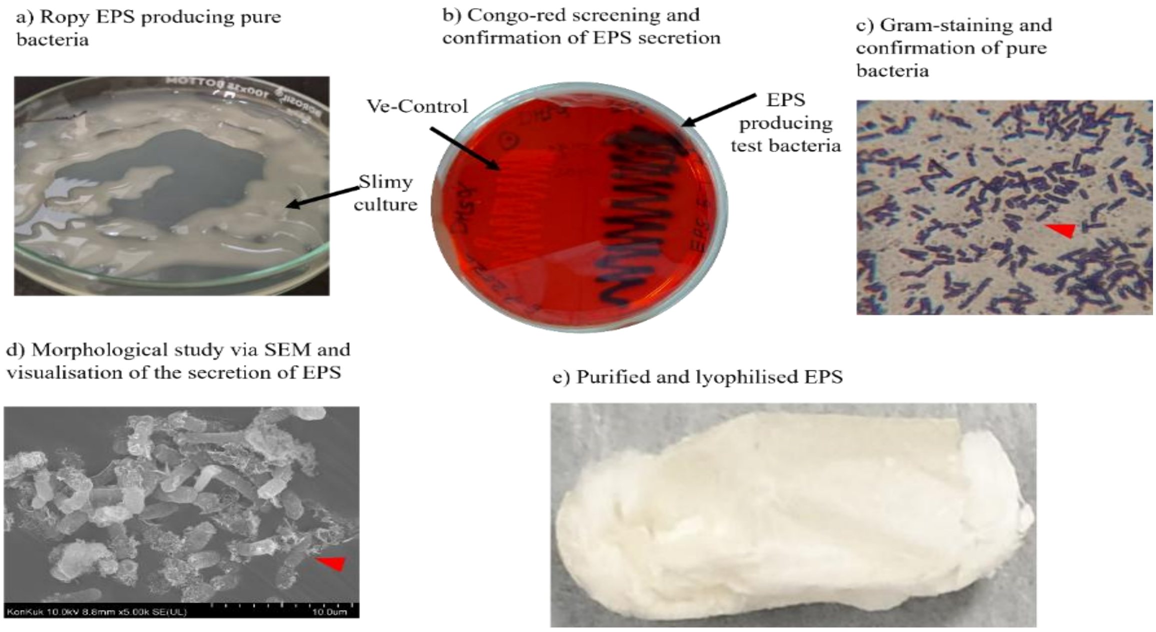

Figure 2.Morphological and structural characterization of Exopolysaccharide producing bacteria, and purified Exopolysaccharide.**(A)** Mucoid colony morphology of Bacteria on agar plate; **(B)** Congo Red binding assay demonstrating biofilm/EPS production (indicated by the blac arrow); **(C)** Light microscopy of Gram-stained cells showing rod-shaped morphology (red arrowhead); **(D)** SEM micrograph at 5.00k magnification showing bacilli embedded in a dense exopolysaccharide matrix (red arrowhead); **(E)** Physical appearance of the purified, lyophilized exopolysaccharide CDB-1.

The 2-diphenyl-1- picrylhydrazyl (DPPH)method was performed based on the capture of the DPPH radical for antioxidants as described previously [46]. About 10 mM DPPH stock solution was prepared by dissolving DPPH in ethanol to a final volume of 20 mL. For the assay, 200 µl of DPPH solution (0.2 mM) was mixed with 0.2 mM 200 µl of EPS solution at various concentrations (0, 0,5, 1, 2, and 4 mg) [46]. The mixture was shaken incubated at 25 °C for 30 min and protected from light. The DPH assay was conducted in triplicate using ascorbic acid as

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positive control and distilled water a negative control [47].

The percentage of scavenging activity for DPPH radical was expressed as:

Scavenging effect of EPS (%) = $[1 - (A_{Sample} - A_{Blank})/A_{control}] \times 100\ \%$

Where A_{sample} is the absorbance of the DPPH solution mixed with the EPS solution, A_{Blank} is the absorbance of a mixture of blank, and $A_{Control}$ was the absorbance of the control reaction.

8. Functional Characterization Of Purified Eps

8.1. Fourier Transform Infrared Spectroscopy (FTIR)

FT-IR analysis was conducted to determine the functional groups present in EPS. The functional groups of the EPS were analyzed via an FTIR (JASCO FT/IR-6800) [49]. The Fourier transform infrared spectrum (FTIR) spectra was obtained utilizing 20 mg of lyophilized EPS was ground and mixed with potassium bromide (KBr) powder. Then to obtain the FT-IR spectrum of EPS was employed. The spectrum was recorded in the range of 4000 cm^{-1} to 500 cm^{-1} [48].

8.2. X-Ray Diffraction (XRD)

X-ray diffraction (XRD) analysis to study the physical properties of EPS were performed on the Bruker D8 Advance model XRD [6]. The radiation generated at 40 kV and 30mA using slow scan. X-rays were diffracted and calculated with Bragg's law ($n\lambda = 2d \sin\theta$). EPS sample placed into sample holder and collected the data as a 2θ range of 20°–80°, at a scan rate of 1.0° per minute, at room temperature [50].

9. Results And Discussion

9.1. *Screening of the Bacterial Isolates*

In the present study, EPS producing isolates was obtained to investigate the potential production of EPS by Bacillus strain, the bacterial strain. The isolates were selected according to their appearance as ropy, irregular, large colonies, a dull surface, and smooth texture or slimy in nature. Colonies that showing slimy appearance were picked and subculture in a nutrient agar medium (NAM). The total isolates were five, collected from the cow dung sample. The EPS-producing isolates were named CDB-1, CDB-2, CDB-3, CDB-4, and CDB-5. Based on the important colony morphology of the isolates shown in (Fig 1a). Among these isolates, one strain (CDB-1) was found to produce EPS in satisfactory amounts [51]. Based on these results, we have focused our investigations on the CDB-1 bacterium isolate. Standard for biochemical test, physiological, and morphological investigations showed that the bacterium strain is a Gram-positive Bacillus species, shown in Fig 1b). In Congo red agar formed pigmented colonies with a dark red centre zone, which confirms that the isolated strain was positive, as shown in (Fig 1c). The isolated strain *Bacillus strain* CDB-1 exhibits typical mucoid characteristics on solid media, a phenotypic hallmark of exopolysaccharide (EPS) secretion. Gram staining and Scanning Electron Microscopy (SEM) confirm the strain as rod-shaped bacilli. High-resolution SEM imaging (Fig. 1D) reveals that individual cells are anchored within a complex, web-like extracellular matrix, which provides structural integrity to the bacterial community [51]. The purified EPS (Fig. 1E) appears as a white, fibrous, and porous material after lyophilization. The CDB-1 bacterium isolate showed positive results in the catalase test, catalase, amylase, cellulase, urease, gelatinase, and Voges–Proskauer, indicating extensive extracellular capabilities (Table 1). Negative results in Indole and Methyl Red tests for Bacillus species [52].

9.2. *Optimization of different media*

The evaluation of different media such as NBM (Nutrient broth media), LB (Luria-Bertani) and P media were explored for high growth biomass and EPS yield over four days which reveals significant differences in their capacity to support the growth and EPS production [53]. Among three media, LB exhibited the highest growth, reaching an OD600 nm of approximately 5.5 by days 4 shown in figure 3. LB media also showed to be the most effective for EPS production, with 0.2/mg/mL on day 4. P media also showed high yield of approximately 0.20mg/mL. Unlike, LB, P media showed

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a more gradual, steady increase in yield from day 2 as compared to days 4, which indicates a stable EPS biosynthesis. In contrast, NAM media, showed lowest EPS yield around 0.15 mg/mL on day 4. Overall, the data suggested that all three media supported for similar biomass however LB provide the most favourable nutrition profile for diverting the carbon flux toward EPS production.

EPS was dissolved in deionized water, and the obtained solution was subsequently dialyzed for 72 h against deionized water. After that solution was lyophilized and precipitated by three-fold volume of absolute 95% ethanol chilled to maintain the solid of CDB-1.

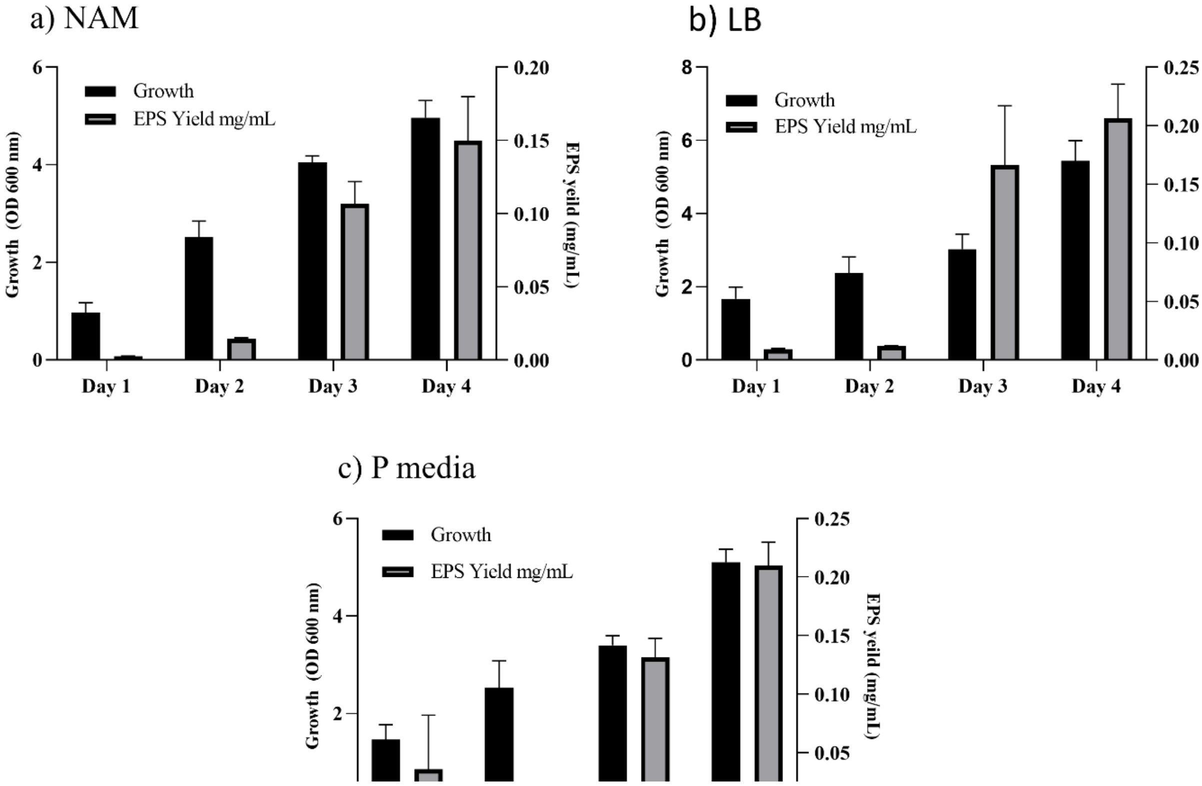

Figure 3.Optimization of EPS production. (A) Effect of NAM on growth and yield; (B) Influence of LB media; (C) Influence of P media.

9.3. Effect of pH on Bacterial Growth and EPS Yield

The optimization study showed that the environmental pH significantly influences both the metabolic activity (growth rate) and the yield of EPS from isolated CBD-1. The maximum growth rate and exopolysaccharide (EPS) production were observed at pH-7, where the yield peaked at approximately 53 mg/L figure.4c). This aligns with general observations that most *Bacillus* species and other microbial strains maximize EPS production at near-neutral pH levels [54]. A neutral pH likely provides the ideal physiological environment for the enzymes involved in the complex exopolysaccharide biosynthesis pathways, such as the Wzx/Wzy-dependent or ABC transporter-dependent pathways. At pH-8, EPS remains high despite nearly 70 % growth drop at pH-9, indicating the stress induced the overproduction for protection, as observed in *Bacillus sp.* [55]. AT pH-6, higher EPS, but the moderate growth compared to pH-9, was observed, where alkalinity disrupts membranes more severally than acidic environment. Bacillus tolerates wide pH but Favor higher acidity over extremes [56].

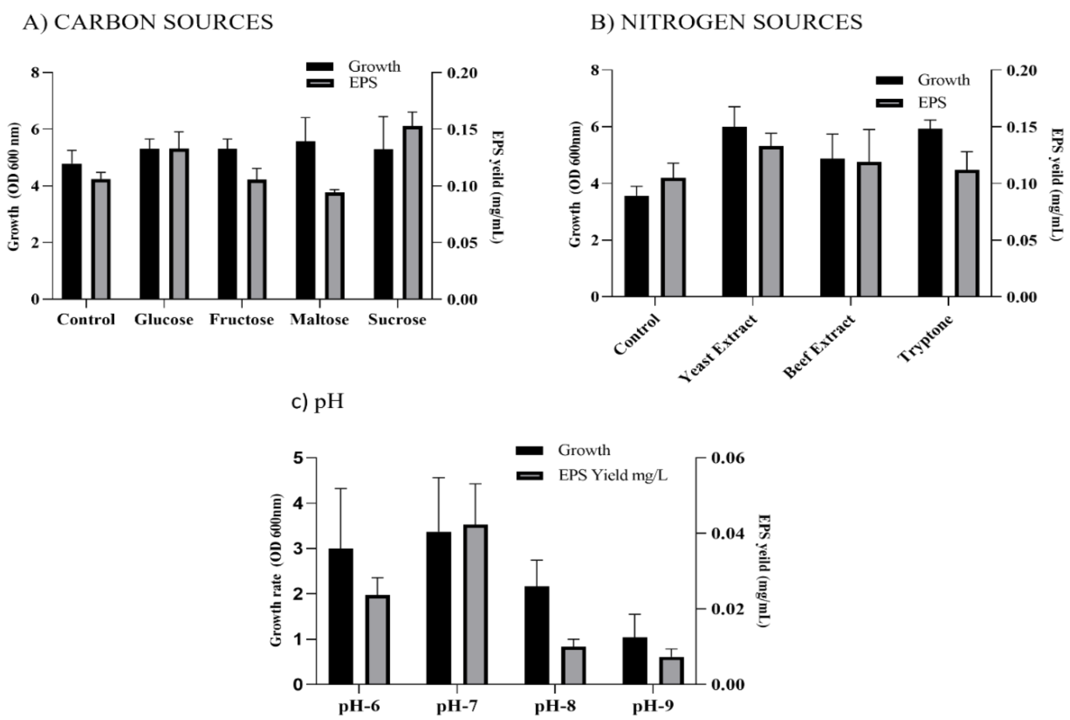

Figure 4..Effect of Different Carbon Sources, nitrogen source and, pH on Biomass Accumulation and Exopolysaccharide Production.

9.3. Effect of different carbon and nitrogen Sources

The biosynthetic efficiency of the *Bacillus sp.* is highly dependent on environmental and nutritional parameter. Optimization study showed that sucrose often excels due to efficient UDP-glucose channelling into synthesis pathway. Sucrose's showed EPS yield nearly 0.15 mg/mL, Figure 3, a), despite comparable growth to glucose reflects its role in priming glycosyltransferase, as seen in Bacillus subtilis studies [57]. Maltose prioritized biomass OD 600nm ~ 5.6 over EPS ~ 0.09 mg/mL, driving flux to glycolysis rather than polymer assembly Figure 3.a). This optimization study showed that sucrose consistently maximizes EPS in Bacillus up to 2-3fold higher than glucose, as it sustains high intracellular sugar nucleotides for Wzy-dependedt polymerization Figure 4. Without catabolite repression. And the growth remains steady OD600 nm ~ 5.3, emphasizing synthesis efficiency over proliferation [33]. In case of nitrogen source optimization, all three nitrogen sources (yeast extract, beef extract, and tryptone) significantly increased both bacterial growth and EPS production compared to the control group [56]. Among all three, Yeast extract stands out as the preferred organic nitrogen source for Bacillus EPS, elevating growth OD 600 nm ~ 6.0 and yielded ~ 0.13 mg/mL, beyond the control and beef extract Figure 3. b) [58, 53).

Table 2. Physicochemical and functional properties of EPS.

Oil Holding	EPS mg/L	Reference
Kerosine oil	40%	Ahmed Z. et al.; 2013
Sunflower oil	110%	Dai Y. et al.; 2024
Groundnut oil	120%	Alvarez V. M. et al.; 2018
Water Holding	125.4mg/L	Seveiri R. M. et al.; 2020
Hydrocarbons		
Xylene	228 mg/L	Nikolova C. et al.; 2024
Benzene	157.1 mg/L	Freitas F. et al.; 2011
Chloroform	120 mg/L	Freitas F. et al.; 2011
Solubility	66.6%	Ahmed Z. et al.; 2013
Solvent	50%	Patil S. V. et al.; 2011

9.4 Emulsification Assay:

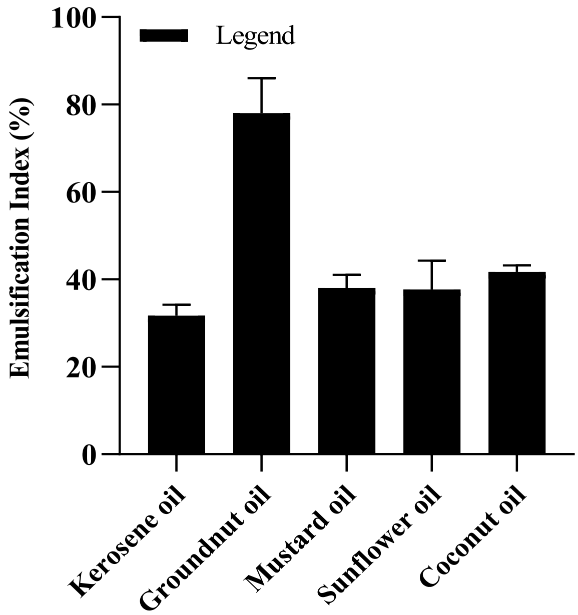

Figure 5.Emulsification Index (E24) of EPS CDB-1 across various vegetable and mineral oil substrates

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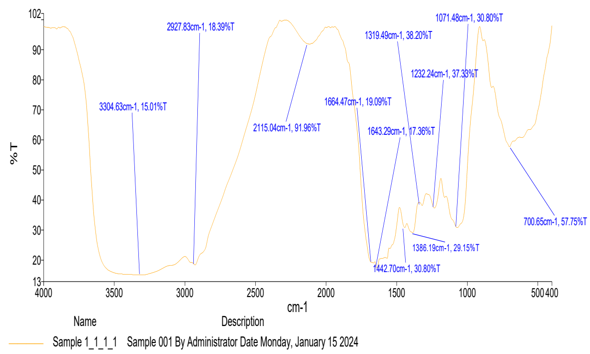
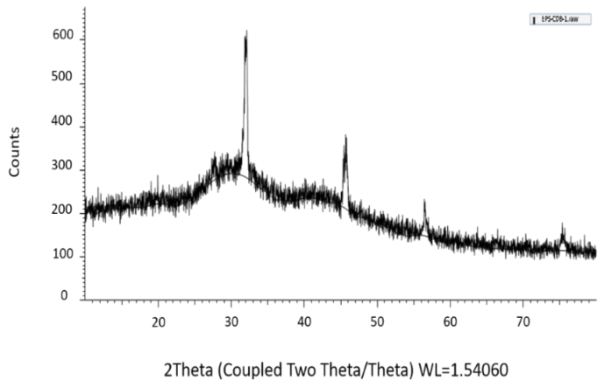
The emulsification index (%) of EPS as shown in (Figure 4), that a Bacillus sp. Produced EPS compared to previous data against vegetable oils and kerosene after 24 Hour [59]. Groundnut oil exhibits the highest stability~ 80%, followed by mustard ~60 %, sunflower ~55%, and kerosene ~40%. This evaluation demonstrated that EPSs shows significant emulsification for edible oils over hydrocarbons, attributed to amphiphilic polysaccharide chains adsorbing at oil-water interfaces [59]. The EPS exhibit a strong affinity for both lipid and aqueous phases, which is a critical characteristic for stabilizer. The high oil-holding capacity (up to 120%) (Table-2). suggested a porous structural arrangement and the presence of non-polar moieties within the heteropolysaccharide. Xylene shows the highest interaction yield was observed with xylene at 228 mg/L compared to finding by [45]. Benzene interaction with EPS was recorded at 157.1 mg/L, consistent with benchmarks set by [44]. A yield of 120mg/L was observed, aligning with the metabolic responses reported by [44]. The EPS reached a solubility of 66.6%, which is a high value for microbial polysaccharides and compares favourably to the results of [59]. Also, the biopolymer maintained a stability of 50% in the presence of solvents, matching the structure resilience documented by [57].



9.5. *Antioxidant Activity*

Natural exopolysaccharides are increasingly recognised as viable sources of natural antioxidants. EPS-CDB-1 from Bacillus demonstrates promising antioxidant activity via DPPH assay, scavenging free radicals in a time and dose-dependent manner. In this study, the antioxidant potential of EPS-CDB-1 was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay across a concentration range of 200, 400, 600, 800 and 1000 ul/mL) and the bleaching absorption of DPPH and free radical was assessed after incubation, by spectrophotometer at a wavelength of 517nm [60,58]. The scavenging study showed that, the DPPH assay confirmed that EPS-CDB-1 possesses significant dose-dependent antioxidant properties. At a concentration of 1000 ug/mL, it achieved a scavenging activity of 75% (Figure 5). Notably, at the lowest tested concentration (200 ug/mL), its activity (48%) was nearly identical to the lowest tested concertation (200 ug/mL), its activity (48%) was nearly identical to the ascorbic acid control (50%).



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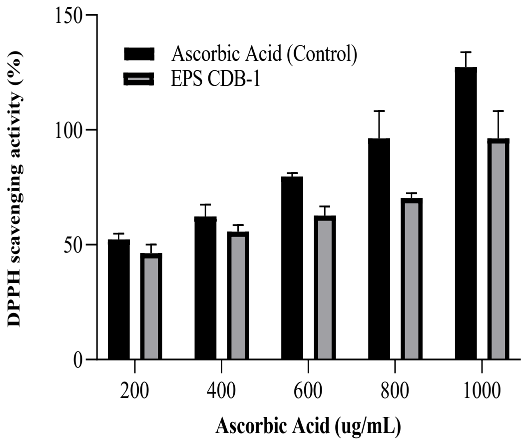

Figure 6.X-Ray diffraction of EPS-CDB-1 and crystalline structure analysis.

Figure 7.Comparative DPPH radical scavenging activity (%) of EPS CDB-1 and standard ascorbic acid (Control).

9.6. Structural Characterization of EPS by FT-IR and XRD

The FTIR spectrum of the exopolysaccharide CDB-1 displays several characteristic absorption bands typical of complex polysaccharides, ranging from 4000 to 400 cm^{-1} (Figure 6). The spectrum showed a broad band at 3304.63 cm^{-1} which corresponded to the vibration of the hydroxyl group, a C-H stretch a 2927.83 cm^{-1}, aliphatic groups, and sharp C-O-C glycosidic linkage peak at 1071.48 cm^{-1}. Peaks at 1664.47cm^{-1}(C=O stretching suggest the presence of uronic acid or amide groups, indicating an acidic heteropolysaccharide. The XRD diffraction exhibited a semi-crystalline profile (Figure 7), characterized by a broad amorphous halo and distinct sharp reflection at 32°, 46°, and 57° (2Θ). Therefore, EPS had semi crystalline and amorphous morphology. The crystalline domains acted as a backbone and improved the properties of EPS biopolymer.

10. Conclusion

This study successfully isolated and characterized CDB-1, a novel heteropolysaccharide produced by *Bacillus subtilis* (strain CDB-1) sourced from cow dung. Our structural analysis confirms that CDB-1 possesses a unique chemical framework that contributes to its significant biological potential, most notably its robust DPPH free-radical scavenging activity. A critical finding of this research is the profound impact of carbon source optimization on the EPS profile. By substituting several carbon sources like glucose, sucrose, and lactose, and

nitrogen sources like we demonstrated that medium composition influence biomass or yield but fundamentally alters the microscopic morphology and functional groups of the resulting polysaccharide. This suggests that the properties of CDB-1 can be "tuned" during the fermentation process to meet specific

industrial requirements. The functional versatility of the isolated exopolysaccharide is demonstrated through its interaction with various liquid phases, which indicates the potential for industrial and environmental application. Ultimately, the antioxidant capacity and structural versatility of CDB-1 position it as a high-value biopolymer. These results provide a strong foundation for the utilization of *B. subtilis* CDB-1 in food, pharmaceutical, and cosmetic industries.

Competing interests

The authors report no conflict of interest.

Availability of data and materials

In texts, all the accessible websites and organizations have been provided. Authors declare that all data were generated in-house and no paper mill was used.

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