Utilization of Agricultural Waste for the Production of Xylooligosaccharides Using Response Surface Methodology and Their In Vitro Prebiotic Efficacy

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UTILIZATION OF AGRICULTURAL WASTE FOR THE PRODUCTION OF XYLOOLIGOSACCHARIDES USING RESPONSE SURFACE METHODOLOGY AND THEIR *IN VITRO* PREBIOTIC EFFICACY

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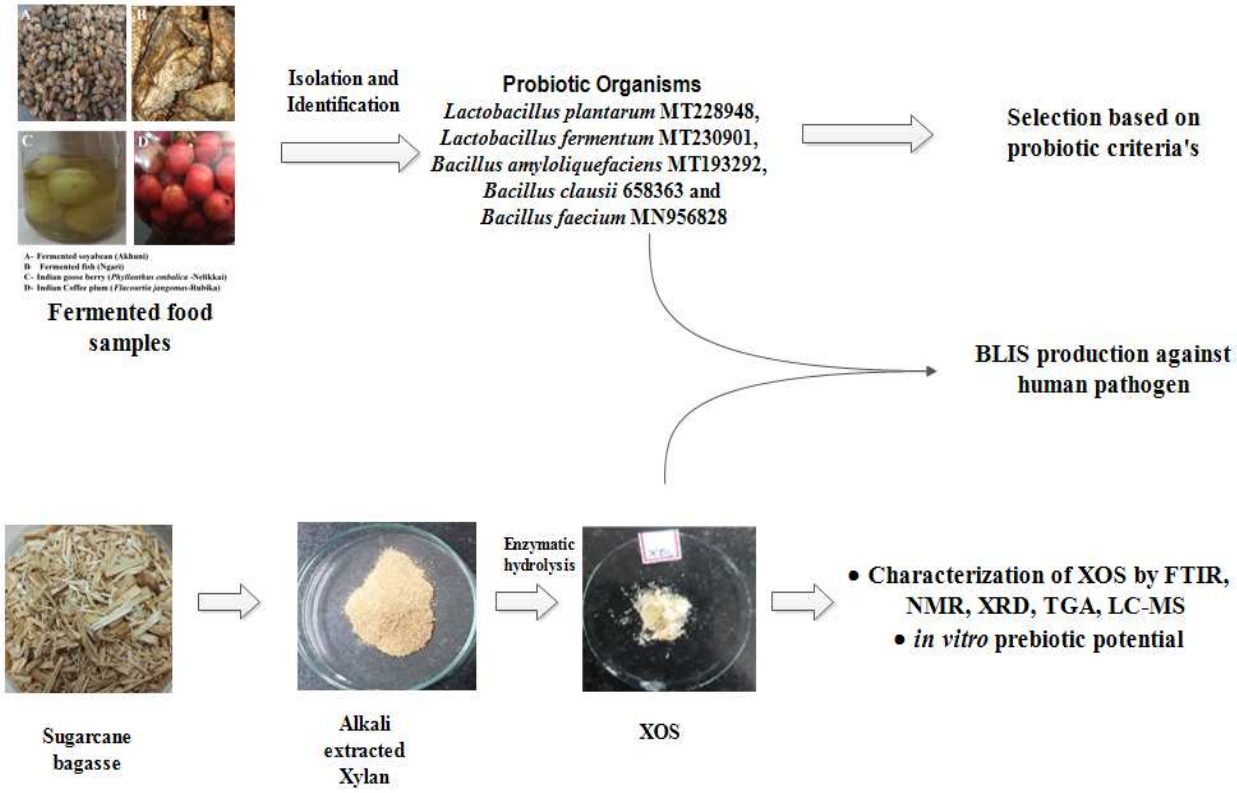
Graphical abstract



Abstract

Air pollution is a prominent problem recently faced in various parts of India due to the burning of stubbles (coconut husk, corn cob, paddy stubbles, sugarcane bagasse, etc.) which are rich in a lignocellulosic component that can be converted into a prebiotic known as Xylooliogsaccaride (XOS). They can be produced by autohydrolysis, acid hydrolysis and enzymatic hydrolysis of xylan. In the present study, Xylan was extracted from sugarcane bagasse using two alkalis (NaOH and KOH) and the yield was compared. Xylooligosaccharide produced by enzymatic hydrolysis and their factors influencing the yield were optimized using Response Surface Methodology. Xylan and Xylooligosaccharide was characterized by FTIR, NMR, XRD, TGA and ESI-MS. Xylooligosaccharides was investigated for their prebiotic potential by *in vitro* study. The maximum (Relative yield of 86%) yield of xylan was observed in 20% of NaOH. Xylan peaks at 3762cm^{-1}, 3347 cm^{-1}, 2917cm^{-1} represents the OH and CH stretching of xylan. The main signals at 4.26 (H-1), 3.19 (H-2), 3.59 (H-3), 3.63 (H-4) and 3.98 (H-5) ppm determines the existence of xylan. The higher amount of XOS is pH 4.75, temperature 45°C, enzyme 4U/ml and for time of 16h. The spectrum of 5.0-5.40ppm and 4.30-4.60ppm represents the α anomeric and β anomeric protons in XOS. They are resistant digested and the reaching percentage to the intestine is 95% unhydrolyzed. The maximum prebiotic index was noted in *L.plantarum* (1.92) and *L.fermentum* (1.61). The highest prebiotic index and score was observed in *L.plantarum* (1.9) and *L.fermentum* (17). The maximum bacteriocin production of *Enterococcus faecium* against *E.fecalis* (13mm) and *Streptococcus pyogenes* (11mm). Therefore, utilization of agricultural residues for a value-added product not only shows a great impact on environmental issues but also could double the farmer's income

Keywords: Xylooligosaccharide, Xylan, Sugarcane bagasse, prebiotic, probiotic, Fermented foods, bacteriocin.

1. Introduction

Sugarcane (*Saccharum officinarum L.*) is a perennial crop that grows predominantly in subtropical and tropical regions. Sugarcane bagasse (SB) is a fibrous residue of cane stalk left after the crushing and extraction of juices. Sugarcane bagasse is one of the most abundant lignocellulosic materials in the agro-industrial residues (Cardona et al. 2010) consisting of cellulose, hemicellulose and lignin. A total of $54x10^8$ dry tons of sugarcane is processed annually around the world and 1 ton of sugarcane generates 280 kgs of bagasse (Cerqueira et al. 2007). About 50% of these residues are used for generating power and heat to run the sugar, ethanol and distillery plants. The remaining bagasse are piled up which may cause spontaneous combustion of stored bagasse (Lavarack et al. 2000 and Pandey et al. 2000). Xylooligosaccharides (XOS) are the prebiotic component obtained from the plant biomass. Biomass of plant origin is one of the renewable and cheapest raw materials for sustainable development. That could be a promising initiator for the production of biofuel and bioenergy along with value-added biomolecule (Prebiotic). Prebiotics, as the name, implies "Pre- before; bio-life" it is evolved before life evolved. But it came to light in 1995 with the definition given by Gibson and Roberfroid (1995) as "Nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and or activity of one of the limited number of bacteria in the colon" (Samanta et al. 2014).

XOS is the synthesized prebiotics from various agricultural residues viz., corncob sugarcane bagasse, stalks of cotton, tobacco and shells of pistachios, walnut and groundnut, etc., they are hydrolyzed products of xylan a polysaccharide which is synthesized by various methods. These agricultural wastes are dumped in the field or burned after harvesting (Agrupis and Markawa 1999). By utilizing these wastes will protect the environment from pollution as well as increases the economic status of farmers and generates employment (Akpinar et al. 2009).

From a nutritional point of view, XOS is known as nondigestable (ND) as they are not degraded in the stomach and reaches the lower bowel, to be utilized by the microbiota residing there (Okazaki et al. 1990; Roberfroid 1999; Collins and Gibson 1999; Vazquez et al.2000). They have also helped in reducing cholesterol and maintains gut health. They are moderately sweet, inhibit the retrogradation of starch, and improves the sensory and nutritional properties of food and are stable for a wide range of pH and temperature (Vorgen 1998). XOS is noncarcinogenic and regulates insulin secretion from the pancreas, besides increasing mineral absorption from the large intestine. It affects bowel function through its mild laxative ability. The prebiotic consumption gradually raises the ability to stimulate the growth of indigenous *Bifidobacterium* and *Lactobacillus* in the hindgut which in turn suppress the growth and activity of harmful or putrefactive bacteria and reduces the concentration of toxic substances in the gastrointestinal tract (Samanta et al. 2007, 2010). XOS predominantly increases the population of *Bifidobacterium* and *Lactobacillus* which results in the production of SCFA by the prebiotic fermentation which helps in important physical events viz., Calcium absorption, bowel function, lipid metabolism and reduces the risk of colon cancer (Rycroft et al. 2001).

The Prebiotic potential is attributed by the utilization and nourishment of probiotic via fermentation thrive to maintain the gut microflora diversity by eliminating the harmful pathogen (Gibson et al. 2004). The prebiotic index and score can be calculated by comparing the stimulated growth by prebiotic on beneficial microbial diversity and other intestinal pathogens (Huebner et al. 2007).

Bacteriocins can be defined as extracellularly released peptides or protein molecules which have low molecular weight with a bacteriostatic mode of action of closely related species. Bacteriocins are classified into three classes based on their structure and function (Klaenhammer 1993). The action or effectiveness of the probiotics depends mainly on the type of strain and the amount consumed. Prevention of growth of the pathogenic organisms by occupying all the adhesion sites as pathogenic organisms also need to adhere and attach to the epithelial cells of the intestine. The action might also be due to the synthesis of the acids and generating the acidic environment and prevents the growth of pathogens. The immunological benefits conferred by probiotics are by prevention of allergies due to activation of macrophages and thereby increasing antigen presentation and increases secretion of immunoglobulin A.

In this research, the xylan was extracted from the sugarcane bagasse by alkali extraction and xylooligosaccharides has been produced by enzymatic hydrolysis and studied for its *in vitro* prebiotic potential and their bacteriocin activity against pathogens

2. Materials and methods

2.1 Strains used

The probiotic organisms were isolated from fermented foods viz., fish (Ngari), soyabean (Akhuni), Indian goose berry and Indian coffee plum, identified and submitted in NCBI were used in this study viz., *Lactobacillus plantarum* MT228948, *Lactobacillus fermentum* MT230901, *Bacillus amyloliquefaciens* MT193292, *Bacillus clausii* 658363 and *Bacillus faecium* MN956828.

2.2 Sample collection and processing of the sample

Sugarcane bagasse was collected from the local Chinnalapatti market, Dindigul district, Tamil Nadu. The collected Sugarcane Bagasse was washed with hot water to remove the dirt and dried in a hot air oven at 60±2°C. The dried sample was powdered with a mechanical blender and stored in an airtight container until further usage.

2.3 Compositional analysis of Sugarcane bagasse

The composition of sugarcane bagasse (Cellulose, Hemicellulose and Lignin) was analyzed gravimetrically (Ayeni 2015). The physicochemical parameters Moisture analysis (ASTM D2216 1993), Ash content (ASTM D2866, 2000) and Lipid content (Soxhlet method) of sugarcane bagasse were quantified. All estimations were carried out in triplicates.

2.4 Extraction of xylan by alkali (KOH and NaOH) treatment.

Xylan was extracted from sugarcane bagasse by alkali treatment coupled with steam treatment according to Samanta et al. (2012) with slight modification. The sugarcane bagasse was treated with alkaline viz., KOH and NaOH in a series of concentrations from 4% to 40%. Sugarcane bagasse was soaked overnight in alkali with a solid to liquid ratio of (10: 1) and they are autoclaved for 20 min at 121°C. The solid-liquid fractions were centrifuged at x10000 rpm for 15 min. The supernatant was neutralized to pH6 using 1N glacial acetic acid and 2 volumes of ice-cold ethanol (70%) were added and allowed for precipitation. The aliquots were centrifuged at x10000 rpm for 20 min and the pellets were washed twice with distilled water, lyophilized, homogenized and stored at 20°C until further usage. The maximum recovery of xylan from the sample (True yield) and hemicellulose (Relative yield) can be calculated using the following formulae (Jnawali et al. 2018)

$$\textbf{True Recovery}(\%) = \frac{\textbf{Dry weight of extracted xylan (g)}}{\textbf{Weight of the sample (g)}} \textbf{x 100}$$

$$\textbf{Relative Yield}\ (\%) = \frac{\textbf{True yield}\ (\%)}{\textbf{Xylan contents present in original samples}\ (\%)} \textbf{X100}$$

2.5 Optimization of Xylooligosaccharides (XOSs) by Response Surface Methodology (RSM)

The optimization of XOS production was carried out by Response Surface Model (RSM) -Central Composite Design (CCD). The extracted sugarcane bagasse xylan was subjected to enzymatic hydrolysis using commercial xylanase enzyme extracted from *T. viridae* (Sigma, Bangalore). The experiments were carried out in triplicates with 29 runs by varying pH (4 to 5.5), temperature (40°C to 55°C), enzyme (4U to 20U) and incubation time (8 to 24 hours). Two percent of substrates were added to 10ml of sodium citrate buffer and 1ml of enzyme and incubated in shaking waterbath at x150g for appropriate temperature and time. The aliquots were drawn from the enzymatic hydrolysis and the mixture was heated to 100°C to inactivate the enzyme and the hydrolysate was filtered with Whatman No1 filter paper. Three volumes of ice-cold ethanol were added to the filtrate to precipitate the traces of unhydrolyzed xylan (Samanta et al. 2014) and it was vacuum filtered using G3 sintered crucible filter and the filtrate was analyzed for its non-reducing sugar (XOS) by Lane and Eyon chemical method The optimization study was designed using by Design expert software version 11.

2.6 Detection and purification of Xylooligosaccharides

The crude xylooligosaccharide was purified by the Activated charcoal column chromatography method according to Chapla et al. (2012) with slight modification. Briefly, the activated charcoal was added to the crude XOS with a solid to liquid ratio of 1:10 and they were incubated at 25°C for 30 min at 150 rpm in a cooling shaking incubator**.** After incubation, the charcoal mixture was washed with 5 volumes of distilled water by vacuum filtration, as the monosaccharides in the mixture solution get washed off and the XOS adheres to the pores of activated charcoal. The charcoal containing XOS mixture is packed into the column with bed volume 1-2 cm length with 2.3cm width as the stationary phase and 90% ethanol was used as the eluent. Elution was carried out at room temperature with gravitational force at a flow rate of 5ml/hour. 6 fractions of 5ml each were collected and Thin Layer Chromatography (TLC) was performed. The desired fractions were pooled together and concentrated using a Rotary Vacuum evaporator and lyophilized and stored at 4°C until further use.

2.7 Characterization of Xylan and Xylooligosaccharides

2.7.1 Fourier Transform Infra-Red (FTIR) analysis

Surface functional groups of Xylan and Xylooligosaccharides extracted from Sugarcane bagasse were unraveled by FTIR (Perkin-Elmer infrared spectrophotometer, India). The xylan and Xylooligosaccharides were mixed with KBr (spectroscopic grade) separately and pellets were prepared in the size of about 10-30 mm diameter and 1 mm in thickness (Jayapal et al. 2013). The samples were scanned in transmission mode with a resolution of 4cm^{-1} in the 4000-400 cm^{-1} range and the functional groups were compared with previously published works of literature.

2.7.2 X-ray Powder Diffractions for Xylan and XOS

To determine the physical nature of Xylan and Xylooligosaccharides, X-ray diffraction (XRD) was analyzed using a powder diffractometer (PANalytical/XPert 3, New York). The structural property of Xylan and

Xylooligosaccharides was identified using Bragg's law by measuring the line spacing in diffraction pattern (d) and angle of incidence (θ) where λ is the wavelength of the monochromatic X-ray beam.

$$\mathbf{d} = \frac{\boldsymbol{\lambda}}{\mathbf{n}\,\mathbf{sin\theta}}$$

The crystallinity index (CI) of Xylan and Xylooligosaccharides was also evaluated by calculating the ratio of area under the crystalline peaks and total area of the scattered diffractogram using followed formulae (Singh et al. 2011).

$$\mathbf{CI} = \frac{\boldsymbol{\varepsilon A_{Crystal}}}{\boldsymbol{\varepsilon A_{Crystal}} + \boldsymbol{\varepsilon A_{amorphous}}}$$

2.7.3 NMR analysis for XOS

Approximately 10mg of xylan and Xylooligosaccharides samples were dispersed in Dimethyl Sulfoxide (DMSO) and Deionized water (Peng et al. 2010) and these solutions were used to record the ^{1}H and ^{13}C spectra. The Acquired time (AQ) is 4.089 seconds. The number of scans was 16 (NS) and the delay between transients was 2 seconds. Data were processed using the Bruker Topsin NMR software (Bruker, Avance III HD Nanobay 400 MHz FT-NMR SPECTROMETER, California)

2.7.4 Thermogravimetric Analysis (TGA)

The thermal stability of the xylan and Xylooligosaccharides component was determined by Thermogravimetric analysis (NETZSCH, NJA – STA 2500 Regulus, Germany) (Bian et al. 2010). 10 mg of dried xylan and Xylooligosaccharides samples were dried in a desiccator before experimenting. 2mg of samples were loaded in the crucible and heated up to 600°C from room temperature at a rate of 10°C/min with a continuous flow of nitrogen.

2.7.5 Liquid Chromatography-Mass Spectroscopy (LC-MS)

The macromolecules in the sample were analyzed by 6530Q-TOF LCMS (Agilent, United States). 2µl of XOS sample diluted in methanol was injected into the column and ionized by electron spray ionization source in a positive ion charge mode. The scan was performed for the mass charge range (*m/z*) between 100-1000 (Xiao et al. 2018).

2.8 Prebiotic attributes

2.8.1 Resistance to acid hydrolysis

The resistance to gastrointestinal tract fluids was studied according to Wang (2009) and Winchienchot et al. (2010). Artificial human gastric juice was mimicked by using hydrochloric acid buffer containing (in g/L) NaCl, 8; KCL, 0.2; $Na_2HPO_4.2H_2O$, 8.25; $NaHPO_4$, 14.35; $CaCl_2.2H_2O$, 0.1; $MgCl_2.6H_2O$, 0.18. This buffer was adjusted to pH 1 to 5 using 5 M HCl. This buffer (5 ml at each pH) was added to the sample solution (1% w/v, 5 ml) and incubated in a water bath (37 ± 1°C) for 6 hours. Sample (1ml) was taken periodically at 0, 0.5, 1, 2, 4 and 6 hours and tested for reducing sugar content using the dinitrosalicylic acid (DNS) and also total sugar content using the Anthrone method. In this experiment, Inulin was used as a control. Percentage of the sample was calculated based on reducing sugar released and total sugar content of the sample as below:

$$\textbf{\% hydrolysis} = \frac{\textbf{Reducing Sugar released}}{\textbf{Total sugar content} - \textbf{Initial reducing sugar content}} \textbf{X100}$$

2.8.2 Prebiotic efficiency

The capability of probiotics to utilize prebiotics for their growth as a carbon source was determined according to Agte et al. (2010) protocol with slight modification. Probiotic cultures *Lactobacillus* sp. and *Bacillus* sp. were grown in their specific medium viz., *Lactobacillus*- MRS broth and *Bacillus*-Nutrient broth by altering the carbon source of the synthetic medium with XOS and EPS each. The utilization of prebiotics was analyzed by the growth of probiotic cultures using the visible spectrophotometer at 600nm every 12 hours after incubation for a day.

2.8.3 Prebiotic Index

The prebiotic index is a growth comparison of probiotic on control media and prebiotic substituted media was analyzed according to Rodriguez et al. (2019). Probiotics were inoculated into the sterilized control media and carbon sources substituted with prebiotic and incubated at 37 ±2°C for 24 hours and it was calculated using the following formula by reading the growth at 600nm

$$Prebiotic\ Index = \frac{A_{600nm}\ of\ probiotic\ growth\ in\ prebiotic\ subtituted\ medium}{A_{600nm}\ of\ probiotic\ growth\ in\ control\ medium}$$

2.8.4 Prebiotic activity score

The utilization of prebiotics by probiotic cultures and an indicator *E. coli* were determined following the protocol of Huebner et al. (2007) by comparing their growth at 0^{th} and 24 hours of incubation using UV- Visible spectrophotometer at 600nm. The cultures grown in media without prebiotics were used as control. The prebiotic score was calculated using the following formula:

$$\boldsymbol{Prebiotic\ activity\ score}$$

$$= \frac{\textbf{probiotic growth at 24th hrs with Prebiotics} - \textbf{Probiotic growth at 0th hr with Prebiotics}}{\textbf{probiotic growth at 24th hrs with glucose} - \textbf{Probiotic growth at 0th hr with glucose}}$$

$$- \frac{\boldsymbol{E.coli}\ \textbf{growth at 24th hrs with Prebiotics} - \textbf{E. coli growth at 0th hr with Prebiotics}}{\boldsymbol{E.coli}\ \textbf{growth at 24th hrs with glucose} - \textbf{E. coli growth at 0th hr with glucose}}$$

2.9 Bacteriocin production from probiotic with prebiotic

The probiotic cultures grown in prebiotics substituted medium were screened for its bacteriocin activity using the agar well diffusion method. Briefly, the isolates were inoculated in 50ml of respective prebiotic substituted medium and incubated overnight at 37°C for 24 hours. The isolates were centrifuged in the cooling centrifuge at 4°C at the rate of x5000g for 20 minutes. The supernatant was filtered through a 0.22µm membrane filter to remove the bacterial cell to obtain Cell-Free Supernatant (CFS) and adjust to pH 6. The pathogens were swabbed onto the nutrient agar plate and 6mm diameter wells were cut using a sterile well diffuser. Consequently, 100µl of pH neutralized CFS were added to wells and plates were incubated at 37°C for 12 hours and examined for the presence of zone, measured zone using zone scale.

3. Results and Discussion

3.1 Compositional Analysis of Sugarcane bagasse

Agricultural residues being dumped dumped or burned in fields, both activities lead to environmental problems; hence these residues can be potentially converted into a value-added prebiotic component Xylooligosaccharide. The Plant biomass are mainly composed of cellulose, hemicellulose and lignin components. In this research, the composition of sugarcane bagasse was analyzed and composed of cellulose (36±0.02%); hemicellulose (25±0.03%), lignin (20.23±0.04%), Ash content (1.23±0.15%) and wax less than 1 (Fig. 1). Similarly analysis of sugarcane bagasse had been carried out by Ayeni *et al*. (2015) and Bon and Ferrara (2007).

3.2 Alkali extraction of xylan from sugarcane bagasse

Treating lignocellulose-rich agricultural residues to alkali results in swelling of cellulose and rupturing of cell walls and high temperature softens the protective shielding lignin layer (Lavarack et al. 2002). Extraction of xylan with alkali like NaOH and KOH does not require any special instrument and it is an affordable and simple method. The xylan has been steadily increased when incrementing of concentration up to 20% of NaOH or KOH giving a true yield of 22 % and 20% and relative yield of 86% and 78%. By comparing the xylan yield among the alkali used, the maximum xylan yield was observed in 20% NaOH combined with steam-treated sugarcane bagasse (Table 1). Jayapal et al. 2013 have compared the xylan extraction with two different alkali. The relative recovery for KOH is 6 to 53% and 12 to 85% for NaOH. Samanta et al., 2012 also compared the alkali extraction of xylan from natural grass (*Sehima nervosum*) and the true yield of KOH is 2.47% to 16.52% and NaOH is 3.75 to 25.12% and the maximum relative yield for KOH is 23.43% and 83.38% for NaOH.

3.3 Optimization and Production of XOS from xylan using Response Surface Model (RSM).

RSM quadratic model was adopted to maximize the yield of XOS production and minimize the undesirable product (xylose) formation. The XOS production was estimated by standard chemical method (Lane and Eyon 1923). The correlation and interaction of the independent variables were determined by the Box Bohnken method (Table 2). The effect of the model was analyzed by regression coefficient, Analysis of variance (ANOVA) and response surface plots (Fig 2).

The coefficient of the factors was determined by the R^2 value, this value has to be above 0.80 to good fit a model and they elucidate the accuracy of the response of the model. The regression coefficient for XOS production was significant ($P<0.05$) with an R^2 value of 0.925 that determines the 92.5% accuracy. The results recommend the quadratic equation for XOS recovery from Sugarcane bagasse xylan as follow:

$$XOS = 0.6244 - 0.0143A - 0.00206B - 0.1766C - 0.0264D - 0.0295AB + 0.0050AC + 0.0493BC + 0.0328BD + 0.0088CD - 0.0059A^2 - 0.0071B^2 + 0.2073C^2 + 0.0000D^2$$

Where A- pH, B- Temperature, C- Enzyme, D- Time

Analysis of variance for the current model is significant with $p<0.001$and the lack of fit Not significant (0.4087) as the designed model perfectly fits the yield of the XOS. The maximum yield was observed in the following runs 12, 19, 20, 26 and 29 with 1.57±0.29, 0.99±0.24, 0.95±0.21, 0.98±0.31 and 1.04±0.33 respectively.

Response surface plots elucidate the interaction between independent variables by plotting 3D surface curves against two variables by keeping the other two variables at their central level. The central level for the independent variables is pH (4.75), temperature (45°C), enzyme (4 U/ml) and time (16 h). When the pH and temperature are decreased to (40 to 4°C) the XOS yield was a maximum of 0.634 mg/ml (Figure 2A). The low pH (4) and enzyme concentration (4 U/ml) enhances the XOS yield 1.013mg/ml and the yield declines when the enzyme concentration has increased (Figure 2B). The interaction between pH and Time doesn't have much impact on the production of XOS (Figure 2C). The XOS yield was gradually decreased when the temperature is decreased and the enzyme concentration increased (Figure 2D). When the time and enzyme concentration increased, the XOS yield has been reduced (Figure 2E). Interaction between the temperature and time has shown less impact in enhancing the XOS production. Hence the ideal condition concluded for the maximum yield of XOS is pH 4.75, temperature 45°C, enzyme 4 U/ml and Time 16 h. Jayapal et al. (2013) have produced XOS at pH 4, using enzyme at 2.65 U/ml, time 8 hours and temperature 40°C whereas Samanta et al. (2014) produced XOS at pH 3.53, Temperature 51.46°C, enzyme 24.7 U/ml and time 12 hours.

3.4 Characterization of Xylan and XOS

3.4.1 Fourier Transform Infra Red (FTIR) analysis of xylan and XOS

FTIR was employed to study the functional groups present in the Xylan and XOS which corresponds to a signature molecule (Fig. 3). The FTIR spectrum for the sugarcane bagasse xylan peaks at 3762cm^{-1}, 3347 cm^{-1}, 2917 cm^{-1} represents the OH and CH stretching of xylan (Peng et al. 2010, Samanta et al. 2012, Jayapal et al. 2013, and Hesam et al. 2020). The short narrow bend denotes the o acetyl group in the hemicelluloses chain, 1640 cm^{-1} due to the presence of residual water. 1373 cm^{-1} and 1219$cm^{-1}$are due to the CH, OH, or CO stretching and bending vibrations of the hemicelluloses. 1033 cm^{-1} and 896 cm^{-1} denotes the1-4 β configuration of xylan (Hasem et al. 2020). The spectrum at 1635cm^{-1} represents the CH streaking of Xylooligosaccarides (Peng et al. 2010). Asymmetric and symmetric (C=O) stretching vibration of the Carbohydrate groups made small vibration at 3289 cm^{-1}. A peak at 1286 cm^{-1} elucidates the C=O and C-O stretching. A small vibration at 1054 cm^{-1} is due to the presence of 4-O methyglucuronoxylan type oligo and polymers (Kacurakova et al. 1998).

3.4.2 XRD analysis of xylan and XOS

The XRD profile for xylan was represented in Fig 4. Various peaks 2 theta values ranging from 4 to 90 spectrum were observed. The narrow sharp and short broad peaks represent crystalline and amorphous phases. 9.35, 8.99, 11.14, 18.72, 18.90, 22.6, 22.34, 26.64, 26.65, 29.7, 29.36, 30.82, 33.88, 36.42, 40.89 and 44.68 implies the interplanar spacing (d spacing) of 9.60, 7.81, 4.64, 4.12, 3.97, 3.80, 3.56, 3.33, 3.177, 3.01, 2.8, 2.75, 2.72, 2.67, 2.53, 2.46, and 2.43. The CI index of xylan is 0.45 (45%). Xylan consists of nearly equal proportions of a crystalline and amorphous phase. The 2theta values of XOS are 9.35, 18.9 and 28.4 represents the d spacing values 9.49, 4.71 and 3.13 respectively. The CI index of the XOS is 0.030 (3%). XOS has a majorly amorphous phase with a trace of crystalline structure. Lyophilization (freeze-dried) method was adopted for processing the XOS into powder. During the process, they may let the sample absorb water leads to crystals formations in an amorphous sample (Zhang et al. 2019)

3.4.3 ^{1}H and ^{13}C NMR spectrum characterization of XOS

The protons in the xylan were analyzed by ^{1}H NMR and illustrated in Fig. 5A The main signals at 4.26 (H-1), 3.19 (H-2), 3.59 (H-3), 3.63 (H-4) and 3.98 (H-5) ppm imply the β- D xylopyranosyl residues originated from 4-o-methyl α-D GlycpA acid (1→2). The protons of arabinofuranosyl determine the 5.1 to 5.4ppm and the minor signal at 5.40ppm illustrates the Xylopyranosyl residues. The strong signal at 2.5ppm and 1.630ppm state the Methylene and groups in the solvent (methanol). The ^{13}C NMR spectrum of Xylan (Fig. 5B) represents the (1-→4)linked β-xylan. Peaks at 102.2 (C-1), 73.04(C-2), 74.43(C-3), 75.78(C-4), 63.98 (C-5) ppm. 102.2ppm peak represents the β configuration of the backbone of the xylan confirmed by ^{1}H NMR.

The spectral region ranging between 4.30-5.40ppm in ^{1}H NMR confirms the presence of XOS in the sample (Fig 5C). The spectrum of 5.0-5.40ppm and 4.30-4.60ppm represent the α anomeric and β anomeric protons. 5.32 ppm is the characteristic signal of α-L-arabiofuranosyl (α-L-Araf) residue (1-→2) linked with the monosubstituted β-D xylopyranose residue. The signals at 5.07ppm and 4.50 ppm illustrate the reducing end of X α and X β. 5.20ppm was due to the attachment of 4-o-methyl glucuronic acid to xylose through α(1-→2) linkage. 4.45 - 4.35ppm is due to the protons in the internal and nonreducing end of xylosyl residues. The heterogeneity structure of the XOS was analyzed by ^{13}C NMR. The signals at 91.67 ppm and 96.54 ppm determine the reducing end of α and β C-1. The major four signals at 72.74 (C-2), 73.48 (C-3), 76.39 (C-4), 63.05 (C-5) represent the nonreducing end of the β D xyl residues (Fig 5D). 80.76, 77.47, 84.82, 62.8ppm represents the C-2, C-3, C-4, C-5 of αL-Ara units. The signals at 101.4 and 101.7 ppm represent the internal and non-reducing terminals confirmed by ^{1}H NMR.

3.4.4 LC-MS/MS Analysis of XOS

LC-MS/MS data elucidate the structure, molecular weight and distributions of acetyl groups in the XOS (Fig 6 and Table 3). The sharp narrow peak at 305 *m/z* and 317 *m/z* indicates the presence of disaccharide(xylobiose-$C_{10}H_{18}O_9$) of two pentoses with Na^+ ions respectively. The peak at 361 *m/z* indicates the two pentoses with acetyl and Na^+ ions presence. The spectrum at 462 *m/z* indicates the trisaccharide-xylotriose ($C_{15}H_{26}O_{13}$) with 3 pentose units with Na^+ ions. 615 *m/z* peak represents the tetrasaccharides -xylotetrose ($C_{20}H_{34}O_{17}$) made up of four pentose units with Na^+ ions and 672 *m/z* represents the presence of four pentose units with two acetyl groups in the XOS (Xiao et al. 2018).

3.4.5 TGA analsyis of XOS

In the TGA profile of XOS, the gradient temperature increase elucidates the difference in sample weight due to the presence of volatile groups. For XOS, a weight loss of 17.11% was observed between 100°C -300°C due to the evaporation of water vapors in the sample. After this, the sample may undergo a pyrolysis process where the sample is partially decomposed into ash and the sample loses its weight of 17.90% between 300°C -400°C and the sample would have completely decomposed (14.04%) between 500°C to 600° C into ash by the combustion process (Fig 7). Differential Thermogravimetric analysis (DTG) represents the maximum degradation (T_d) at a temperature that determines the stability of the sample. The maximum degradation of the sample was observed at 147°C.

3.5 Selection attributes of Prebiotics

3.5.1 Acid Indigestability

The development of prebiotics has focused on the non-digestibility of oligosaccharides (Wang 2009) and to ensure them to reach the colon to benefits the diversity of niche and probiotic microorganisms residing there (Gibson et al. 2004). Prebiotics extracted from sugarcane bagasse were hydrolyzed with artificial gastric juice, the degree of hydrolysis decreased when the pH of the juice increases. Hydrolysis percentage of prebiotics was compared with reference prebiotic (inulin). The hydrolysis of prebiotics ranges from 5.3%, 3.9%, 3.89%, 2.43%, 2%, 1.4% in varied pH (1-6) whereas, the hydrolyzed percentage of Inulin is 52%, 34%, 21.5%, 18% and 15.3%. Maximum hydrolysis (5.3%) was observed in pH 1 at 6 hours of incubation in gastric juice. Hence when comparing with the reference prebiotic, XOS is less digested and the percentage of prebiotic reaching to the intestine is 95% unhydrolyzed and the results are shown in Fig. 8. The degree of hydrolysis at pH 1, 2, 3 and 5 was 4.08%, 2.3%, 1.66%, 0.85% and 0.02% in oligosaccharides extracted from Pitaya fruits (Wichenchot et al. 2010).

3.5.2 Prebiotic efficiency on Probiotics

The efficacy of prebiotic is determined by the selective stimulation of probiotic growth and their metabolism when other commensal microorganisms are available in the intestinal region The growth of all probiotic organisms has increased when the incubation time increases (Fig. 9). In 24 hours *L.plantarum*, *L.fermentum* and *B.amyloliquefaciens* are showed maximum XOS utilization in optical density of 0.99, 0.97, 0.89. Madhukumar and Muralikrishna 2012, evaluated the optical density (A_{600nm}) growth 0.296 and 0.604 of *L.plantarum* NDRI strain 184 in Bengal gram husk and wheat bran XOS at 24 h incubation. Yu et al. 2015 have also reported that the corncob utilization by *L. plantarum* QH251 and SC52 was 0.62 and 0.62 at 600nm.

3.5.3 Prebiotic Index and Score

The prebiotic index is the measurement of growth comparison of probiotic bacteria utilizing the prebiotics and the MRS medium containing glucose as the carbon source in 24 hours of incubation (Table 3A). The value below and near to one is determined as the low efficiency of prebiotic on probiotic utilization. The maximum prebiotic index was noted in *L.plantarum* (1.92), *L.fermentum* (1.37) and *B.amyloliquefaciens* (1.61) and the minimum was observed in *B.clausii* (0.21). Huebner et al. (2007) derived a prebiotic activity score based on the cell density values of probiotics on prebiotic.

The prebiotic score can be calculated by comparing the growth difference between probiotic bacteria in media with glucose and substituted with prebiotics and Reference bacteria (*E.coli*) in media with glucose and prebiotic substituted media (Table 3B). The score below or near one elucidates that *E.coli* dominates the growth of probiotic bacteria. The score above one implies that the probiotic bacteria has suppressed the growth of other commensals bacteria with prebiotic as carbon source. *L.plantarum* and *L.fermentum* have highest prebiotic score of 12 and 17 whereas least was observed in *B.clausii* (0.9) and *E.faecium* (0.57)

3.6 Bacteriocin produced by probiotics on utilizing the XOS as a carbon source

Bacteriocin produced by the probiotic organisms on growing in MRS media (Table 4A. Media substituted with XOS (Table 4B and Fig. 10) as a carbon source were tested against the pathogens. Comparatively, the bacteriocin produced on utilization of prebiotic has the high ability to inhibit the pathogen. Maximum growth inhibition was

observed by *Enterococcus faecium* against *E.fecalis* (13mm) and *Streptococcus pyogenes* (11mm). *Lactobacillus plantarum* against *E.fecalis* (11mm) and *L. monocytogenes* (12mm). *Bacillus clausii* have shown growth inhibition against *L.monocytogenes* (12mm). All probiotic bacteriocins produced, have shown inhibition against *E.coli.* Yu et al. (2015) have reported that bacteriocin from *Lactobacillus plantarum* S2 shown antibacterial activity against *Shigella flexneri* and *E.coli* moderately (3-6mm in diameter). Least growth inhibition (0-3mm in diameter) against *Salmonella typhimurium* and *Staphylococcus aureus* in all the triplicates.

4. Conclusion

Xylooligosaccharides can be produced in a single step method by autohydrolysis. But we adopted alkali extraction and enzymatic hydrolysis of xylan that is advantageous as it does not leave any toxic traces in the environment, minimal production of xylose (undesirable component) and cheap method (not laborious). Upon comparing various alkali, NaOH has a greater effect on xylan extraction from sugarcane bagasse. Bio process variables such as temperature, pH, enzyme concentration and reaction time have been optimized for XOS production using RSM. As it is known that XOS is an emerging prebiotic component these days, utilization of agricultural wastes as a source for its production shall open new insights for zero waste technology that can improve gut health with proliferation of residential and probiotic flora. Production of XOS from agricultural residues shall improve socio economic status globally by converting the trash into cash.

5. Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: All the authors have read the manuscript and approved for its submission

Availability of data and materials: All the datasets are included in the manuscript

Competing interests: The authors declare that they have no competing interests

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Author’s Contributions

NK has conceptualized and designed the experiments. NK, LG and AN carried out the experiment. VK have helped in analyzing the data. Wrote the manuscript with support from KT and DRA. DRA have supervised the whole experiment.

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Figure captions

Figure 1 Compositional analysis of Sugarcane bagasse

Figure2 Optimization of external factors for the enzymatic production of Xylooligosaccharides using Response Surface Methodology

Figure 3 FTIR characterization for alkali extracted xylan and enzymatically produced Xylooliogsaccharide

Figure 4 XRD pattern of alkali extracted xylan and enzymatically cleaved Xylooligosaccharide products

Figure 5 ^{1}H and ^{13}C NMR spectra for alkali extracted xylan (A, B) and its enzymatically cleaved Xylooligosaccharides (C, D)

Figure 6 ESI-MS/MS characterization of enzymatically produced Xylooliogsaccharides

Figure 7 TGA characterization of enzymatically produced Xylooliogsaccharides

Figure 8 Acid Indigestability of XOS

Figure 9 Prebiotic efficacy of XOS produced from sugarcane bagasse and Inulin (Commercial prebiotic)

Figure 10 Bacteriocin activity from probiotic bacteria grown in prebiotic substituted medium against human pathogens

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553 biotechnology. 25: 1084-1092.

Table 1 Comparisons between different alkali treatment on the extraction of xylan from sugarcane bagasse

Alkali Concentration (%)	NaOH			KOH		
	*Xylan (g) Mean ± SD	True yield (%)	Relative yield (%)	*Xylan(g) Mean±SD	True yield (%)	Relative yield (%)
4	0.11 ± 0.02	11	44	0.04±0.00	4	16
8	0.12 ±0.01	12	48	0.07±0.00	8	31
12	0.09 ±0.01	13	52	0.13±0.00	14	54
16	0.21±0.03	16	64	0.16±0.01	16	64
20	0.21±0.00	22	86	0.19±0.00	18	72
24	0.19±0.00	20	76	0.15±0.07	20	78
28	0.23 ±0.060	14	56	0.16±0.01	17	66
32	0.24 ±0.128	13	52	0.14±0.00	14	56
36	0.24 ±0.133	13	52	0.12±0.04	13	50
40	0.24 ±0.045	12	48	0.07±0.01	7	28

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Table 2 **Optimization of external factors for the enzymatic production of Xylooligosaccharides using Response Surface Methodology (RSM)**

Factors				Xylooligosaccharides (mg/ml)	
A: pH	**B:Temperature (°C)**	**C:Enzyme (U/ml)**	**D: Time (h)**	**Observed Value**	**Predicted Value**
4.00	45	12	8	0.65	0.66
4.00	40	12	12	0.662	0.65
4.75	45	12	12	0.74	0.64
4.75	45	4	24	0.96	0.98
4.75	45	20	8	0.69	0.68
4.75	45	20	24	0.63	0.64
4.75	45	12	12	0.53	0.64
4.75	40	12	8	0.67	0.69
4.75	45	12	12	0.56	0.63
4.75	50	12	8	0.58	0.59
4.75	40	12	24	0.53	0.58
4.75	40	4	12	1.16	1.11
4.75	45	12	12	0.69	0.63
5.5	45	20	12	0.63	0.64
4.75	50	12	24	0.56	0.60
5.50	45	12	8	0.66	0.63
4.00	50	12	12	0.67	0.63
4.00	45	12	24	0.66	0.60
4.00	45	4	12	0.99	1.04
4.75	50	4	12	0.95	0.93
5.50	40	12	12	0.64	0.67
5.50	50	12	12	0.53	0.54
4.75	50	20	12	0.68	0.66
5.50	45	12	24	0.64	0.58
4.75	45	12	12	0.67	0.63
5.50	45	4	12	0.98	1.00
4.00	45	20	12	0.62	0.6671
4.75	40	20	12	0.69	0.6446
4.75	45	4	12	1.04	1.03

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Table 3 LC MS/MS analysis for XOS

Peak No	Compounds other than XOS (m/z)	XOS (m/z)	No.of Pentose units	Na+adducted XOS (m/z)	Acetyl Adducted XOS (m/z)
1		294	2 (Xylobiose)	305	
2		294	2(Xylobiose)	317	
3		294	2(Xylobiose)	305	361
4	406	-	-	-	-
5	436	-	-	-	-
6		441	3(Xylotriose)	462	
7	569	-	-	-	-
8		588	4(Xylotetrose)	615	-
9		588	4(Xylotetrose)		672
10	701	-	-	-	-

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Table 4A Evaluation of prebiotic index of Xylooligosaccharides

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Probiotic organisms	**Prebiotic Index ± SD* for XOS**
***Lactobacillus plantarum* MT228948**	1.926829 ±0.24
***Lactobacillus fermentum* MT230901**	1.372263±0.33
***Bacillus amyloliquefaciens* MT193292**	1.619565±0.37
***Bacillus clausii* MN658363**	0.21148±0.42
***Enterococcus faecium* MN956828**	1.426702±0.53

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Table 4B Evaluation of Prebiotic Score by comparing the growth of Probiotics and *E. coli* in the prebiotic (XOS) substituted and synthetic medium

Probiotic organisms	Prebiotic Score ± SD*	
	24 h	48 h
***Lactobacillus plantarum* MT228948**	12.437±0.64	00.737±0.59
***Lactobacillus fermentum* MT230901**	17.289±0.34	00.979±0.52
***Bacillus amyloliquefaciens* MT193292**	04.210±0.40	00.905±0.72
***Bacillus clausii* MN658363**	00.900±0.43	01.021±0.57
***Enterococcus faecium* MN956828**	00.575±0.25	00.634±0.65

Table 5A Bacteriocin activity from probiotic bacteria grown in synthetic medium

Test Pathogens	Zone of growth inhibition (mm in diameter)				
	***Lactobacillus plantarum* MT228948**	***Lactobacillus fermentum* MT230901**	***Bacillus amyloliquefaciens* MT193292**	***Bacillus clausii* MN658363**	***Enterococcus faecium* MN956828**
***Escherichia coli* MTCC 2622**	-	-	-	17.5±0.073	11.5± 0.0053
***Staphylococcus aureus* MTCC 7278**	16.5±0.045	-	16.5± 0.020	13±0.067	15.5±0.0075
***Enterococcus faecalis* MTCC 439**	-	12.6± 0.057	-	17.5±0.039	15±0.00
***Listeria monocytogenes* MTCC 657**	-	-	18.5±0.065	12.5±0.049	-
***Streptococcus pyogenes* MTCC 442**	5.5±0.070	4.9±0.820	-	-	5±0.034

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Table 5B Bacteriocin activity from probiotic bacteria grown in prebiotic substituted medium

Test Pathogens	Zone of growth inhibition (mm in diameter)				
	Lactobacillus plantarum **MT228948**	***Lactobacillus fermentum*** **MT230901**	***Bacillus amyloliquefaciens*** **MT193292**	***Bacillus clausii*** **MN658363**	***Enterococcus faecium*** **MN956828**
Escherichia coli **MTCC 2622**	11±0.63	11±0.57	7±0.28	8±0.08	10±0.57
Staphylococcus aureus **MTCC 7278**	10.2±0.12	9.5±0.045	10.5±0.02	0	0
Enterococcus faecalis **MTCC 439**	11±0.57	10±1.52	12±0.52	0	13±0.04
Listeria monocytogenes **MTCC 657**	12±0.72	11±0.57	0	12±0.5	0
Streptococcus pyogenes **MTCC 442**	6±0.22	-	-	7±0.03	11±0.034

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Figures

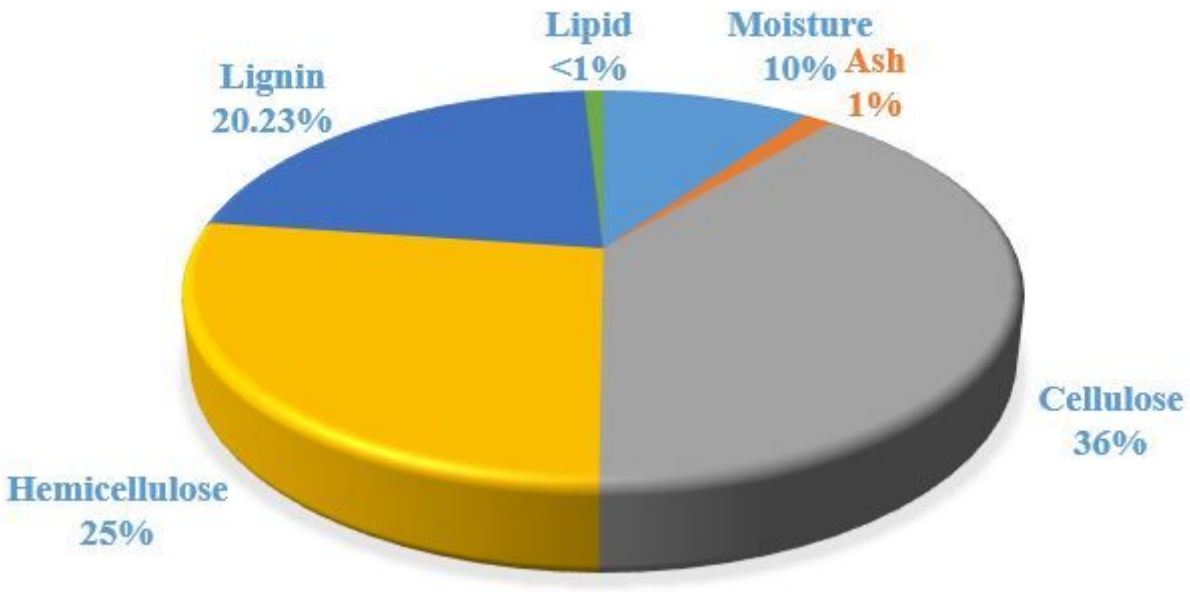


Figure 1

Compositional analysis of Sugarcane bagasse

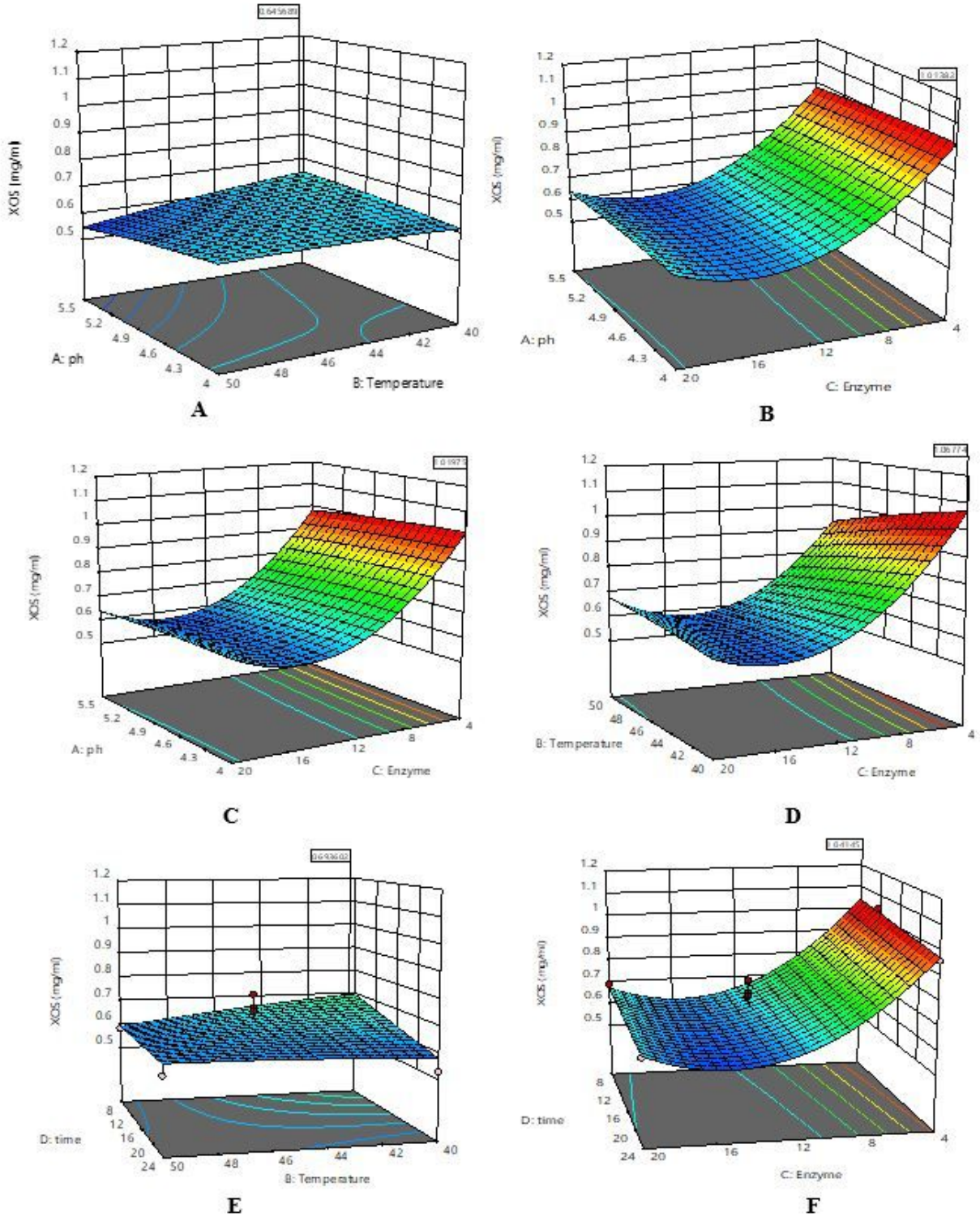


Figure 2

Optimization of external factors for the enzymatic production of Xylooligosaccharides using Response Surface Methodology

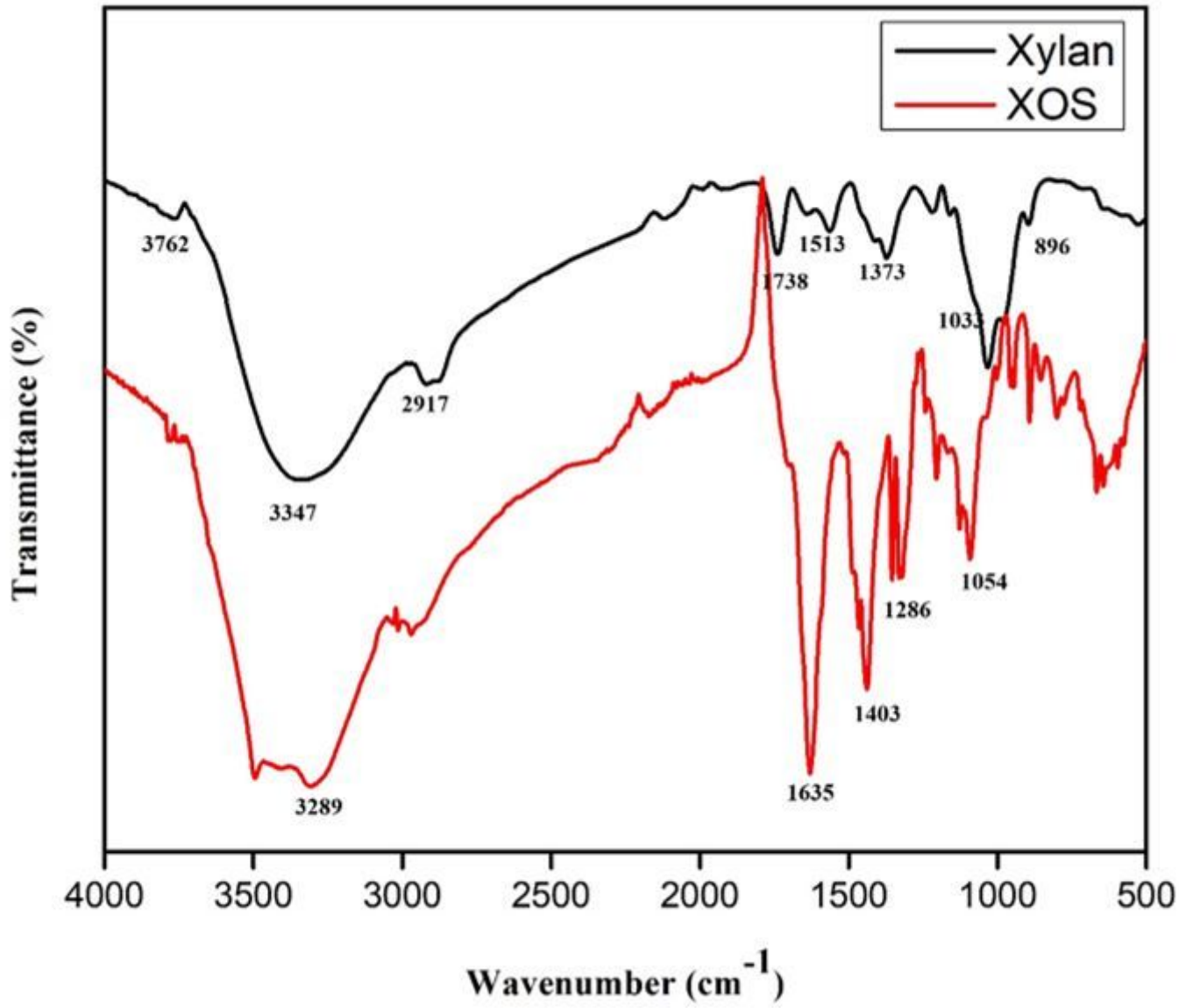


Figure 3

FTIR characterization for alkali extracted xylan and enzymatically produced Xylooliogsaccharide

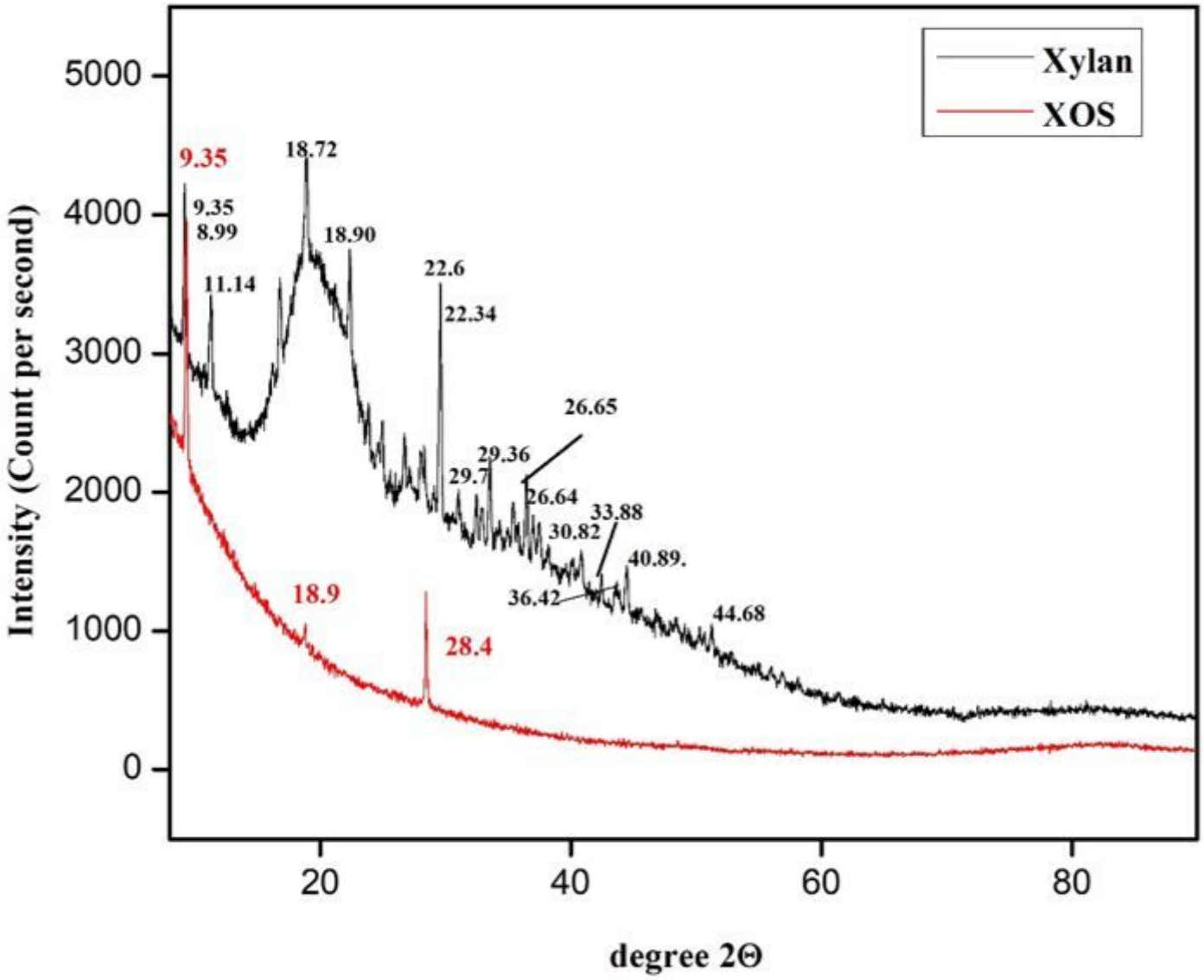


Figure 4

XRD pattern of alkali extracted xylan and enzymatically cleaved Xylooligosaccharide products

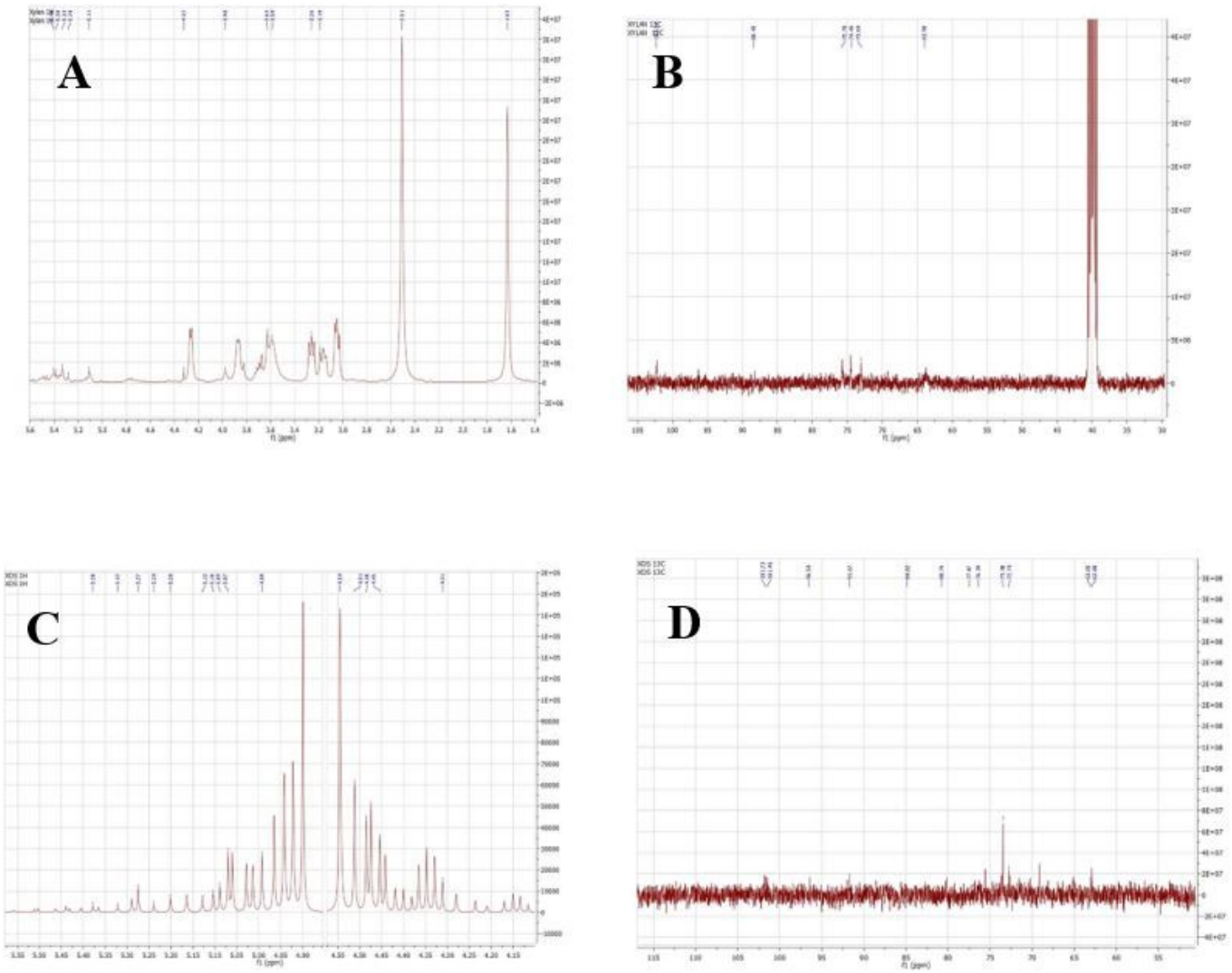


Figure 5

1H and 13C NMR spectra for alkali extracted xylan (A, B) and its enzymatically cleaved Xylooligosaccharides (C, D)

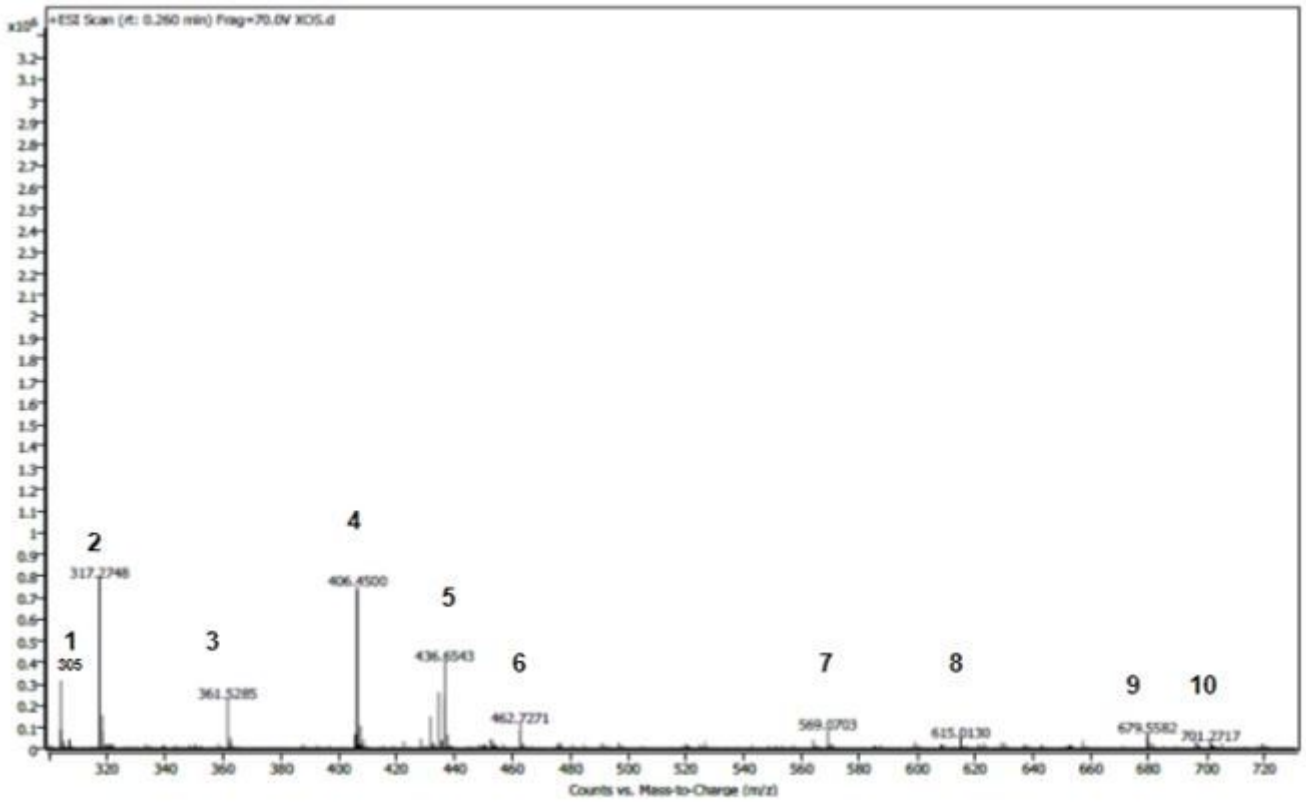


Figure 6

ESI-MS/MS characterization of enzymatically produced Xylooliogsaccharides

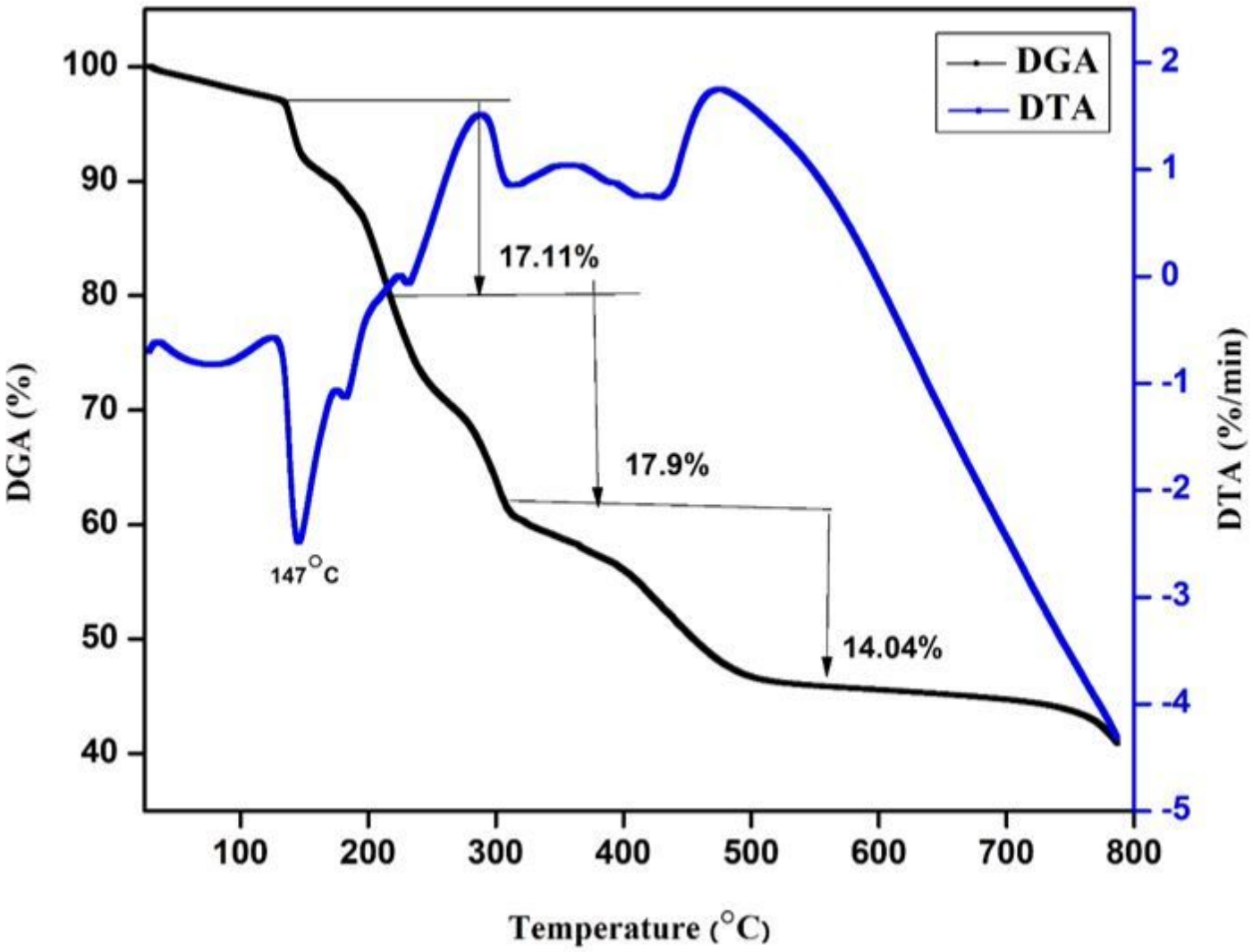


Figure 7

TGA characterization of enzymatically produced Xylooliogsaccharides

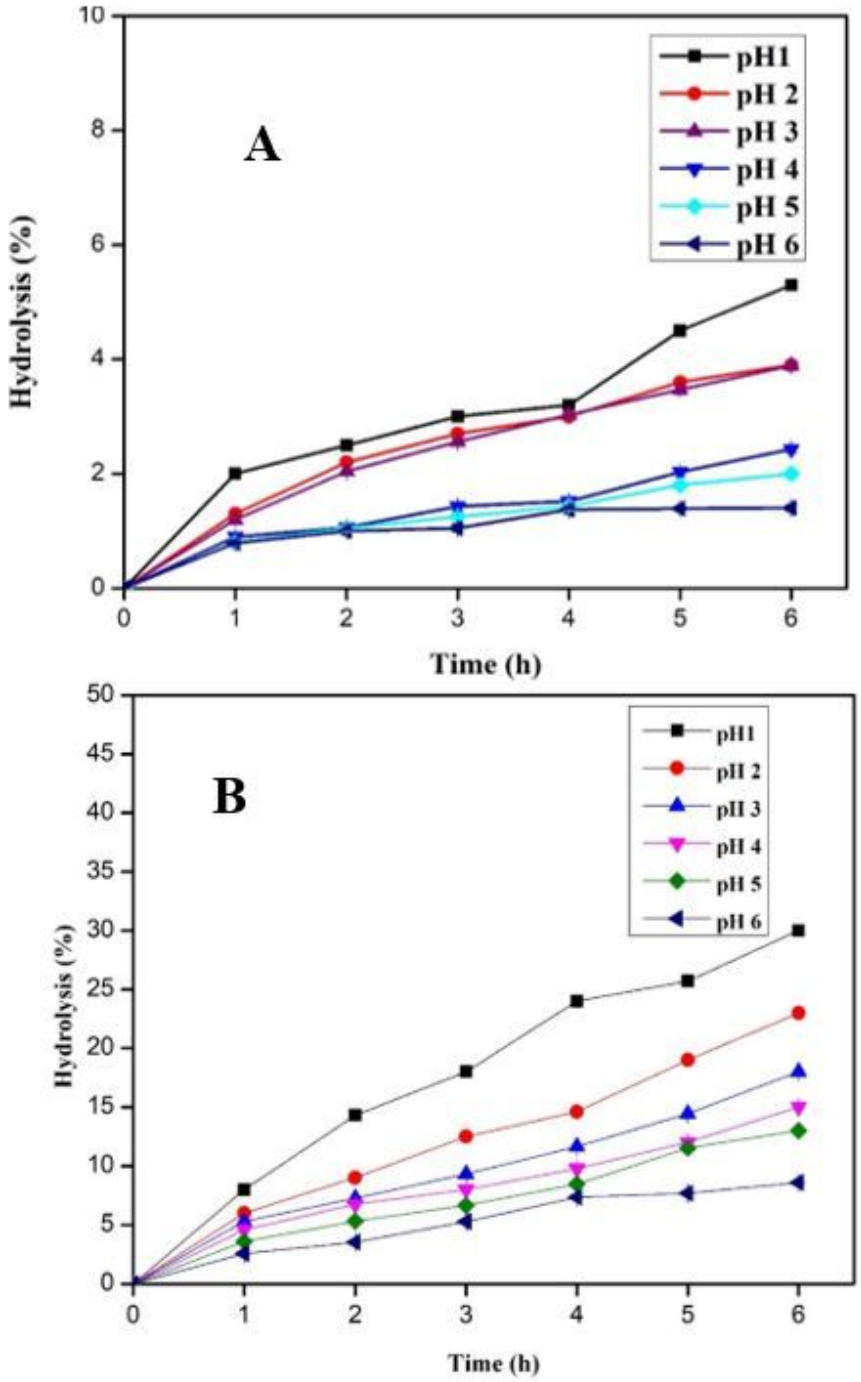
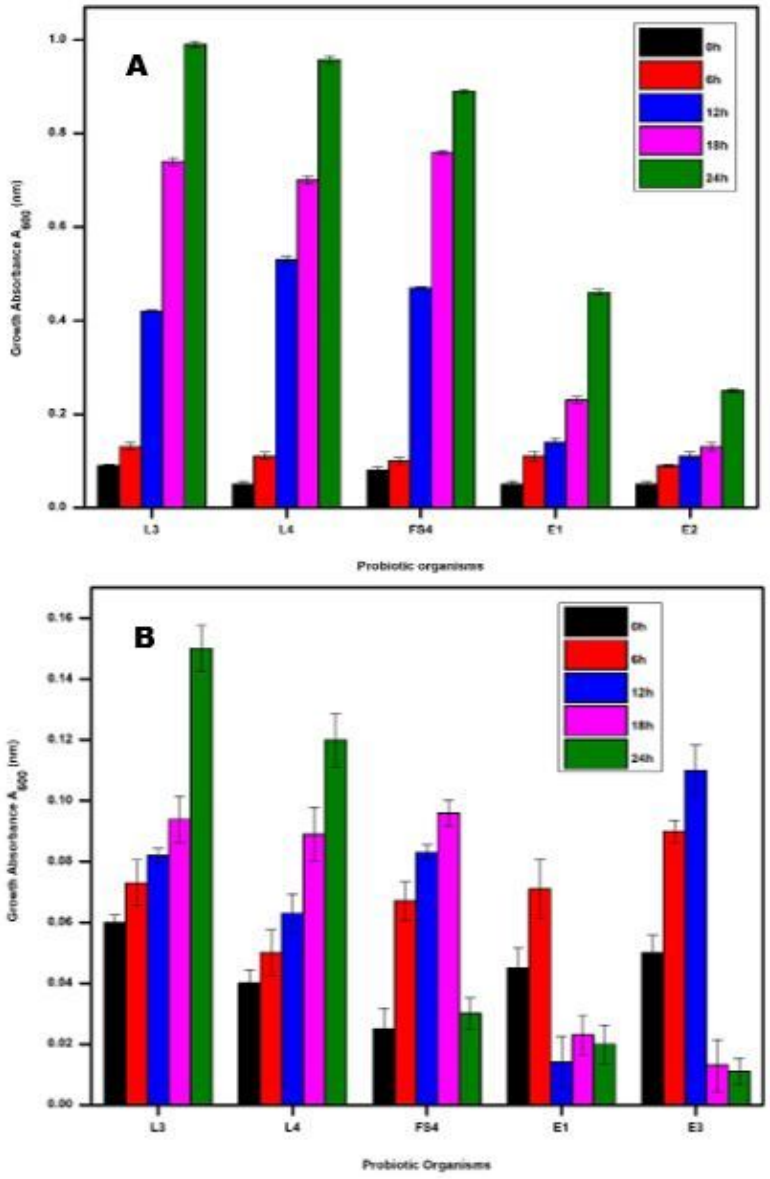


Figure 8

Acid Indigestability of XOS



A Prebiotic efficacy of XOS

B Prebiotic efficacy of Inulin

L3 *Lactobacillus plantarum* **MT228948**

L4 *Lactobacillus fermentum* **MT230901**

FS4 *Bacillus amyloliquefaciens* **MT193292**

E1 *Bacillus clausii* **MN658363**

E3 *Enterococcus faecium* **MN956828**

Figure 9

Prebiotic efficacy of XOS produced from sugarcane bagasse and Inulin (Commercial prebiotic)

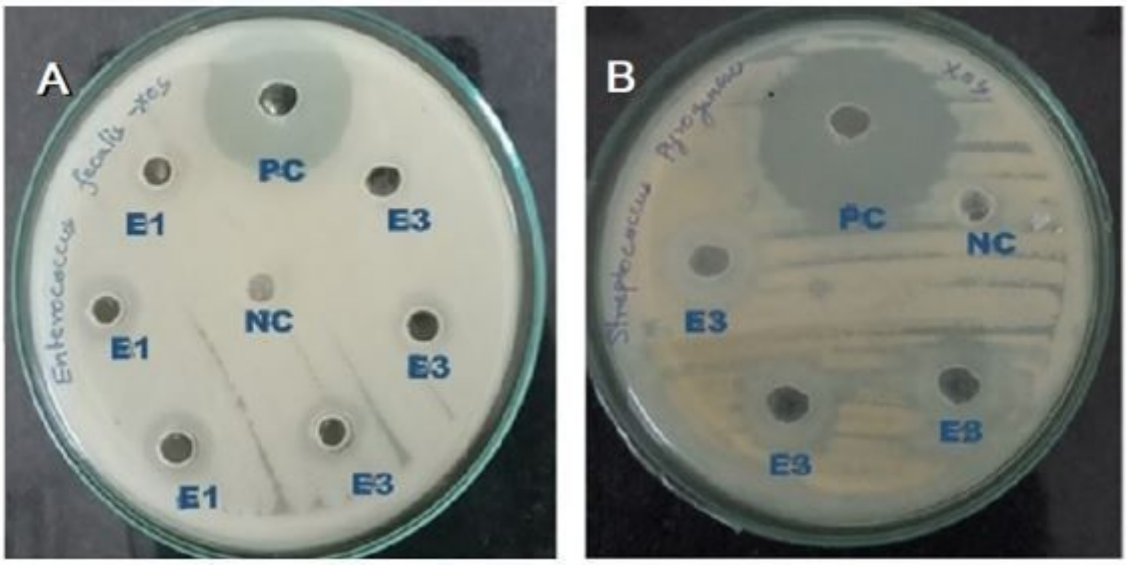


Figure 10

Bacteriocin activity from probiotic bacteria grown in prebiotic substituted medium against human pathogens Note: A and B probiotic organisms against Enterococcus fecalis and Streptococcus pyogenes, E1 Bacillus clausii MN658363; PC Positive Control; E3 Enterococcus faecium MN956828; NC Negative Control

Supplementary Files

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- Supplementary.docx