

Characterization of bioactive porphyran oligosaccharides produced using porphyranase from *Bacillus* sp. NIOA284

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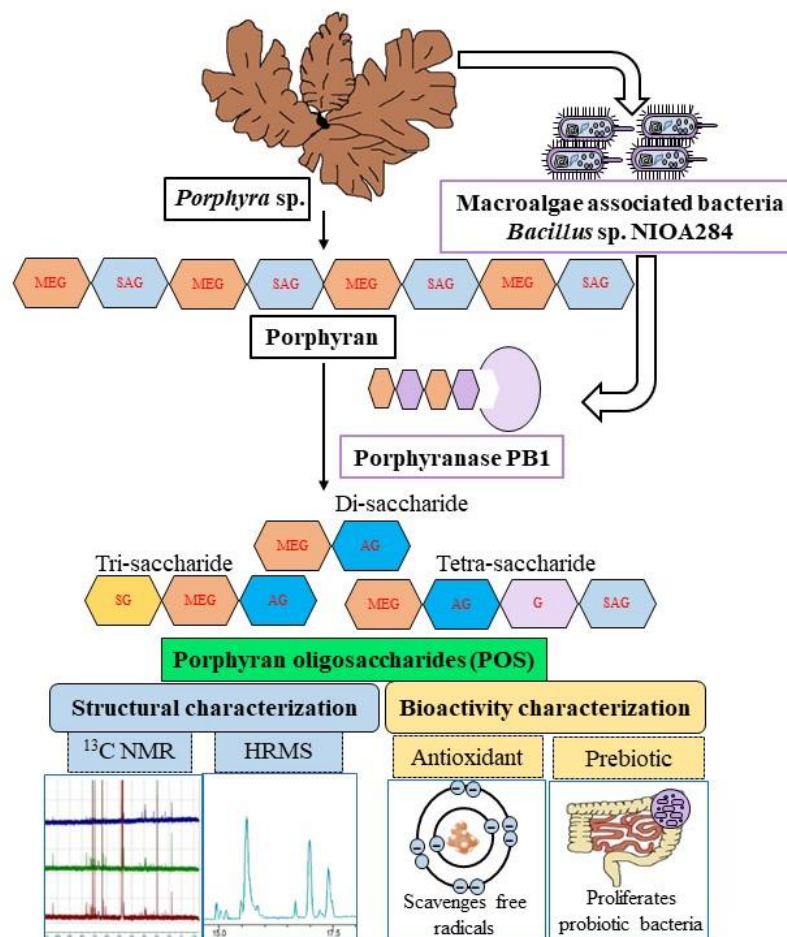
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Graphical Abstract



Abstract

Macroalgae associated bacteria are the major source of polysaccharide hydrolyzing enzymes for efficient bioutilisation of macroalgal biomass. In this study, extracellular porphyranase, PB1 isolated from a macroalgae associated bacterium, *Bacillus* sp. NIOA284 was purified and characterised for hydrolysis of porphyran into porphyran oligosaccharides. The enzyme, PB1 showed the highest activity at the optimum conditions; the temperature of 35 °C, pH 7 and 0.4 NaCl with 10 mM CoCl₂. The structural characterization of the enzymatically produced POS using NMR spectroscopy and HRMS spectrometry showed that the enzyme acted on porphyran as β -porphyranase and produced di, tri and tetra saccharides. The bioactive potential of POS was determined based on its radical scavenging and prebiotic activity. Results show that POS exhibited higher antioxidant activity than parent porphyran and fructooligosaccharides. It was also capable to proliferate the beneficial gut bacterium *Lactobacillus acidophilus* and *L. bulgaricus*, it recorded a prebiotic activity score equivalent to model prebiotic compound fructooligosaccharides. Results show that the enzymatically produced POS has combined antioxidant and prebiotic activity. This highlights the significance of macroalgae associated bacteria as a source of enzymes capable of hydrolysing polysaccharides into simple bioactive oligosaccharides for efficient management of chronic disorders caused due to oxidative stress.

Keywords Macroalgae associated bacteria; Porphyran; Porphyranase; Porphyran oligosaccharides; Antioxidant; Prebiotic

1. Introduction

Red macroalgae or Rhodophytes are the phylogenetically oldest division of marine macrophytes, which includes approximately 7000 species across the world. This division includes the economically most important macroalgae or seaweeds, such as *Kappaphycus*, *Eucheuma*, *Gracilaria*, *Gelidium* and *Porphyra* [1,2]. They are composed of specific sulfated galactan such as carrageenan, agar and porphyran as polysaccharides; and unique pigments such as phycocyanin, allophycocyanin and phycoerythrin, which are phycobiliproteins. They have high polyphenol, protein, polyunsaturated fatty acid and mineral content, due to which they have vast biological and commercial applications [3,4]. Red macroalgae such as *Kappaphycus*, *Eucheuma* and *Gracilaria* are the major carrageenolytic and agarolytic algae cultivated worldwide and account for 33.6 and 10.5% of total seaweed production, respectively [5]. However, *Porphyra*, which is one of the major macroalgae that has been used since ancient times in Chinese and Japanese medicine and as food, has not been fully explored for its commercial and aquaculture potential. It is popularly called as 'Zicai' in China, 'Nori' in Japan, 'Kim' in Korea and is used in the diet of many other countries, including the United Kingdom, Ireland, the USA and Canada. It is one of the most extensively used macroalgae worldwide as a Japanese delicacy, "Sushi". *Porphyra* is reported to have a very high nutritional composition due to their dietary fiber, a trace mineral, nicotinic acid, vitamin B1 and palmitoyl cholesteryl mannose glycoside content [6,7]. There are nearly 133 species of *Porphyra*; a few species, *P. yezoensis*, *P. tenera*, *P. haitensis*, *P. pseudolinearis*, *P. dentate* and *P. angusta* are being cultivated worldwide and are now expanding in Korea, China and India [8,9,10].

The cell wall of *Porphyra* is mainly composed of a unique polysaccharide, porphyran. It is primarily made up of alternating units of 3-linked methylated β -D-galactose (MeG) and sulfated 3, 6-anhydro- α -L-galactose (SAG). This composition accounts for up to 70% of the *Porphyra* cell wall and the remaining 30% is constituted of agar comprising of 3, 6 anhydro- α -L-galactose (AG) and β -D-galactose (G) units [11,12,13]. Due to the complex monomers of the polysaccharide porphyran, it has a relatively higher molecular weight, increased viscosity and low solubility. This limits its application in the nutraceutical and pharmaceutical industries [14,15]. However, the enzymatically depolymerized porphyran, i.e., porphyran oligomers, are shown to have far-reaching applications in food industries [16,17,18]. Enzymatically produced red macroalgal oligosaccharides of agar and carrageenan using microbial agarase and carrageenase are well documented for their applications and various biological activities [19,20,21,22,23]. In contrast, only a few porphyranase enzymes have been characterized to hydrolyze porphyran into their oligosaccharides. However, their bioactive potential for the management of chronic diseases due to oxidative stress is not well explored [16,24,25].

Porphyranase (EC 3.2.1.178) is the specific glycoside hydrolase (GH) enzyme that can catalyse the breakdown of the glycosidic bonds in porphyran [13,24,25,26]. The Carbohydrate-Active enZYmes (CAZy) database groups it as β -porphyranase within GH16 and GH86 families [12,27,28]. It specifically recognizes the β -1, 4 linkages between MeG and SAG in porphyran and hydrolyses porphyran to produce porphyran oligosaccharides (POS). β -porphyranase has so far been reported from different marine sources, including seawater and macroalgae-associated bacteria (MAB) belonging to Proteobacteria (*Pseudoalteromonas atlantica*), Bacteroidetes (*Bacteroides plebeius*, *Zobellia galactanivorans*, *Wenyngzhuangia fucanilytica*) and Actinobacteria (*Arthrobacter* sp.) [12,13,24,25,29]. However, purification and production of porphyranase are not explored thoroughly for commercial applications due to their narrow specific activity. Therefore, it is crucial to constantly identify and isolate microorganisms that exhibit extracellular porphyranase activity.

MABs are the major source of these specific polysaccharide hydrolyzing enzymes and from our previous study, we have shown that of the 400 MABs studied, only 5%, i.e., about 19 of the MABs, are shown to hydrolyse porphyran [30]. This includes *Bacillus* sp. NIOA29, NIOA34, NIOA181, NIOA183, NIOA284, NIOA321, NIOA323, NIOA329, NIO388, *Exiguobacterium* sp. NIOA96, *Vibrio* sp. NIOA169, *Pseudomonas* sp. NIOA319, *Oceanobacillus* sp. NIOA379, *Staphylococcus* sp. NIOA118, NIOA174, *Marinobacter* sp. NIOA327, *Acinetobacter* sp. NIOA328 and *Micrococcus* sp. NIOA354 [30]. Among these MABs, *Bacillus* sp. NIOA284 recorded the highest extracellular porphyranase activity in a minimal media with 0.5% porphyran as the sole carbon source. In this study, we have purified and characterized porphyranase from the potential macroalgae-associated bacterium, *Bacillus* sp. NIOA284, isolated from the surface of red macroalgae *Porphyra* for the production of bioactive POS. Characterizing it will help to understand the importance of microbial enzymes to produce bioactive algal oligosaccharides, which can be explored for functional food and healthcare applications.

2. Materials and methods

2.1. Materials

Fine biochemical, hydrogen peroxide (H₂O₂), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and fructooligosaccharides (FOS) from chicory were purchased from Sigma Aldrich, USA. Polyacrylamide, Diethylaminoethyl (DEAE)-Sepharose and Superdex[®]75 gel filtration columns and protein molecular weight marker were also purchased from Sigma Aldrich, USA. Dextrose-free Man-Rogosa-Sharp (MRS) medium was bought from Sisco Research Laboratory, India. Porphyran used in this study was obtained through aqueous

extraction of *Porphyra* sp. collected from the Goa coast [30]. Type specimens of probiotic cultures *Lactobacillus acidophilus* and *L. bulgaricus* and pathogenic strain of enteric *Escherichia coli* were procured from CSIR-NCL, National Collection of Industrial Microorganisms, Pune, India.

2.2. Production and purification of porphyranase from *Bacillus* sp. NIOA284

The potential macroalgae-associated bacterium, *Bacillus* sp. NIOA284 (NCIM 5816) was selected for this study as it showed the highest porphyranase activity of about 200 U mg⁻¹ [30]. The isolate, NIOA284 was grown in a selective medium for the production of the extracellular porphyranase. It contained porphyran (0.5%) as the only carbon source and it was amended with salts such as dipotassium phosphate (0.2%), sodium chloride (3%), magnesium sulfate heptahydrate (0.1%) and iron (II) sulfate heptahydrate (0.001%) in distilled water at pH 7. The isolate was aerobically cultured in 250 mL selective medium and incubated at 28 °C and 150 rpm for 24 h, then centrifuged at 8000 rpm for 20 minutes to extract the extracellular enzyme-containing supernatant. This cell-free supernatant was precipitated with saturated ammonium sulfate, it was further subjected to dialysis in phosphate buffer (50 mM, pH 7) to concentrate the enzymes from the supernatant. The enzymes were purified through the DEAE Sepharose Ion-Exchange chromatography column and eluted using a linear gradient of NaCl (0.2 - 1 M). The eluted fractions (5 mL each) were collected and active fractions were combined and concentrated on a vacuum concentrator (Fig. S1a). The enzymes were further purified and eluted using phosphate buffer (50 mM, pH 7) through gel permeation chromatography on a Superdex[®]75 column as 5 mL fractions (Fig. S1b). The active fractions were lyophilized and utilized for further analysis.

2.3. Biochemical characterization of porphyranase (PB1)

Extracellular porphyranase PB1, from the potential, macroalgae-associated bacterium *Bacillus* sp. NIOA284 was characterized for its optimum activity at different parameters such as temperature, pH and varying NaCl concentrations. The effect of different temperatures ranging from 20 - 60 °C on porphyranase activity was analysed by incubating the PB1 with 0.5% porphyran at selected temperatures for 1 h. Similarly, the effect of pH on porphyranase activity was also determined at optimum temperature in appropriate buffers (50 mM) for each of the pH ranges, such as acetate buffer (pH 3 - 6), phosphate buffer (pH 6 - 7), Tris-HCl buffer (pH 8 - 9) and carbonate-bicarbonate buffer (pH 10 - 11). The effect of different NaCl concentrations (0.001 M - 1 M) on porphyranase activity was evaluated at pH 7 and 35 °C for 1 h. The relative activity at different conditions were determined by comparing the enzyme activity obtained at 28°C, pH 7 and without NaCl addition which was used for production as extracellular enzymes as 100% control activity. The impact of different metal ions

as salts of LiCl, KCl, MgCl₂, CaCl₂, BaCl₂, NiSO₄, CoCl₂, CuCl₂ and FeCl₃ at 10 mM concentration on porphyranase activity was determined to understand their role as cofactors. The influence of chelating agents such as ethylene diamine tetra acetic acid (EDTA), inhibitors such as urea, PMSF, β -mercaptoethanol and detergents including sodium dodecyl sulfate (SDS), Triton X-100, Tween 20, and Tween 80 at 0.1% concentration on porphyranase activity was determined. The relative activity was determined by comparing the enzyme activity obtained without the addition of metal ions, inhibitors or detergents as 100% control activity.

2.4. Enzymatic hydrolysis of porphyran using PB1

Purified porphyranase, PB1 from NIOA284 at 10 $\mu\text{g mL}^{-1}$ concentration was used for the production of POS at 35 °C to breakdown porphyran (1%) in phosphate buffer (50 mM, pH 7) with 0.4 M NaCl and 10 mM CoCl₂. The K_m and V_{max} were determined based on Lineweaver–Burk equation at these optimum conditions. The enzymatic hydrolysis of porphyran was studied at the start of the incubation at 0 h and 3, 6, 9 and 12 h time intervals and the hydrolysed product was analysed on aluminum silica gel 60 mm Thin Layer Chromatography (TLC) plates. The separation of the products was carried out on TLC plates using an n-butanol: acetic acid: water (2:1:1) solvent mixture as a mobile phase. The TLC plate was stained for visualization, with 10% H₂SO₄ in ethanol and baked at 100 °C for 10 minutes. The enzymatically produced POS using PB1 was purified on Sephadex® G-100 medium and fractionated using phosphate buffer (50 mM, pH 7.0) and the fractions with high reducing sugar content were combined for further analysis [30].

2.5. Structural characterization of enzymatically produced POS

2.5.1. ¹³C NMR analysis

The enzymatically produced and purified POS using PB1 was treated with D₂O and freeze-dried for its structural analysis. ¹³C NMR analysis of POS was recorded using BRUKER AVANCE III HD NMR 500 MHz in Liquid State at a concentration of 20 mg mL⁻¹ in D₂O to investigate its composition and conformation.

2.5.2. High-Resolution Mass Spectrometry analysis

The enzymatically produced and purified POS (0.1 mg mL⁻¹) was analysed using High-Resolution Mass Spectrometry (HRMS) on Q-TOF spectrometer (Bruker Impact HD, USA) to determine the number of sugar moieties as described earlier [30]. The MS spectra obtained were used to determine the molecular weight of POS based on the m/z ratio and published literature [24,25].

2.6. Bioactivity of enzymatically produced POS: Antioxidant and prebiotic activity

The antioxidant activity of the enzymatically produced POS was evaluated and compared with porphyran and FOS based on their ability to scavenge model radicals DPPH and ABTS. The activity was evaluated at different concentrations (2 - 10 mg mL⁻¹) of POS and compared with porphyran and FOS. The radical scavenging activity (%) was estimated using gallic acid as a positive control [31,32]. The prebiotic potential of POS was evaluated using the probiotic cultures *Lactobacillus acidophilus* (NCIM2285) and *Lactobacillus bulgaricus* (NCIM2056) and enteric culture of *Escherichia coli* (ATCC8739) and compared with the porphyran and FOS was used as a model prebiotic compound as described earlier [30].

2.7. Statistical analysis

All the experimental analyses were performed in triplicate (n = 3) and the average values are represented with standard deviations. One-way analysis of variance (ANOVA) and Student's t-test at $P \leq 0.05$ was used to calculate the statistical significance.

3. Results and discussion

3.1. Purification of porphyranase PB1 from *Bacillus sp.* NIOA284

The enzymatic breakdown of the polysaccharides requires specific glycoside hydrolase enzymes such as agarase, carrageenase, porphyranase, laminarinase and fucoidanase, or polysaccharide lyase, which includes alginate lyase and ulvan lyase. Among these, enzymes such as porphyranase, laminarinase, fucoidanase and ulvan lyase are seldom studied, novel and are shown to be the inherent property of only a small percentage of MAB [25,28]. From our recent studies, we have identified novel MABs producing specific enzymes such as porphyranase, alginate lyase and ulvan lyase [30]. In this study, the potential porphyranase producing strain, *Bacillus sp.* NIOA284 was grown in a selective medium containing porphyran as a sole carbon source and porphyranase activity of 188 U mg⁻¹ was recorded (Table 1). The sequential purification of porphyranase was carried out, initial ammonium sulfate precipitation increased the specific activity to 368 U mg⁻¹. Further, the consecutive DEAE anion exchange and gel permeation chromatography increased the specific activity of porphyranase to 562 and 1983 U mg⁻¹, respectively (Table 1). The porphyranase specific activity and purification fold increased with each purification step. The purity of the porphyranase enzyme after each of these steps was confirmed through SDS-PAGE and the porphyranase PB1 used was 10.5 fold purified (Table 1). The molecular mass of the purified porphyranase PB1 was confirmed to be between 50 to 55 kDa against the standard molecular weight marker (Fig. S2).

3.2. Biochemical characterization of porphyranase PB1

The purified PB1 enzyme was tested at different physicochemical conditions and in the presence of metal ions and chelating agents or inhibitors or detergents to determine the optimal conditions for the enzymatic hydrolysis of porphyran. The impact of temperature (20 to 60 °C), pH (3 to 11), and NaCl concentration (0.001 to 1 M) on the PB1 activity was determined. The PB1 showed >50% activity at all tested temperatures and about 70 – 95% of relative activity was recorded at 25 to 45 °C, with the highest activity at 35 °C with about 94% of relative activity (Fig. 1a). The PB1 activity was also measured in various buffers representing the pH range from 3 - 11 at 35 °C. The PB1 retained >70% of its activity at pH 6 with decreasing activity towards both acidic pH (3 - 5) and alkaline pH (8 - 11) ranges. The highest activity was recorded in 50 mM phosphate buffer at pH 7.0 (Fig. 1b). The enzyme, PB1 showed the highest enzymatic activity at a 35 °C temperature and pH 7.0, similar to previously reported porphyranase activity [24,25]. The effect of NaCl concentrations on PB1 activity was determined and results show that >55% of porphyranase activity was retained at all tested NaCl concentrations (0.001 M - 1 M). The highest porphyranase activity of nearly 100% was recorded at 0.4 M NaCl concentration, which is equivalent to 24 ppt (Fig. 1c). The effect of different NaCl concentrations tested showed that the enzyme activity was higher at low salt concentrations. *Porphyra* sp. is generally known to grow at the end of the monsoon season when there is a salinity drop by 5 ppt from 30 to 35 ppt [9].

The effect of different metal ions (10 mM) and chelating agents or detergents (0.1%) on the PB1 activity was also estimated at optimum conditions of 35 °C temperature, pH 7.0 and in the presence of 0.4 M NaCl concentration. Metal ions such as Li⁺, K⁺ and Fe³⁺ did not show any impact on porphyranase activity. However, Ni²⁺ and Cu²⁺ declined the PB1 activity up to 51 and 61%, respectively. The PB1 activity increased in the presence of Co²⁺, Ca²⁺ and Ba²⁺ and recorded the highest (225%) activity in the presence of Co²⁺ metal ion (Fig. 2a), which could act as a cofactor for the enzyme. The effect of chelators such as EDTA, inhibitors like urea, PMSF, β-mercaptoethanol and detergents SDS, Triton X100, Tween 20 and Tween 80 on PB1 activity was also investigated. Porphyranase activity was significantly inhibited by EDTA and SDS; this may be due to the depletion or chelation of the essential metal ions present in the minimal media used for the growth and production of PB1 by NIOA284. Competitive inhibitors such as urea and protease inhibitor PMSF did not show any impact on PB1 activity, however, reducing agent β-mercaptoethanol showed activation of the PB1 with 110% activity retention (Fig. 2b). Detergents, Triton X-100, Tween 20 and Tween 80 did not interfere with the activity and more than 90% of activity was retained. Most of the previous studies on porphyranase activity for hydrolysis of porphyran have used crude bacterial extracts and have not

determined the enzyme kinetics [12,13]. In this study, the kinetic parameters K_m and V_{max} of the extracellular porphyranase PB1 were estimated as 0.05 mg mL^{-1} and 3.80 U mg^{-1} , respectively. These values represent the affinity of the substrate to the enzymes and their ability to hydrolyse half of the substrate show that the PB1 from MAB, *Bacillus* sp. NIOA284 has the potential for industrial applications.

3.3. Enzymatic production and structural characterization of POS

The purified porphyranase, PB1, was used to hydrolyse porphyran into POS at the optimum conditions obtained from this study at a temperature of $35 \text{ }^\circ\text{C}$, pH 7 and 0.4 M NaCl concentration with 10 mM CoCl_2 . The hydrolysis pattern and production of POS were monitored on a TLC plate with porphyran substrate and porphyranase PB1. The results showed that porphyran was hydrolyzed into two different oligomers with different degrees of polymerization (Fig. S3). However, there were no monomers visualised from the TLC plate, which indicates that porphyranase PB1 was a random endo-acting enzyme. The enzymatically produced POS was purified and characterized using NMR spectroscopy and HRMS spectrometry to determine its structural composition and molecular mass.

3.3.1. NMR analysis

The ^{13}C NMR spectrum of the enzymatically produced POS was elucidated and the peaks were obtained from 15 to 105 ppm and the peak at 20 ppm was identified to be for the methylation of galactose (Fig. 3a). As porphyran is composed of MeG and SAG, the signal at 100.9 and 101.9 ppm are attributed to the presence of AG and G, respectively. The resonance at 92.2 and 96.2 ppm is attributed to α - and β -anomeric carbon in the reducing end of the galactose, respectively [11,33]. These results show that the enzymatically produced POS are mainly composed of α - and β - anomeric forms of galactose, indicating the production of oligosaccharides consisting of galactose with varying degrees of polymerization. The additional peaks at 98.0 and 58.0 ppm confirm the presence of AG and methylation at C6 of galactose, respectively. The peaks at 74.7 and 75.0 ppm were assigned for C4 and C5 of SAG, respectively. The peaks at 60.9 and 61.2 ppm represent the methylation at C3 and C6 of galactose, respectively. The multiple signals between 68.5 ppm to 70.9 ppm represent the presence of numerous -C-OH bonds. The signal at 90.0 ppm was not detected, which is the characteristic of hydrolysis of α (1-3) linkage in the porphyran chain, which confirms the cleavage site of the enzyme is β (1-4) and the enzyme acted on it is β porphyranase [21].

3.3.2. Mass analysis

The results of HRMS spectrometric analysis of enzymatically produced POS showed multiple peaks at various m/z ratios for different degrees of polymerization (Fig. 3b). The HRMS analysis of

enzymatically produced POS showed a significant peak at 475 m/z, indicating the presence of disaccharides composed of MeG and SAG, representing that the β -porphyranase cleave the β -1 \rightarrow 4 glycosidic bond in porphyran [12,13]. The additional peak at 365 m/z is due to the β -1 \rightarrow 4 glycosidic cleavage between the G and AG, producing MeG and AG disaccharides. Similarly, the peak at 665 m/z represents trisaccharides units of SAG, MeG and SAG, formed due to the β -1 \rightarrow 4 glycoside bond cleavage [21,34]. The peak at 273, 365, 606, 665 and 701 m/z implies that the oligosaccharides also consist of monosaccharide sulfated galactose (SG), a disaccharide of MeG-AG, trisaccharides of SG-MeG-AG, SG-MeG-SG and tetrasaccharides of MeG-AG-G-SAG, respectively. The HRMS spectrum represents the enzymatic breakdown of porphyran with the production of di-, tri- and tetrasaccharide, with the composition of basic sugars, G and AG. These results of NMR and mass analysis suggest that the hydrolytic product were oligosaccharides of porphyran and the enzyme acted as β -porphyranase.

The structural configuration and the degree of polymerization of the oligosaccharides greatly determine the biological activity. Though there are a few reports on porphyranase from marine bacteria and their oligosaccharide production, the biological activity of these oligosaccharides has not been investigated. This is the first study on the production and characterization of bioactive POS, using porphyranase, PB1 obtained from a *Porphyra* associated bacterium, *Bacillus* sp. NIOA284 (Table 2).

3.4. Bioactivity of enzymatically produced POS

3.4.1. Antioxidant activity

The DPPH and ABTS assays were used to assess the antioxidant capacity of POS in comparison with its polymer porphyran, commercial prebiotic FOS and a model antioxidant compound, gallic acid [35]. The results show that POS exhibited good DPPH radical scavenging activity with inhibition of about 20% DPPH radicals than parent polysaccharide porphyran with <4% inhibition of DPPH radicals and FOS did not show any radical scavenging activity at tested concentrations up to 10 mg mL⁻¹ (Fig. 4a). Similarly, the ABTS radical scavenging activity results showed that the POS could scavenge ABTS radicals efficiently with >90% radical inhibition at 10 mg mL⁻¹ concentration which was higher than the porphyran with <40% radical scavenging activity and equivalent to gallic acid. POS showed concentration-dependent ABTS radical scavenging activity with a low IC₅₀ value of 4.72 mg mL⁻¹ than porphyran (Fig. 4b). Antioxidant activity is very vital and has ubiquitous applications as it can scavenge free radicals and reduce oxidative stress. The POS produced using PB1, exhibited very good, antioxidant potential, with higher radical scavenging activity than the parent polysaccharide porphyran and FOS. These enzymatically produced POS showed similar DPPH and ABTS radical activity to the

previous studies on enzymatically produced alginate oligosaccharides [31,36]. However, some of the earlier studies on enzymatically produced ulvan oligosaccharides and agaro oligosaccharides did not report any antioxidant activity [20,30].

3.4.2. Prebiotic activity

The prebiotic compounds are indigestible oligosaccharides that can selectively proliferate the probiotic bacteria *Bifidobacterium* and *Lactobacillus*, which is known to have a beneficial effect on the human gut and suppress the growth of pathogenic enteric bacteria [37]. Though there are reports on the prebiotic activity of FOS, the algal oligosaccharides are the least explored [35]. The enzymatically produced POS from this study showed good prebiotic potential and it significantly promoted the growth of probiotic *Lactobacillus* strains, equivalent to the model prebiotic compound FOS and higher than parent porphyran. Previous studies have shown that gut probiotic bacteria and their metabolites can regulate oxidative stress and protect the cells from oxidative damage [38]. Recent studies on hydrolysed porphyran have also showed that it is capable of proliferating the gut microbiota including *Bacteroides*, *Lactobacillus* and *Muribaculum* species and increasing the level of small-chain fatty acids to manage ulcerative colitis [39]. In this study, the prebiotic activity of enzymatically produced POS was measured based on colony forming units of probiotic cultures, *L. acidophilus*, *L. bulgaricus* and enteric pathogenic culture *E. coli* and it was compared with porphyran and FOS. The results show that *L. acidophilus* and *L. bulgaricus* exhibited significantly different growth patterns in the presence of porphyran, POS and FOS (Fig. 5a). The growth of *L. acidophilus* was higher in POS (Abs 1.40) than in porphyran (Abs 0.8) and FOS (Abs 1.09). The POS was also able to promote the growth of *L. bulgaricus* higher than porphyran and equivalent to FOS. The prebiotic activity score of POS was estimated based on colony forming units obtained from probiotic cultures and the growth of enteric bacteria *E. coli* and compared with porphyran and FOS. The results exhibited that POS increased the growth of probiotic isolates and suppresses the growth of enteric pathogens. POS recorded a prebiotic activity score of 7.39 and 7.22 with *L. bulgaricus* and *L. acidophilus*, respectively, which was higher than porphyran (6.91 and 7.16) and was equivalent to commercial prebiotic FOS (7.26 and 7.10). The difference in the growth of probiotic and enteric bacteria and their prebiotic activity score in the presence of POS, porphyran and FOS recorded was supported by statistical significance (Fig. 5b). These POS showed prebiotic activity equivalent to enzymatically produced ulvan oligosaccharides reported previously [30]. The results from this study highlight the significance of macroalgae associated bacteria as a source of enzymes capable of hydrolysing polysaccharides into simple bioactive oligosaccharides with combined antioxidant and prebiotic activity. Macroalgal biomolecules with cumulative bioactivities such as antioxidant, antiglycemic, antiobesity, anti-inflammatory and

prebiotic activity are in great demand in the healthcare sector. As they can protect the cells from oxidative stress and protect the individuals, against various chronic disorders reported frequently in recent times among all age groups [16,28,40].

4. Conclusion

The current generation has been greatly impacted due to the consumption of unhealthy diets and sedentary lifestyles. This results in an increase in the blood glucose level, causing oxidative phosphorylation and the formation of chronic glycation products, all of which result in the production of excess reactive oxygen species. This overwhelms the natural physiological stability in an individual and causes, cellular and molecular damage affecting the DNA, protein and lipid biosynthesis. Oxidative stress also alters the natural gastrointestinal microflora and causes an imbalance in the probiotic and enteric gut microbiota ratio, resulting in gastrointestinal disorders. This study highlights the significance of macroalgae associated microbes as a source of enzymes capable of hydrolysing complex polysaccharides into simple bioactive oligosaccharides for efficient management of chronic metabolic and gastrointestinal disorders caused due to oxidative stress.

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Figure Legends

Fig. 1. The effect of temperature, pH and NaCl concentration on the activity of porphyranase, PB1 **(a)** temperature (20 - 60 °C), **(b)** pH (pH 3 - 11) and **(c)** NaCl concentration (0.001 - 1 M).

Fig. 2. Influence of **(a)** metal ions and **(b)** chelating agents, inhibitors or detergents on the relative activity (%) of porphyranase, PB1; control was determined without any amendment.

Fig. 3. Structural characterization of enzymatically produced porphyran oligosaccharides (POS) based on **(a)** NMR spectrum; R and nr denote residues at the reducing and non-reducing end, respectively and **(b)** High Resonance Mass Spectrometry (HRMS) spectrum shows residues of galactose (G), anhydrogalactose (AG), sulfated galactose (SG), sulfated anhydrogalactose (SAG) and methylated galactose (MeG) in di-, tri- and tetrasaccharides.

Fig. 4. Antioxidant activity of porphyran oligosaccharides (POS) in comparison with porphyran, fructooligosaccharides (FOS) and gallic acid model antioxidant compound based on **(a)** 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and **(b)** 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity.

Fig. 5. Prebiotic potential of porphyran oligosaccharides (POS) in comparison with porphyran and fructooligosaccharides (FOS) based on **(a)** growth profiling of probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and enteric *Escherichia coli* and **(b)** prebiotic activity score of probiotic bacteria; a, b, c represents statistical significance (at $p \leq 0.05$).

Table 1 Purification of porphyranase, PB1 from *Bacillus* sp. NIOA284.

| Purification Step | Volume (mL) | Total protein (mg) | Total activity (U) | Specific activity (U mg⁻¹) | Purification fold | Yield (%) |
|---------------------------------------------------------------|------------------------|-----------------------------------|-----------------------------------|------------------------------------------------------|------------------------------|----------------------|
| Culture supernatant | 250 | 49.7 | 9335.0 | 187.8 | 1.0 | 100 |
| (NH ₄) ₂ SO ₄ precipitation | 10 | 5.0 | 1851.1 | 370.2 | 1.97 | 19.8 |
| Ion exchange | 5 | 1.3 | 719.7 | 553.6 | 2.95 | 7.7 |
| Gel permeation | 1 | 0.1 | 198.3 | 1983 | 10.56 | 2.1 |

Table 2 Studies on porphyranase used in the production of porphyran oligosaccharides (POS).

| Microbial source of porphyranase | Commercial name of the enzyme | Degree of polymerization in the POS | Structural characterization | Bioactivity | References |
|-----------------------------------------|--------------------------------------|--------------------------------------------|------------------------------------|------------------------|-------------------|
| Proteobacteria | | | | | |
| <i>Pseudoalteromonas atlantica</i> | NA | Dimer, Tetramer | MALDI-MS, ESI-MS | NA | [13] |
| Bacteroidetes | | | | | |
| <i>Zobellia galactanivorans</i> | β -porphyranase 16A | Tetramer, Octamer | NMR | NA | [12,29] |
| <i>Wenyinzhuangia fucanilytica</i> | NA | Dimer, Tetramer, Hexamer | LC-MS | NA | [24,25] |
| <i>Bacteroides plebeius</i> | β -porphyranase 86A | Hexamer | PACE | NA | [41] |
| Actinobacteria | | | | | |
| <i>Arthrobacter</i> sp. S-22 | NA | Dimer, Tetramer | HPLC, FT-IR, NMR | Antiobesity | [16] |
| Firmicutes | | | | | |
| <i>Bacillus</i> sp. NIOA284 | NA | Dimer, Trimer, Tetramer | HRMS, NMR | Antioxidant, Prebiotic | This study |

Matrix Assisted Laser Desorption/Ionization-mass spectrometry (MALDI-MS), Electrospray ionization mass spectrometry (ESI-MS), Liquid chromatography–mass spectrometry (LC-MS), carbohydrate polyacrylamide gel electrophoresis (PACE), High-performance liquid chromatography (HPLC), High Resonance mass spectrometry (HRMS) and Not applicable (NA)

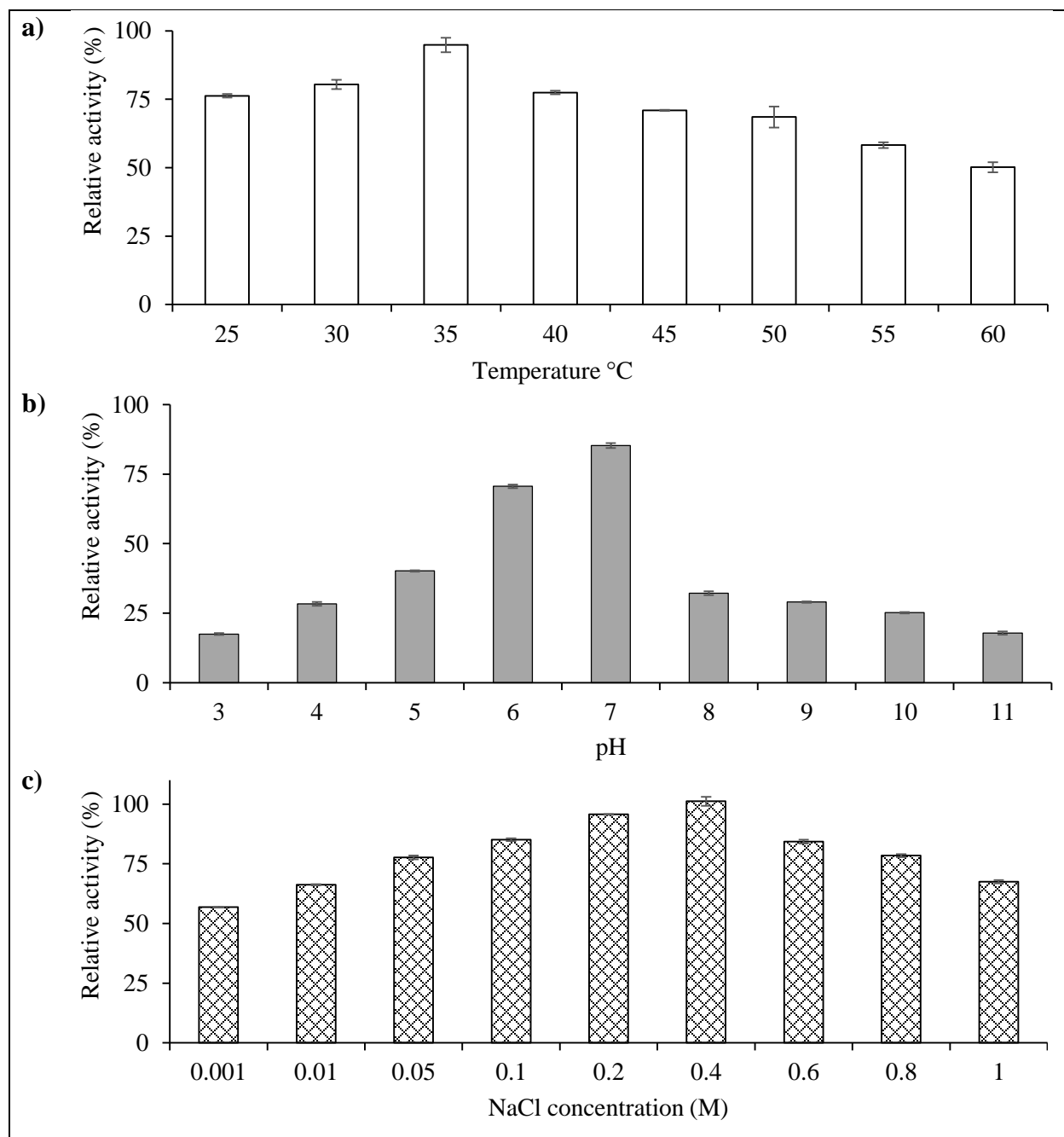


Fig. 1. The effect of temperature, pH and NaCl concentration on the activity of porphyranase (**a**) temperature (20 - 60 °C), (**b**) pH (pH 3 - 11) and (**c**) NaCl concentration (0.001-1 M).

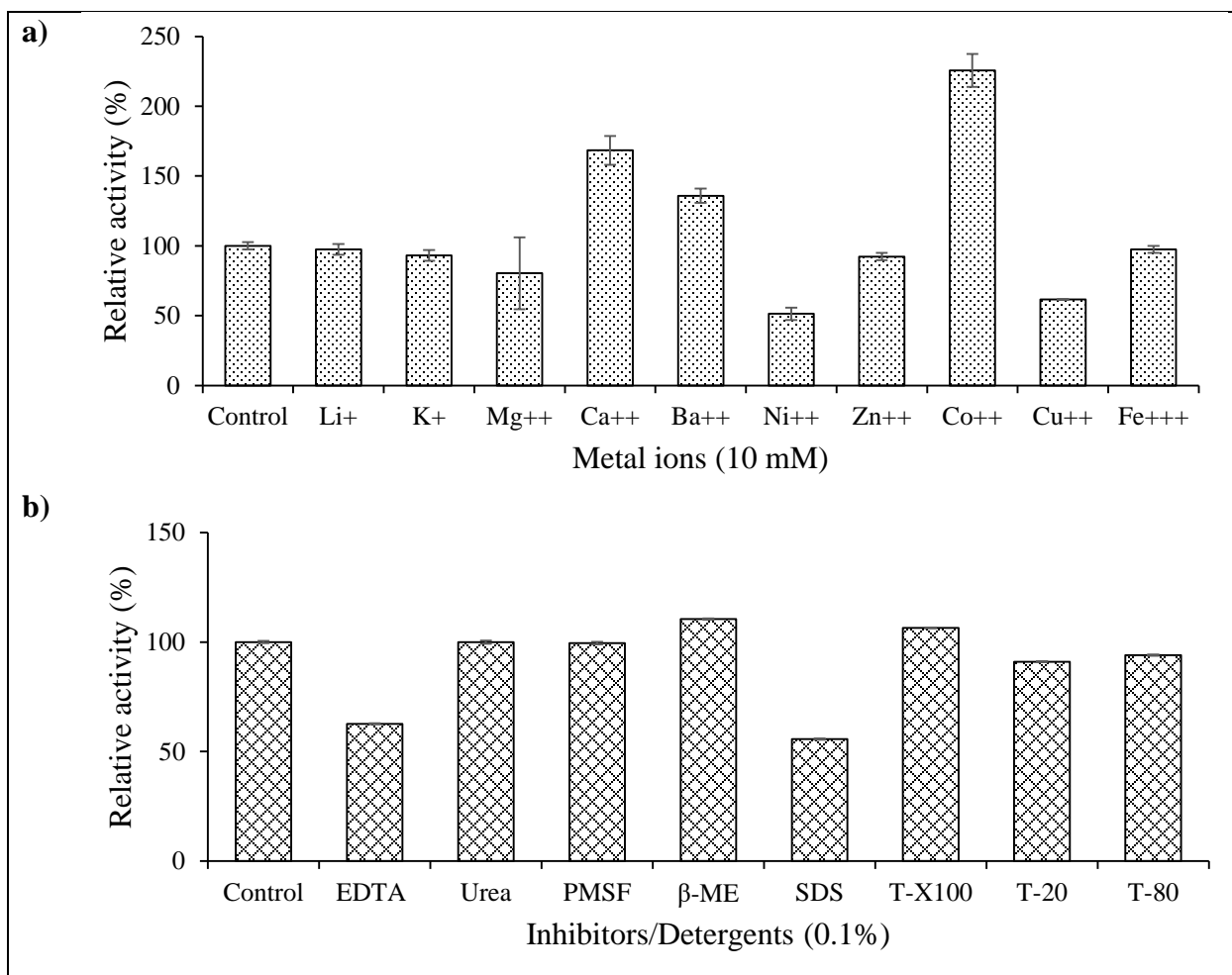


Fig. 2. Influence of **(a)** metal ions and **(b)** chelating agents, inhibitors or detergents on the relative activity (%) of porphyranase, PB1; control was determined without any amendment.

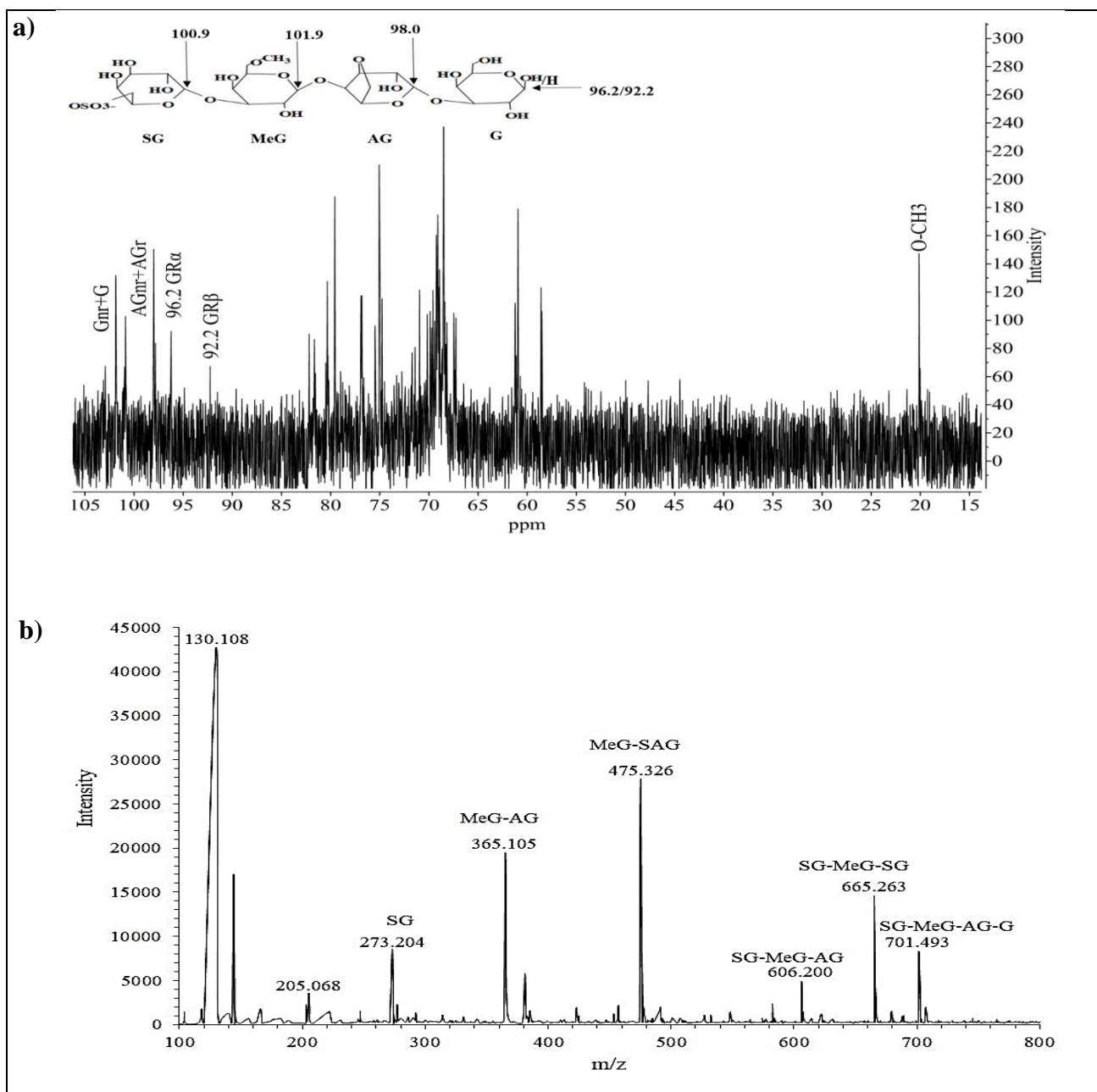


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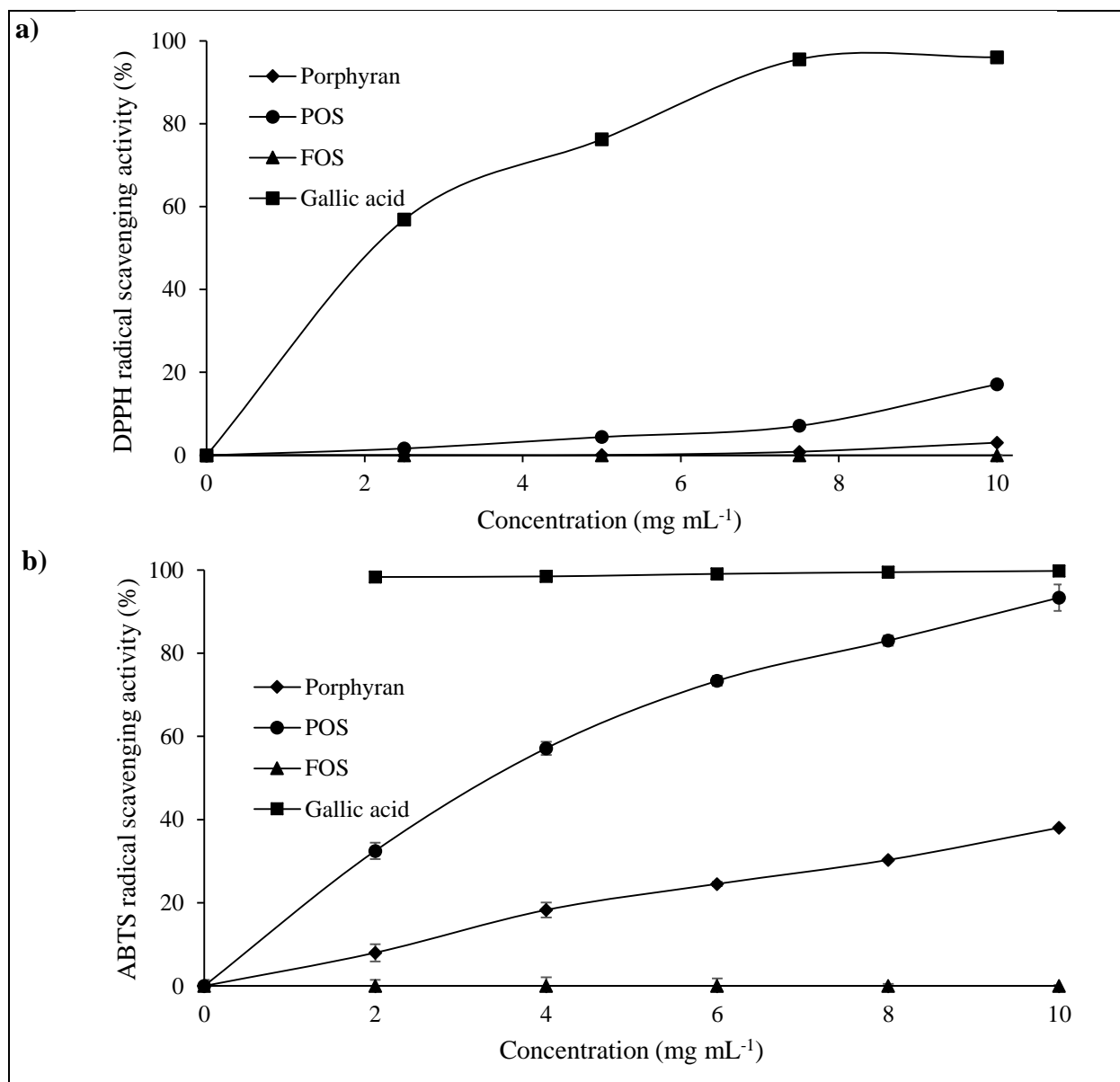


Fig. 4. Antioxidant activity of porphyran oligosaccharides (POS) in comparison with porphyran, fructooligosaccharides (FOS) and gallic acid model antioxidant compound based on (a) 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and (b) 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity.

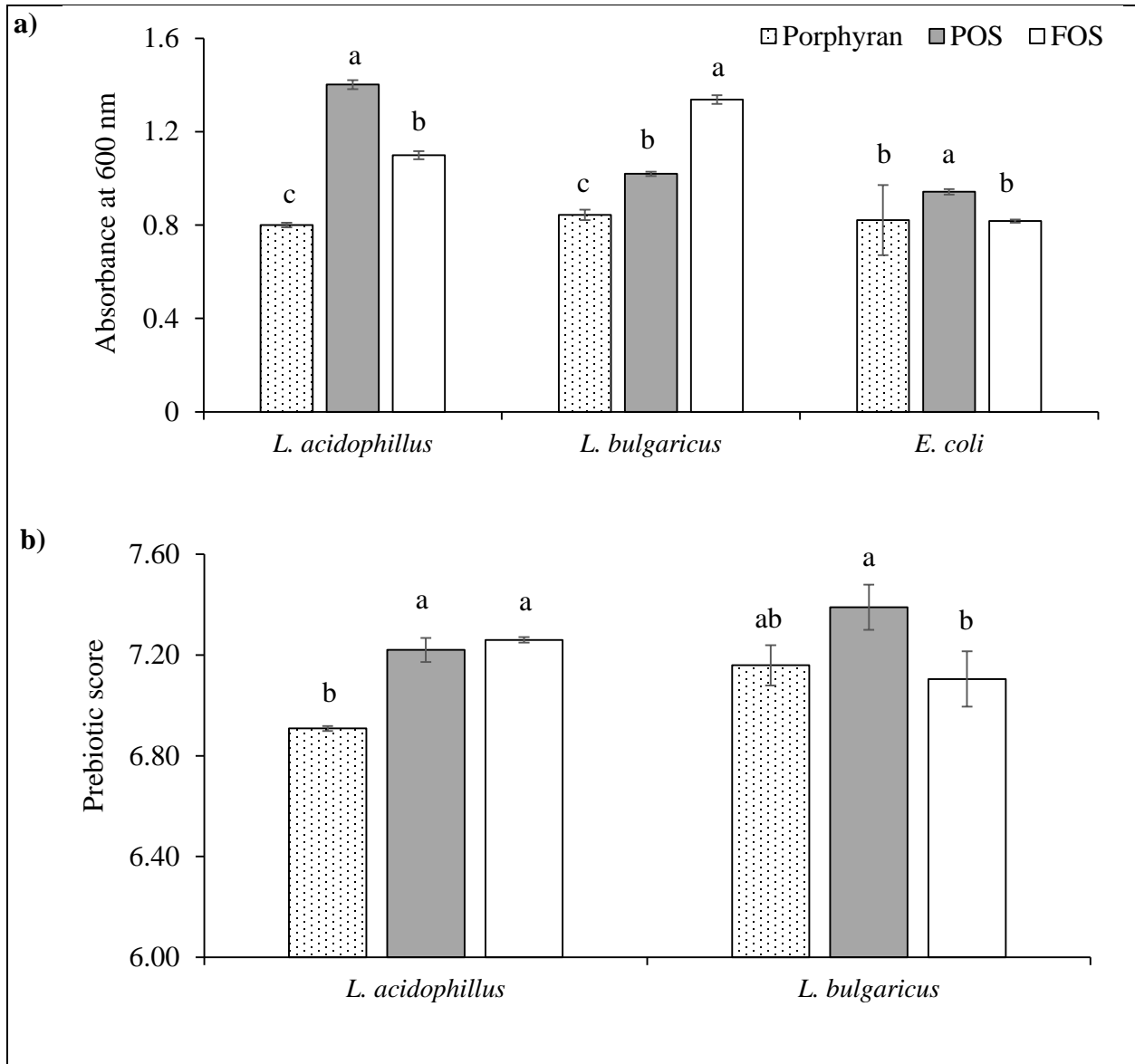


Fig. 5. Prebiotic potential of porphyran oligosaccharides (POS) in comparison with porphyran and fructooligosaccharides (FOS) based on **(a)** growth profiling of probiotic bacteria, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and enteric *Escherichia coli* and **(b)** prebiotic activity score of probiotic bacteria; a, b, c represents statistical significance (at $p \leq 0.05$).

Supplementary Material

Enzymatic production of bioactive porphyran oligosaccharides

**Characterization of bioactive porphyran oligosaccharides produced using porphyranase
from *Bacillus* sp. NIOA284**

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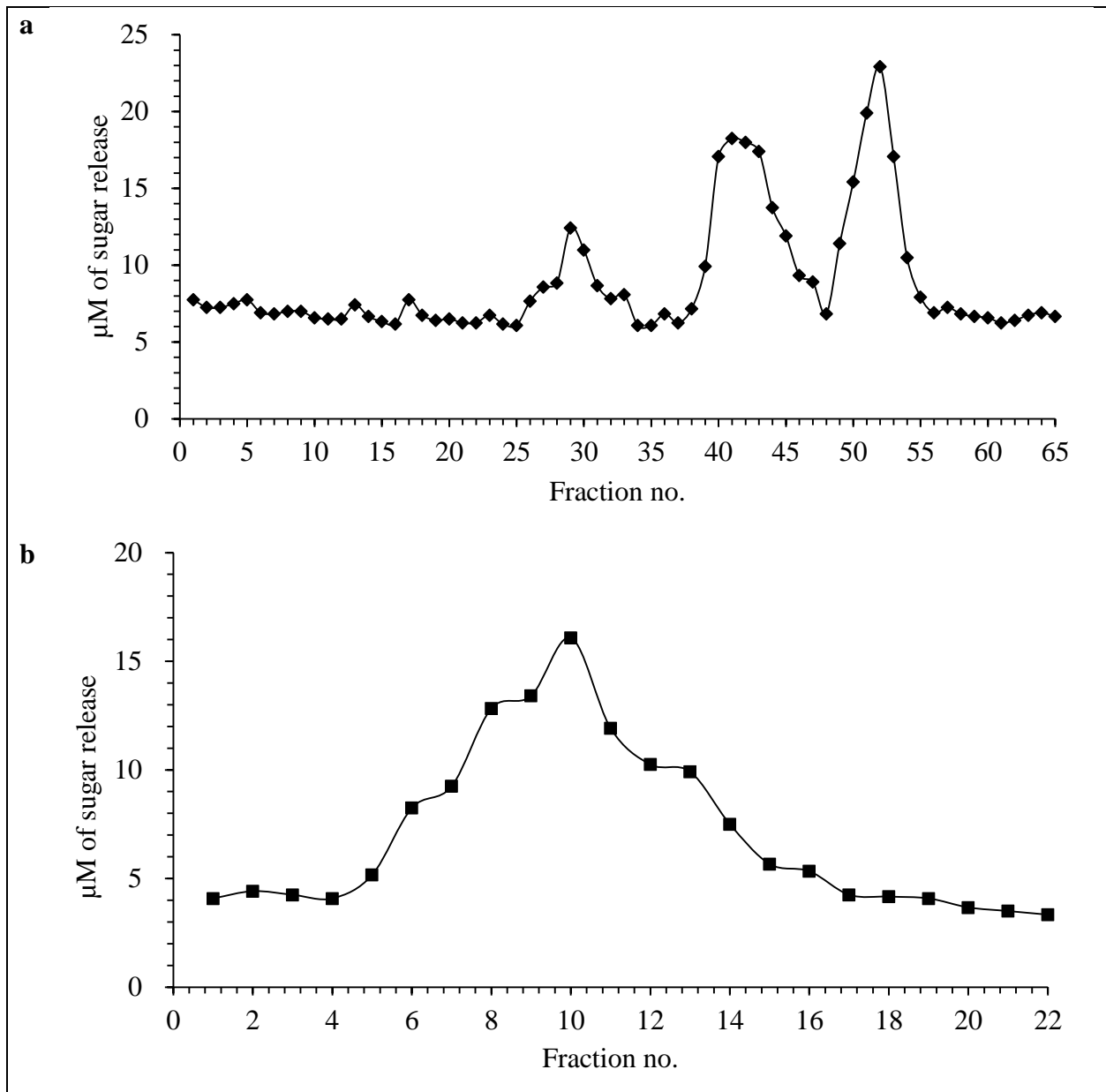


Fig. S1. Enzyme activity based on sugar release of purified porphyranase fractions collected from (a) DEAE Sephadex ion exchange chromatography column eluted with NaCl gradient (0.2 to 1 M) and (b) Superdex 75 gel permeation column eluted with sodium phosphate buffer (pH 7.0, 50 mM).

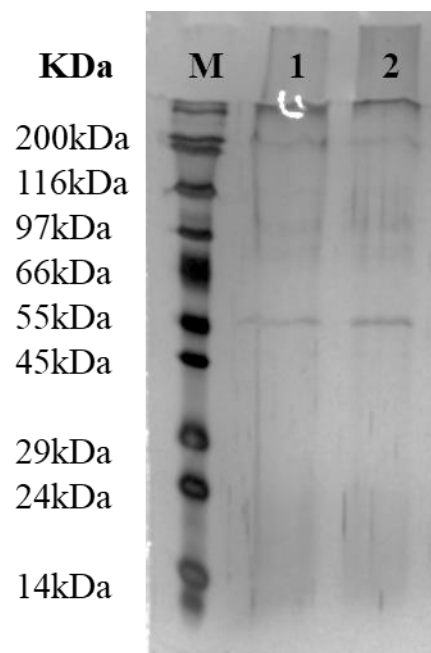


Fig. S2. Molecular weight analysis of purified porphyranase enzyme on SDS-PAGE, Lane M: protein marker; Lane 1: eluted through Sephadex ion exchange column and Lane 2: Superdex 75 gel permeation column.

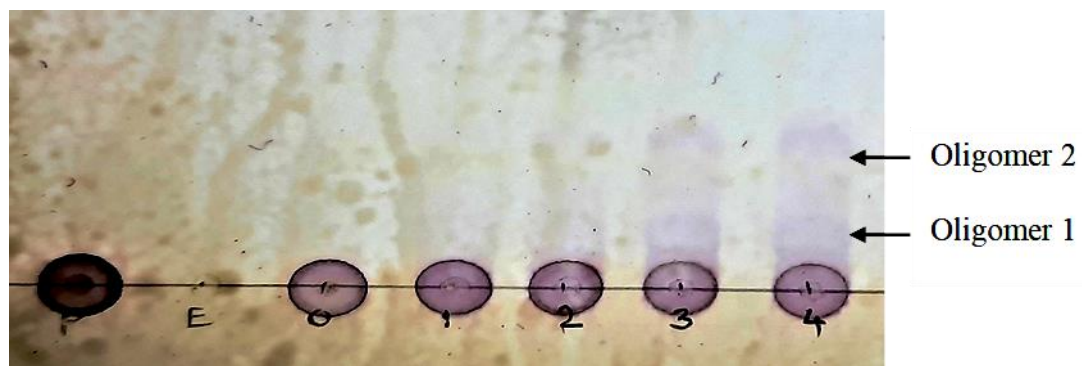


Fig. S3. Structural characterisation of porphyran oligomers produced using porphyranase, PB1 at different time intervals on Thin Layer Chromatography, Lane P: Porphyrin; Lane E: porphyranase, PB1; Lane 0: 0h; Lane 1: 3h; Lane 2: 6h; Lane3: 9h and Lane 4: 12h hydrolysed sample.