

Indian Science Technology and Engineering facilities Map (I-STEM)

Invites application for

1. **Partial financial support for using R&D facilities in other institutions through the I-STEM web portal to Users from Academic Institutions and Start-ups (Format-1)**

Format for requesting support for accessing and using facilities through the I-STEM web portal.

(i) Details of the Personal Information

- a. Name of the Researcher/Faculty Member: **Dr. Debsankar Das**
- b. Name of the Institution/Start-up: **Prabhat Kumar College, Contai**
- c. Name of the Department: **Chemistry**
- d. Degree registered for/Product being developed (for start-up):-
- e. Mob. No.: **6294522012**
- f. Email ID: **das_deb81@yahoo.co.in**

(ii) Project/Research topic Details:

- a. **Project Title/Research Title:** Isolation, purification, structural characterization and prebiotics evaluation of oyster mushroom (*Pleurotus djamor*) polysaccharides.
- b. **Sub area:**
 1. Extraction of polysaccharide from mushroom
 2. Purification by dialysis and gel permeation chromatography
 3. Determination of sugar composition
 4. Linkage analysis of sugar moieties by GC-MS of alditol acetates of partially methylated polysaccharide
 5. FT-IR study of polysaccharide
 6. Detail structural characterization of polysaccharide by 1D and 2D NMR
 7. Prebiotic activity study
- c. **Area Description:** Hot water extraction and alkali extraction of polysaccharide from edible mushroom, *Pleurotus djamor* and purification by gel permeation chromatography using sepharose-6B. Structural characterization of polysaccharide by GC-MS of alditol acetates of partially methylated polysaccharide, FT-IR study, 1D (1H and 13C) and 2D NMR (DQF-COSY, TOCSY, NOESY, ROESY, HMQC, HMBC and DEPT-135) and Prebiotic activity evaluation.
- d. **Aims & Objective:**
 - Isolation of polysaccharide fractions from fruit bodies of *Pleurotus djamor* through hot water extraction procedure.
 - Purification of isolated polysaccharides.

- Fractionation by using gel permeation chromatography.
- Detailed Structural characterization.
- Testing of polysaccharide for their prebiotic potential in relation to the selected probiotic strains as *Bifidobacterium sp.*, *Lactobacillus sp.*, compared to that of a pathogenic strain of *E.coli*.

e. Duration (MM/YYYY): 2 (Two) years

TASK	1 ST YEAR			2 ND YEAR		
	Q1	Q2	Q3	Q1	Q2	Q3
2.Isolation, purification and fractionation of mushroom polysaccharide						
3.Characterization of polysaccharide by chemical analysis						
4.Complete structure elucidation of polysaccharide using 1D and 2D NMR						
5. Testing of prebiotic activity of polysaccharide in vitro						

f. Project Summary (1000 words):

Current trend in food science and technology indicates development of prebiotics, selectively fermentable ingredients that can modulate the human colonic microbiota and improve the host health in return. While inulin and fructo-oligosaccharides (FOS) remain among the most established and studied prebiotics, a better understanding on the benefits of prebiotics has urged a need for exploring new sources. Rich in polysaccharides such as chitin, hemicelluloses, β -glucan, mannans and xylans, mushrooms seem to be a potential prebiotic candidate. The present project is aimed to evaluate prebiotic property structural characterization of edible mushroom by isolating polysaccharide from oyster mushroom *Pleurotus djamor*. Structural characterization will be done by employing gas chromatography-mass spectrometry (GC-MS), methylation and FT-IR. NMR techniques e.g., ^1H , ^{13}C , DQF-COSY, TOCSY, NOESY, ROESY, HMQC, HMBC and DEPT-135 will also be employed for structural elucidation. Prebiotic activity of the polysaccharides will be evaluated by *in vitro* fermentation in anaerobic batch cultures of *Lactobacillus sp.* and *Bifidobacterium sp.* followed by short chain fatty acids (SCFAs) analysis by HPLC. Simultaneously, non-digestibility assay can predict the hydrolysis percent of the candidate

molecule under artificial gastrointestinal conditions. This study can help to understand the structure-function interplay of a polysaccharide and its related prebiotic activity, thus contributing to ever-evolving nutraceutical technology.

g. Technical Details

1. Methodology:

➤ Isolation of the polysaccharides:

The polysaccharide will be extracted from the fruit bodies of edible mushroom *Pleurotus djamor* using hot water as solvent.

➤ Purification and fractionation

The obtained polysaccharide will be dialyzed against Milli Q water using 10,000 kDa MWCO membrane (Thermo-Fischer, USA). The purification and fractionation is carried out by passing through column (90×2.1 cm, GE Healthcare) with water as eluent (0.75 mL /min). Ninety fractions (2 mL each) will be collected and monitored spectro-photometrically at 490 nm with the phenol-sulfuric acid assay.

➤ Molecular weight determination

The column Sepharose-6B will be standardized by loading different dextran molecular weight markers (Range: 40-2000 kDa). From the separation pattern of dextran marker, a standard curve will be plotted by taking the log of molecular weight against fraction number. GPC purified polysaccharide will be used for further chemical characterization and prebiotic activity studies.

➤ Determination of monosaccharide by HPTLC

For determination of monosaccharide composition of polysaccharide, 3 mg of purified polysaccharide will be taken and hydrolysed in 4M TFA at 120° C in oil bath for 4 hours. Excess acid will be completely removed by co-distillation with water and methanol. Hydrolysed sample will be finally collected in methanol for further analysis. For qualitative analysis, High Performance Thin-Layer Chromatography (CAMAG, Switzerland) will be done using a LINOMAT-5 sample applicator. Chromatogram will be developed on silica gel 60 F₂₅₄ TLC plates (MERCK, Germany) using 2-propanol: ethyl acetate: acetic acid: water (4:2:2:1) as mobile phase. Sugars will be visualized with orcinol-sulphuric acid as spray reagent.

➤ Monosaccharide composition analysis by GLC

The monosaccharide composition of pure polysaccharide will be determined following alditol-acetate derivatization method, as described by Blakeney *et al.* (1983). After hydrolysis of polysaccharide into monomeric sugars by TFA and removal of excess acid by evaporation, H₂O

and NaBH₄ will be added to the reaction vessel. Sodium borohydrate (SRL) facilitates the aldehyde groups in monomeric sugar residues to reduce into alditols. These alditols will be acetylated using acetic anhydride and pyridine towards the formation of volatile alditol-acetate forms of the component monosaccharides. The alditol-acetate will be extracted in ethyl acetate (Merck, India) phase and finally collected in chloroform (Merck, India) to be analyzed by GLC.

➤ **Methylation analysis of polysaccharide for linkage determination**

The polysaccharide will be methylated according to the methods of Ciucanu and Kerek (1984). The polysaccharide (5 mg) will be dissolved in 0.5 mL of distilled DMSO. After addition of solid finely grounded NaOH dust and stirring for 30 mins, 0.5-1 mL CH₃I will be added and stirred for 1-1.5 hours. The methylated material will be isolated by partition between CHCl₃ and H₂O (5:2, v/v) and the CHCl₃ solution of methylated product will be dried on water bath to yield solid product. After hydrolysis with HCOOH for 1 hour, and removal of excess acid by co-distillation with water, the hydrolyzed methylated product will be acetylated by Ac₂O-pyridine (1:1) mixture at 100 °C for 1 hour. The alditol acetates of methylated sugars will be analyzed by GC-MS (Thermo Scientific, USA).

➤ **FT-IR analysis**

Functional group identification and type of linkage (α or β) analysis of polysaccharide will be done using Fourier Transform Infra-Red Spectrum. The dried polymer will be grinded with Potassium bromide (KBr) powder at a ratio of 1:50 and pressed into pellets of halide salt or disc for FT-IR spectral measurement in the frequency range of 4000–600 cm⁻¹.

➤ **Complete structure elucidation of polysaccharide by NMR Spectroscopy (1D and 2D)**

Nuclear Magnetic Resonance (NMR) is the most powerful and non-destructive technique for identification of monosaccharide composition, elucidation of α or β anomeric configurations, linkage patterns and sequence of sugar units in the repeating unit of the polysaccharide. Different types of 1D (¹H, ¹³C, DEPT-135) and 2D (COSY, DQF-COSY, TOCSY, NOESY, ROESY, HMQC and HMBC) NMR techniques will be adapted to characterize the structure of the polysaccharide.

➤ **Prebiotic activity**

(i) Effect of *in vitro* hydrolysis in artificial gastric juice and pancreatic amylase

Hydrolysis profile of isolated polysaccharide fractions can be checked in artificial gastric juice and pancreatic α-amylase as previously reported by Gnoth et al., 2000 and Weichenhot et al., 2010, with required modifications. In brief, for the digestion experiments, 10 mg/mL of the freeze-dried polysaccharide will be prepared in PBS (pH 7.0) and incubated in artificial human gastric juice (hydrochloric acid buffer containing (in g/l): NaCl, 8; KCl, 0.2; Na₂HPO₄·2H₂O, 8.25;

NaHPO₄, 14.35; CaCl₂·2H₂O, 0.1; MgCl₂·6H₂O, 0.18. The pH of the buffer was adjusted to 1, 2, and 3 using HCl (0.2 mol/L) in a temperature-controlled water bath at 37°C for 4 h. Aliquots will be taken out periodically at 0, 1, 2, 3 and 4 h to measure reducing and total sugar content. For amylase digestion studies, pH of the solutions will be readjusted to pH 7.0 with NaHCO₃ (0.2 mol/L). 100 U of the enzyme (porcine pancreas, Type VI-B, Sigma-Aldrich) will be added to the polysaccharide solutions and incubated. As before, sample-reaction mixture aliquot will be taken periodically at 0, 2 and 4 h to determine reducing sugar and total sugar content. Following this, the hydrolysis % can be calculated based on the formula below:

$$\text{Hydrolysis \%} = \frac{\text{Reducing sugar released}}{\text{Total sugar content} - \text{Initial reducing sugar content}} \times 100$$

Additionally, the hydrolysates obtained post digestion assays, can be checked qualitatively by analysis in TLC silica gel 60 F₂₅₄ plates (MERCK, Germany).

(ii) Prebiotic activity assay and determination of prebiotic activity score

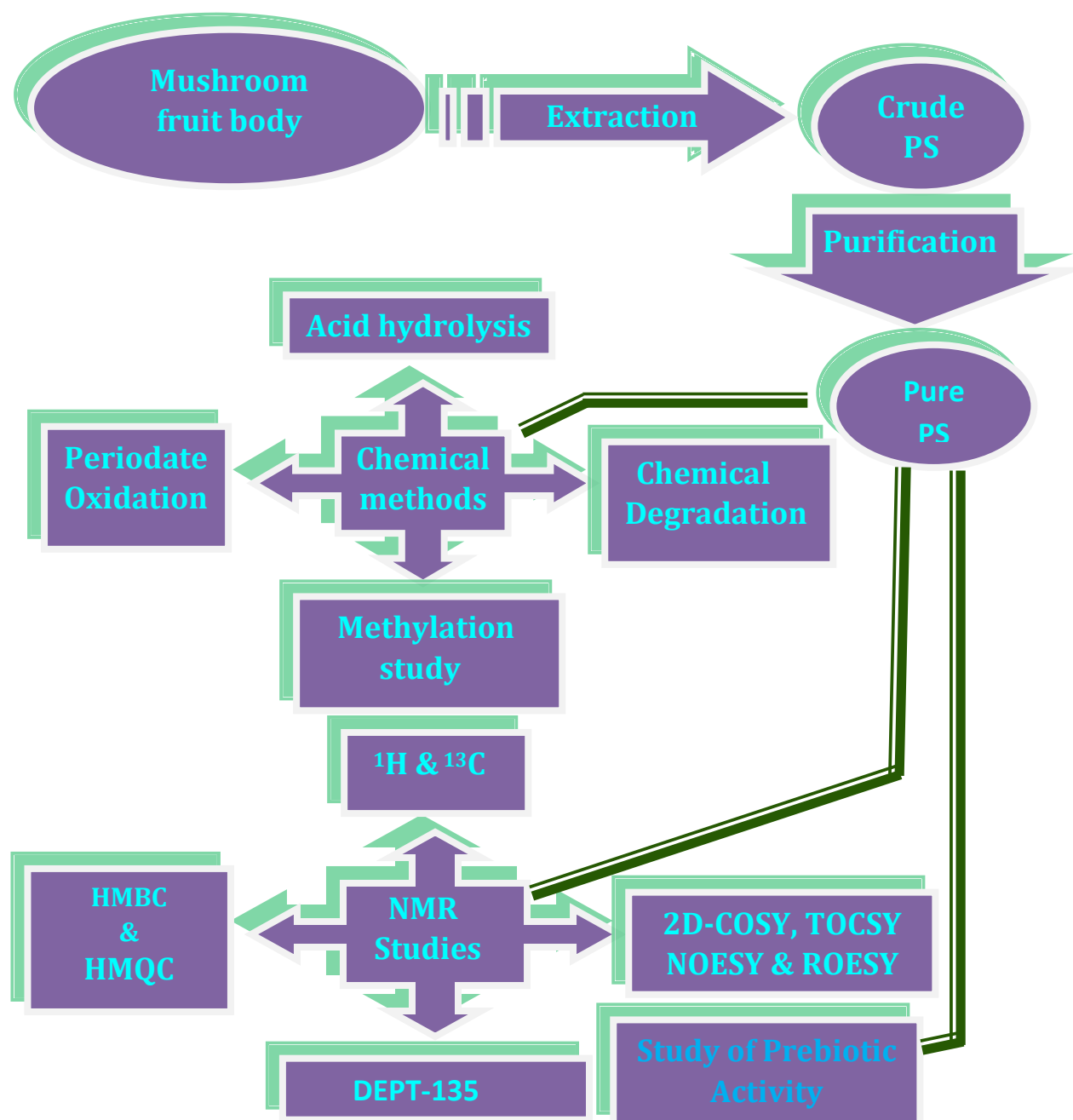
The ability of the isolated polysaccharide to support the growth of selected probiotic strains relative to pathogenic strain/s and also relative to growth on a non-prebiotic substrate, such as glucose will be evaluated. In addition, a quantitative score to describe the extent to which my candidate prebiotic molecule supports the selective growth of lactobacilli and bifidobacteria will be calculated. The prebiotic activity assay parameters will be based on the change in cell biomass after 24 h or 48 h of growth of the probiotic strain on 1% prebiotic or 1% glucose relative to the change in cell biomass of a mixture of enteric strains grown under the same conditions. From the biomass data, a prebiotic activity score can then be calculated for the lactobacilli and bifidobacteria stains. The prebiotic activity score will be determined using the following equation (Huebner *et al.*, 2007).

$$\text{Probiotic activity score} = \left[\frac{(\text{probiotic log CFU ml}^{-1} \text{ on the prebiotic at } 24\text{h} - \text{probiotic log CFU ml}^{-1} \text{ on the prebiotic at } 0\text{h})}{(\text{probiotic log CFU ml}^{-1} \text{ on glucose at } 24\text{h} - \text{probiotic log CFU ml}^{-1} \text{ on glucose at } 0\text{h})} \right] - \left[\frac{(\text{enteric log CFU ml}^{-1} \text{ on the prebiotic at } 24\text{h} - \text{enteric log CFU ml}^{-1} \text{ on the prebiotic at } 0\text{h})}{(\text{enteric log CFU ml}^{-1} \text{ on glucose at } 24\text{h} - \text{enteric log CFU ml}^{-1} \text{ on glucose at } 0\text{h})} \right]$$

(iii) Analysis of SCFAs from fermentation media

Fermentation metabolite analyses will be done by aliquoting samples from batch culture systems and analyzed by HPLC with UV detector for short chain fatty acids (SCFA), including acetate, propionate and butyrate. Briefly, fermentation media will be centrifuged and the supernatants were harvested for analysis of organic acids by HPLC after filtration using 0.20 µm membranes. An Agilent 1200 series HPLC system with a RI detector and Aminex HPX-87H column (BioRad, Hercules, CA, USA) will be used to perform the analyses of SCFAs (Gullónet *al.*, 2016). Other analysis conditions as flow rate will be optimized as necessary.

2. Design & development:



3. Facilities and Instrumentation required: Magnetic stirrer, Freeze, Vacuum pump, Digital balance, water bath, micro pipette, Vacuum Desiccator, Freeze dryer, Cold Centrifuge machine, Laminar air flow, UV-Visible Spectrophotometer, FT-IR spectrometer, Nuclear Magnetic Resonance spectrometer.

4. Consumables (Scientific materials):

Test tube, beaker, Sulphuric acid, Ethanol, Calcium chloride, Dimethyl sulfoxide, Acetic acid, Acetone, Parafilm, Teflon, Tissue Paper, Dropper, Beaker, Round bottom flask, GE Column, Aluminium foil, D-galacturonic acid, D-glucouronic acid, L-rhamnose, L-fucose, D-

mannose, D-glucose, D-galactose, D-xylose, D-arabinose, L-arabinose, Formic acid, Trifluoro acetic acid, Ethylene glycol, Methyl iodide, Chloroform, Phenol, Sodium hydroxide, Dialysis membrane, NMR tube, D₂O, Weighing bottle, Sepharose-6B.

h. Expected output and outcome:

In recent years, much attention has been paid to the physiological functions of food due to increasing awareness for health. The demand from health conscious consumers has led to emergence of various health promoting products in which prebiotics are used as food ingredients because of their ability to modulate human gut microbiota and improve the host health in return. These include enhancement of immune function, improve digestion and elimination of faces as well as reducing the potential of getting irresistible bowel syndrome. In this context, there is need more development of prebiotics. The non-digestible property of polysaccharide by human digestive enzyme enables it to be considered as a potential source of prebiotic. The importance of the proposed work is the searching of new source of prebiotic (polysaccharide) along with structural characterization by 1D and 2D NMR analysis and contribution to ever-evolving nutraceutical technology

i. Industry/Institute Collaboration If, yes -

j. Funding required from I-STEM (with justification:

Details of Requirements & Mode of the Engagement: (Attach the quotation for the consumables)

S.N.	Description of goods	Make	Qty.	Rate	Amount with GST (Rs.)
1.	Parafilm:4"x125'(380020),1NO	Tarson	1	1850	2183
2.	Ethanol (500 ml)	Indian	10	169.44	1999.40
3.	Acetic acid glacial LR. (500 ml.)	Merk	1	265	265
4.	Sulfuric acid 98% Emplura (500 ml.)	Merk	1	248	248
5.	NaOH Pellets LR. (500 gm)	Merk	1	260	260
6.	Trifluoroacetic acid (100 ml.)	Merk	1	1812	1812
7.	NaBH ₄ (100 gm)	Merk	1	1835	1835
8.	CaCl ₂ (500 gm)	Merk	1	214	214
9.	CHCl ₃ (500 ml.)	Merk	1	284	284
10.	DMSO LR. (500 ml.)	Merk	1	498	498
11.	HCOOH (500 ml.)	Merk	1	272	272
12.	Sodium metaperiodate GR (500 gm)	Merk	1	5730	5730

13.	Sepharose 6B	Sigma	2	17500	41300
14.	Ethylene glycol (500 ml.)	Merk	1	350	350
15.	Phenol (500 gm)	Merk	1	430	430
16.	Pyridine (500ml)	Merk	1	756	756
17.	Acetone (500 ml.)	Merk	1	325	325
18.	Mel (GRM 8635) (250 ml.)	Himedia	1	9100	9100
19.	D-glucose (1kg.)	Sigma	1	3300	3894
20.	D-mannose (25 gm.)	Sigma	1	2186	2580
21.	D-arabinose (25 gm.)	Sigma	1	1687	2000
22.	L-arabinose (10 mg.)	Sigma	1	2697	3182
23.	L-rhamnose (10 gm.)	Sigma	1	1720	2030
24.	L- fucose (10 mg.)	Sigma	1	3385	3995
25.	D-xylose (10 mg)	Sigma	1	2000	2360
26.	D-galactose (10 mg)	Sigma	1	1798	2122
27.	D-glucouronic acid (10 mg)	Sigma	1	3400	4012
28.	D-galactouronic acid (25 mg)	Sigma	1	3300	3894
29.	Dialysis membrane (5 m)	Himedia	1	1840	2172
30.	Water Bath	Testmaster	1	8220	9700
31.	Beaker (100 ml)	Borosil	12	960	1132
32.	Beaker (250 ml)	Borosil	10	870	1026
33.	Test tube (18x150 mm)	Borosil	50	950	1121
34.	Beaker (500 ml)	Borosil	10	1800	2124
35.	Column	Borosil	2	1700	2006
36.	Centrifuge tube (15 ml, 500)	Tarson	1	3799	4483
37.	Centrifuge tube (15 ml, 500)	Tarson	1	5900	6062
38.	Ammonium molybdate (500 gm)	Himedia	1	6070	7163

(i) Short term Engagement:

a. Usage of testing/characterization facilities across the country (Characterization Mode)

i. Testing Charges: **For 1D and 2D NMR Spectrum-Rs. 12,000/-**

For GC-MS- Rs. 4000/-

ii. Consumable charges:

iii. Travel Cost (Boarding and Lodging, if any): **Rs. 10,000/-**

b. Usage of fabrication and testing facilities across the country (Fabrication Mode)

i. Fabrication and testing Charges

ii. Consumable charges (if any)

iii. Travel Cost (Boarding and Lodging, if any)

(ii) Medium term Engagement:

a. Execution of project in any one of the Host Institution (Project Mode)

a. Fabrication and or testing Charges

b. Consumable charges (if any)

c. Travel Cost (Boarding and Lodging, if any)

b. Execution of project in Multi Host Institutions (Project Mode)

a. Fabrication and or testing Charges

b. Consumable charges (if any)

c. Travel Cost (Boarding and Lodging, if any)

(Round-trip to the Host Institution by 2nd class (Non-AC) railway via the nearest route)

Details of User's Bank Account:

(Please send a copy of the cancelled cheque/copy of the passbook carrying all the information needed)

Beneficiary Name (User's name): **Debsankar Das** PAN:

Bank Account No.:

Name of the Branch:

IFSC Code:

Note: Proposed proposal must be executed within stipulated duration (max. 6 month) to avoid fund availability issues. Travel form will be provided by the I-STEM office. The users will submit the technical report, original invoice (duly signed by the Head of the Institution) raised by the Host Institution through I-STEM portal only along with a copy of the travel forms and original tickets through post to the Nodal Officer, I-STEM, Indian Institute of Science, Bangalore. A scan copy of all the documents may be sent in advance.