OPTIMIZATION OF XYLAN EXTRACTION PROCESS FROM RICE STRAW FOR PRODUCTION OF AUTOHYDROLYSATES RICH IN PREBIOTIC XYLOOLIGOSACCHARIDES

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Rice straw, an abundant agricultural waste, possesses immense potential to serve as renewable, eco-friendly and non-edible feedstock to generate value-added products. Therefore, the present study aimed to obtain prebiotic neutral xylooligosaccharides (XOS)-rich autohydrolysate from rice straw xylan. The central composite design of response surface methodology was employed to optimize the conditions for the alkaline extraction of xylan, *i.e.* NaOH concentration (6-14%, w/v), reaction time (1-3.5 h) and temperature (50-100 °C). Autohydrolysis of xylan was carried out at 121 °C and 15 psi for varied hydrolysis times (10, 25 and 40 min) and sulphuric acid concentrations (0.1, 0.5 and 1.0M) to obtain XOS-rich autohydrolysate. The optimum conditions were found to be as follows: 11.04% (w/v) NaOH, 3.126 h and 80.146 °C, so that the maximum xylan yield of 19.97% was predicted by the software. This value was quite close to the experimental yield of 19.4%, with 80.83% xylan being recovered per gram of rice straw. The best autohydrolysis treatment for xylan was found to be using 0.1M sulphuric acid for 10 min, which allowed 34.5% of 100 mg xylan to be depolymerized to produce neutral XOS (degree of polymerization up to 7), with xylose, xylobiose and xylotriose constituting 4.45, 10.14 and 7.83 mg, respectively. These autohydrolysates promoted higher growth of *Lactobacillus rhamnosus* and *Lactobacillus casei* than established prebiotic fructooligosaccharides. The study attempts to solve disposal issues of rice straw through production of XOS-rich autohydrolysates in demand on the global nutraceuticals market.

Keywords: rice straw, xylan extraction, optimization, response surface methodology, autohydrolysis, prebiotic xylooligosaccharides

INTRODUCTION

The rice–wheat cropping system is the most widely adopted cropping system in India, but the short window period between the harvest of rice and the cultivation of wheat makes the management of rice straw a major issue. Although rice straw can be used as fuel, organic fertilizer, fodder for livestock, packaging material, raw material for pulp and paper industries, none of these seems to offer a profitable outcome. As a result, farmers most commonly burn approximately 70-80 million tonnes of paddy straw per year to reduce the turnaround time between harvesting the first crop and sowing the second one.[1] Inefficient burning of straw emits greenhouse gases, such as carbon dioxide, nitrous oxides, methane *etc.*, releases smoke and soot particles, causing pollution[2] and triggering global warming, and damages bulk minerals, such as N, P and S, adversely impacting soil properties. Therefore, finding a use for the generated rice straw is of utmost importance.

According to FAOSTAT (2018-19),[3] India is among the top ten global rice producing countries and has the largest harvested area, *i.e.* 44,500,000 hectares under rice, with production of about 172,580,000 tonnes. Every tonne of rice results in production of 1.5 tonnes of straw. Rice straw, an abundant lignocellulosic feedstock, is composed of three structural polymers: cellulose, hemicelluloses and lignin (arranged in the form of a complex network), and non-structural components: extractives and ash (mainly silica). It contains 19-32% hemicelluloses represented by *L*-arabino (4-*O*-methylglucurono) xylan.[4]

Xylan has high potential for use in packaging films and on diagnostic equipment, which is of

high interest in biomedical research.[5] It also finds application as an emulsifier and protein foam stabilizer for processing at high temperatures in the food industry. Apart from these, xylan is also used for production of nanoparticles, thus aiding drug delivery.[6] Research has also been carried out on corn cob xylan for its use as drug carrier for the development of microparticles for colon specific delivery of anti-inflammatory and toxic drugs. Moreover, it finds use in the pulp or paper industry to produce paper with higher tensile strength.[7] Further, the hydrolysis of xylan yields xylooligosaccharides (XOS), which may contain two to nine xylose units associated by the usual β-(1,4) glycosidic linkage. Therefore, xylan serves as an intermediate between the rice straw and XOS.

Different methods have been used for extracting hemicellulosic xylan: *i.e.* alkaline extraction,[8] alkaline peroxide extraction,[9] organic solvent extraction,[10] ultrasound-assisted extraction,[11] steam explosion,[12] autohydrolysis,[13] microwave-assisted extraction,[14] ionic liquid extraction[15] *etc*. The alkaline extraction method is one of the most suitable, well-studied and efficient methods for extracting the hemicellulosic fraction of biomass due to its lower operation cost, comparatively milder reaction conditions and lower structural degradation of the obtained hemicelluloses;[8,16] however, appropriate delignification is necessary to obtain high recovery and yield of hemicelluloses.[17]

The valorisation potential of lignocellulosic materials has significantly attracted the attention of researchers from the past decade throughout the world, with aim to develop methods for adding value to waste by its conversion to biofuels, biopolymers, platform chemicals *etc*. Another important and recent valorization route is the production of prebiotic oligosaccharides. Prebiotics are defined as "non-digestible dietary fibres or oligosaccharides that selectively stimulate the growth and/or activity of one or more gastrointestinal bacteria, thereby improving host health".[18] The gut bacteria (*Lactobacillus* and *Bifidobacterium* species) have enzymes that ferment non-digestible ingredients, resulting in the formation of short chain fatty acids, which ultimately serve as fuel to different tissues and regulate various cellular processes in the body.[18] Prebiotics reduce cholesterol levels in blood, destroy carcinogenic enzymes present in the gastrointestinal tract, facilitate mineral uptake and absorption, improve bowel movements, prevent diarrhea and constipation; thus, improving gastrointestinal health and the immune system.[19] Stowell (2008)[20] classified prebiotics as existing prebiotics, *i.e.* fructooligosaccharides (FOS), galactooligosaccharides, inulin, lactulose *etc*., and emerging prebiotics, *i.e*. isomaltooligosaccharides, XOS, lactitol *etc*. XOS, produced by the hydrolysis of hemicellulosic xylan-rich lignocellulosic feedstock, have been increasingly gaining attention. They carry high potential for usage in the food and pharmaceutical industries as nutraceuticals, providing health benefits and thereby increasing the market demand. XOS also find use as food additives and exhibit an enormous range of biological activities, such as enhancement of growth of advantageous bacteria, calcium uptake and absorption, along with antimicrobial and anti-infection properties.[21]

The method of extraction and the nature of the agricultural residues would affect the degree of polymerization, basic structural units and the nature of the linkages present in XOS.[22] XOS can be produced from various xylan-rich biomass sources by chemical,[23-25] physiochemical[26,27] and by a combination of chemical and enzymatic methods.[17,28,29] Enzymatic methods, although specific, are costly, time-consuming and require specific reaction conditions for storage and handling of enzymes; therefore this approach is preferred only on an industrial scale.[17] On the other hand, physiochemical methods (autohydrolysis and steam explosion), involving direct exposure of biomass, form large amounts of side-products, monosaccharides, along with undesirable dehydration products, so that a series of purification steps are carried out to achieve the highest XOS content.[22]

The literature survey conducted in this study revealed studies investigating dominant factors for optimizing the alkaline extraction process of hemicelluloses from various sources, including poplar,[30] rice husk,[31] grape pomace,[11] birch wood[32] and sugarcane bagasse,[9] using response surface methodology (RSM); however, no work has been found to report on optimizing the hemicellulosic xylan extraction process from rice straw using the central composite design (CCD). XOS have been synthesized from tobacco stalk,[33] rye straw,[34] Bengal gram husk and wheat bran,[18] wheat straw,[35] corncobs,[36] hazelnut shell[26] and sugarcane bagasse[37] for evaluation of their prebiotic potential; however, limited work is available on the prebiotic potency of rice straw-based XOS in the context of human gut bacteria

(*Lactobacillus rhamnosus* and *Lactobacillus casei*).[38]

The present study has been intended to optimize the hemicellulosic xylan yield from rice straw, using the alkaline extraction method. The obtained xylan was autohydrolysed under varied reaction time and acid concentration to obtain XOS-rich hydrolysate, which was then evaluated for significant prebiotic potency.

EXPERIMENTAL

Raw material

Rice straw of the variety Pusa Basmati 1121 was dried (40-50 °C), milled and passed through a 1 mm sieve to obtain a homogeneous powdered material, which was stored in plastic bags at room temperature in an air-tight container till further use.

Reference standards and other materials

Xylose, xylobiose, xylotriose and fructooligosaccharides (FOS) were purchased from Sigma-Aldrich, St. Louis, MO, USA. All other reagents, chemicals and solvents (analytical research grade) employed in the study were purchased from Molychem (Mumbai, India). Filtrations were carried out using Whatman Grade 91 filter paper obtained from Whatman International Ltd., Maidstone, England. Precoated silica gel (60 F_{254}) thin layer chromatography (TLC) aluminium sheets were obtained from M/S Merck, Darmstadt, Germany.

Bacterial cultures of *Lactobacillus rhamnosus* (ATCC 530103) and *Lactobacillus casei* (ATCC 393) were procured from Pulses Microbiology Laboratory, Pulses Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. *Lactobacillus* MRS (Man Rogosa Sharpe) agar and broth were purchased from HiMedia Laboratories Private Limited, Mumbai, India.

Determination of chemical composition of rice straw

The chemical composition of rice straw, *i.e.* percent contents of cellulose, hemicelluloses, lignin, extractives and acid insoluble ash or silica, was determined by the Detergent System method.[39]

Extraction of hemicellulosic xylan from rice straw

Finely powdered rice straw material was treated with warm distilled water to remove dirt and water soluble impurities. The dirt-free material was partially delignified by the treatment with a mixture of 4% NaOH and 96% ethanol (3:7, v/v) with a solid to liquid ratio of 1:30 (w/v), in accordance with thr method proposed by Farhat *et al*. (2017).[8] The partially delignified material was then subjected to alkaline extraction using NaOH (6-14%, w/v), with a solid to liquid ratio of 1:25 (w/v) and stirred continuously at 50-100 °C. After 1-3.5 h, the reaction mixture was vacuum filtered to obtain black liquor. The pH of the liquor was adjusted to 8.5 using 6M HCl to precipitate sodium silicate. After that, the pH of the resulting supernatant was adjusted to 5.5 using 6M HCl, followed by the addition of cold ethanol and the mixture was allowed to stand at room temperature overnight to complete the precipitation of hemicellulosic xylan.[40] Xylan was separated by centrifugation at 4000 rpm for 15 min, washed thoroughly with ethanol and finally dried in an oven at 80-90 °C for about 5 h. The yield (%) and recovery (%) of the extracted xylan were calculated.

Optimization of alkaline extraction process of hemicelluloses using RSM

Experimental design

The CCD of RSM was employed from Design-Expert® software (version 12.0.12.0, Stat-Ease, Inc., Minneapolis)[41] for designing the xylan extraction experiment with NaOH concentration, reaction time and temperature as three independent variables and the yield of hemicelluloses as response. The ranges of NaOH concentration (%, w/v), reaction time (h) and temperature (°C) to maximize the yield (%) of hemicellulosic xylan were set as given in Table 1. Further selection of the face centred design in CCD fixed the value of α (the distance of the axial point from the centre) to ±1, where +1 and -1 represent the extreme values of the specified range for each variables and 0 represents the central point.

Table 1
CCD involving three independent variables

Independent variables	Units	Symbols	-1	0	+1
NaOH concentration	%, w/v	(A)	6	10	14
Temperature	°C	(B)	50	75	100
Reaction time	h	(C)	1	2.25	3.5

The total number of experimental runs (N) produced in CCD is given by the following formula:

$$N = 2^n + 2n + n_r \quad (1)$$

where n represents the number of independent variables and n_r corresponds to the number of replicate trials.

Since the present study involves three independent variables, a total of 18 experimental runs were produced with 8 factorial, 6 axial and 4 replicate points

or centre points. Further, the relationship between the independent variables and the response was modelled in terms of the quadratic equation as follows:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{ii}^2 + \sum_{i}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

where Y is the dependent variable or response; X_i, X_j represent independent variables; β_o is intercept; β_i, β_{ii} and β_{ij} are the coefficients, representing linear, quadratic and interaction effects and ε is the error term.

Statistical analysis

The accuracy of the quadratic model was investigated using the analysis of variance (ANOVA). F and p values were used to interpret the significance of model terms. Also, the lack of fit test and the regression analysis (R^2) were used to determine the model accuracy. The 3D interaction plots (response surfaces) and the optimum conditions to maximize the response were produced by the software.

Structural characterization of hemicellulosic xylan

The FT-IR spectrum of xylan was recorded between 4000-450 cm^{-1} on a Perkin-Elmer FTIR Spectrophotometer; ^1H NMR and ^{13}C NMR spectra were obtained in dimethyl sulphoxide (DMSO) solvent on a Bruker Avance Neo 500 MHz NMR spectrometer, using tetramethylsilane as an internal reference standard.

Production of XOS via autohydrolysis of xylan

Xylan powder was suspended in different concentration of dilute sulphuric acid (0.1, 0.5 and 1.0M), maintaining a solid to liquid ratio of 1:50 for variable times (10, 25 and 40 min) at 121 °C and 15 psi. The mixtures were cooled to room temperature and calcium carbonate was added to neutralize excess acid. Then, the mixture was filtered through Whatman filter paper to obtain autohydrolysates, which were then analysed for the presence of XOS, total reducing sugars, phenolic compounds and furfural.

Determination of XOS

The presence of XOS in the autohydrolysate was qualitatively analyzed using TLC, followed by quantification using high performance thin layer chromatography (HPTLC) (M/s CAMAG, Switzerland). Fine spots of standards (xylose, xylobiose and xylotriose) and xylan hydrolysates were applied on silica gel 60 F_{254} TLC plates (20 cm × 20 cm). The plates were developed using acetonitrile:water (9:1, v/v) as mobile phase and the spots were visualized by spraying orcinol reagent (0.2% w/v) prepared in methanol:sulphuric acid (9:1, v/v). The amounts of xylose, xylobiose and xylotriose in the autohydrolysate were quantified through comparison with the peak areas of the calibration curves of the standards used in the study.

Determination of phenolic compounds

The presence of phenolic compounds in the autohydrolysate was tested by the appearance of dark bluish green colour on addition of a few drops of ferric chloride solution (5%, v/v).[42]

Determination of furfural

The presence of furfural, produced by acid dehydration of pentoses, was detected by the formation of red colour on addition of aniline acetate solution, which was analysed spectrophotometrically at 580 nm.[43]

Determination of total reducing sugars

The total reducing sugars in the xylan hydrolysate were determined using the method reported by Miller (1959),[44] making use of dinitrosalicylic acid reagent. The formation of a reddish brown complex was measured spectrophotometrically at 540 nm on a UV-1800 SHIMADZU UV-VIS spectrophotometer. The concentration of reducing sugars in the xylan hydrolysate was calculated from the standard curve of xylose (1-10 mg/mL).

Degree of polymerization of XOS

The degree of polymerization of XOS, in the autohydrolysate, was recorded on a Waters Q-TOF MICROMASS (ESI-MS) system.

Determination of prebiotic potential of xylan hydrolysate

Pure cultures of *Lactobacillus* species were grown on MRS medium slants stored at 3 °C in the refrigerator. The method reported by Samanta *et al.* (2012),[36] with slight modification, was used to determine the prebiotic potency of XOS. The culture of test bacteria (1%, v/v) in triplicates was added to the MRS broth supplemented with 1% xylose/xylobiose/xylotriose/FOS/XOS samples (produced by autohydrolysis of xylan). Serial dilutions for each of the above prepared samples were carried out until a 10^{-5} dilution of the sample was obtained and spread on medium plates prepared by pouring sterilized MRS medium. The plates were incubated at 37 °C for 24 hours in a biochemical oxygen demand incubator. The prebiotic potency of XOS was determined by enumerating colony forming units (CFU)/mL, by dividing the mean number of colony forming units with the dilution factor for probiotic species after 24 h of incubation. The logarithmic value of CFU/mL was taken for representing data results with ease.

Statistical analysis

The experiment was performed in triplicates and values were represented as mean ± standard deviation. The analysis of variance appropriate in completely randomized design was employed for data analysis using CPCS1 and CD (5%) was calculated.

RESULTS AND DISCUSSION

Chemical composition of rice straw

The amounts of cellulose, hemicelluloses, lignin, extractives and silica in the rice straw were found to be 41 ± 0.4%, 24 ± 0.6%, 9 ± 0.5%, 20 ± 0.8% and 6 ± 0.3%, respectively. The result was supported by Khandanlou *et al*. (2013),[4] who reported rice straw to be composed of 32-47% cellulose, 19-32% hemicelluloses, 5-24% lignin and 13-17% ash. Satlewal *et al*. (2018)[45] also reported 32-44% cellulose, 19-22% hemicelluloses, 6-23% lignin and 8-20% ash content in rice straw.

Extraction of hemicellulosic xylan from rice straw

The alkaline extraction method was employed for extracting hemicelluloses with a high degree of polymerization and with high yields. Alkali causes swelling of cellulose, thus decreasing its crystallinity, breaks the intermolecular hydrogen bonds between cellulose and hemicelluloses, and cleaves the ester linkages between lignin and hemicelluloses; thereby, causing dissolution of hemicelluloses and lignin into the solution. The presence of lignin affects the extraction yield and the purity of the resulting hemicellulosic xylan from rice straw. As a result, partial delignification of rice straw was carried out using a mixture of 4% NaOH and ethanol. Although both hemicelluloses and lignin solubilize in alkali, the use of the mixture of alkali and ethanol for delignification would selectively dissolve lignin; thereby, resulting in its removal.

Optimization of alkaline extraction process of hemicelluloses using RSM

Experimental design

RSM was employed as it saves time, by reducing the number of experimental runs, and effectively explains both the individual effect of variables on the response and the interactive dependency between variables. Table 2 presents the CCD design for hemicellulosic xylan extraction, showing the yield of xylan obtained after the experiment.

Table 2
CCD and results for experimental yield of hemicellulosic xylan

Runs	Factors			Response
	A	B	C	Y
1	6	50	3.50	10.9
2	6	75	2.25	13.4
3	6	100	3.50	12.3
4	6	50	1.00	9.7
5	6	100	1.00	11.9
6	10	75	2.25	19.8
7	10	75	1.00	18.1
8	10	75	3.50	19.8
9	10	75	2.25	19.7
10	10	100	2.25	18.7
11	10	75	2.25	19.5
12	10	50	2.25	16.8
13	10	75	2.25	19.7
14	14	100	1.00	15.7
15	14	75	2.25	17.0
16	14	50	1.00	12.7
17	14	100	3.50	16.2
18	14	50	3.50	14.1

Development of regression model equation for hemicellulose extraction process

The mathematical relation showing the relation of three selected factors, *i.e.* A, B and C, with response Y, for alkaline extraction of hemicellulosic xylan from rice straw, developed for the model, is as follows:

$$Y = 19.60 + 1.75*A + 1.06*B + 0.52*C + 0.1875*AB + 0.0375*AC - 0.2125*BC - 0.413A^2 - 1.78*B^2 - 0.5786*C^2 \quad (3)$$

Statistical analysis and authentication of model

The analysis of variance (ANOVA) was employed to study the impact of factors on the

yield of hemicelluloses (Table 3). The results of the analysis were interpreted in terms of F-test or Fisher's test, p or probability for validating the regression model. In general, the larger value of F makes the model significant (when compared to the model with no independent variables), whereas the smaller value of p (less than the level of significance) makes the model terms significant. Also, a higher value of F for a parameter indicates the greater effect of that parameter on influencing the response.

The F value of 418.08 for the model suggested that the model is significant and there is only 0.01% chance that this large value occurred due to noise. The value of F was found to be maximum for NaOH concentration (579.21), followed by temperature (212.5) and reaction time (51.14); therefore, the concentration of NaOH was found to influence the yield to the largest extent, followed by temperature and time. This was also clear from the perturbation plot (Fig. 1), where the slope for factor A, *i.e.* NaOH concentration, was the steepest, followed by temperature (B) and then reaction time (C). Also, the P value less than 0.05 (level of significance) indicated that the terms A, B, C, AB, BC, A^2, B^2 and C^2 are all significant.

Table 3
Statistical analysis for computed hemicellulosic xylan yield

Source	Sum of squares	Df	Mean square	F-value	p-value	
Model	198.95	9	22.11	418.08	< 0.0001	significant
A-NaOH concentration	30.62	1	30.62	579.21	< 0.0001	significant
B-Temperature	11.24	1	11.24	212.50	< 0.0001	significant
C-Reaction time	2.70	1	2.70	51.14	< 0.0001	significant
AB	0.2813	1	0.2813	5.32	0.0500	significant
AC	0.0112	1	0.0112	0.2128	0.6569	not significant
BC	0.3612	1	0.3612	6.83	0.0309	significant
A^2	50.77	1	50.77	960.20	< 0.0001	significant
B^2	8.57	1	8.57	162.11	< 0.0001	significant
C^2	0.9071	1	0.9071	17.15	0.0032	significant
Residual	0.4230	8	0.0529			
Lack of Fit	0.3702	5	0.0740	4.21	0.1333	not significant
Pure Error	0.0528	3	0.0176			
Cor. Total	199.37	17				
Fit statistic parameters						
R^2					0.9979	
Adjusted R^2					0.9955	
Predicted R^2					0.9859	
Adeq. Precision					58.3652	

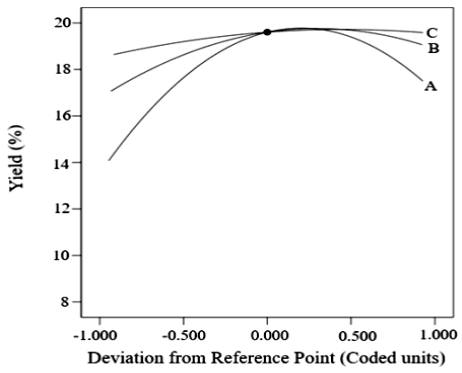


Figure 1: Perturbation plot for the model – generated in Design Expert Software

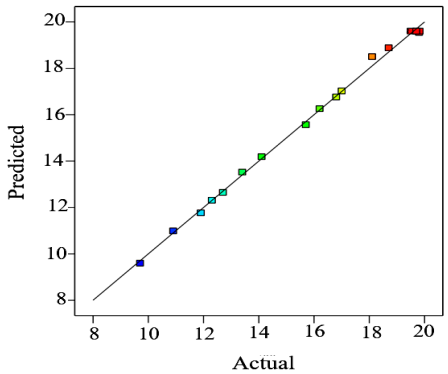


Figure 2: Predicted *vs*. actual yield of hemicellulosic xylan – generated in Design Expert Software

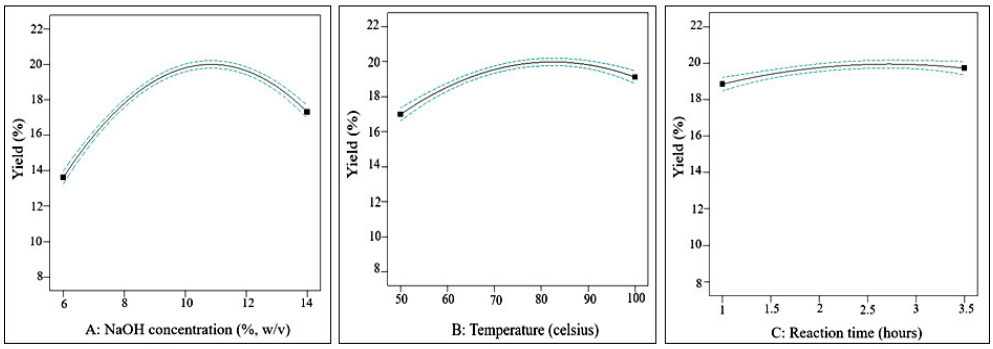


Figure 3: Effect of (a) NaOH concentration, (b) temperature and (c) reaction time on yield of hemicellulosic xylan – generated in Design Expert Software

The lack of fit F-value of 4.21 implies that it is non-significant, so that there is only 13.33% chance that the lack of fit might occur due to noise. Thus, the results suggested that the model is applicable for alkaline extraction of hemicelluloses from rice straw.

The interrelation between the predicted values (generated through computation) and the actual values (observed in experiments) is shown in Figure 2. It suggests that the majority of obtained experimental results agree well with the predicted values for the yield of hemicellulosic xylan. Also, Table 3 demonstrates the fit statistics, *i.e.* the deviation between the observed values and the predicted values for the model. The coefficient of determination (R^2) measures the degree of fitness for the data in the model. In the present study, the R^2 value of 0.9979 implies that the regression model represents the experimental data well. Moreover, the predicted R^2 of 0.9859 is in reasonable agreement with the adjusted R^2 of 0.9955, *i.e.* the difference is less than 0.2. Adequate precision measures the signal to noise ratio, where the ratio greater than 4 is desired for a good fit. The adequate precision in the present study is 58.3625, thereby indicating an adequate signal.

Impact of individual parameters on yield of hemicellulosic xylan

NaOH concentration

The yield (%) of hemicellulosic xylan was found to increase with the increase in NaOH concentration from 6 to 11% (Fig. 3a). This is because the hydroxide ions cause swelling of cellulose; cleave intermolecular hydrogen bonds between hemicelluloses and cellulose; and partially or completely cleave the ester linkages between hemicelluloses and lignin; thereby, causing dissolution of hemicelluloses into the solution.[8] As the concentration of NaOH was increased beyond 11%, a decrease in yield (%) occurred because of the degradation of hemicelluloses at the reducing ends and the removal of acetyl groups, resulting in the production of low molecular weight hemicelluloses. As a result, a NaOH concentration within 11% was preferred for the extraction.

Temperature

The temperature too had a significant impact on the yield (%) of hemicellulosic xylan. With the increase in temperature from 50 to 85 °C, an increase in the extraction yield was observed. This could be explained by the fact that higher temperature decreases the activation energy for the extraction, so that higher yields are obtained in a shorter time.[16] However, an increase in temperature beyond 81 °C resulted in decreased yield of hemicelluloses (Fig. 3b). This might be caused by the degradation of hemicellulosic xylan.

Reaction time

The yield (%) of hemicellulosic xylan was found to increase with an increase in reaction time (although the increase was small). This may be attributed to the fact that increased reaction time would increase the interaction time of rice straw with alkali; thereby, resulting in increased dissolution of hemicelluloses into the solution.[16] The reaction time was found to affect the hemicellulose yield (%) only up to 3 hours, after which there was no significant increase in the extraction yield (Fig. 3c), but prolonged extraction may induce structural changes in hemicelluloses.[9]

Impact of combined parametric interactions on yield of hemicelluloses

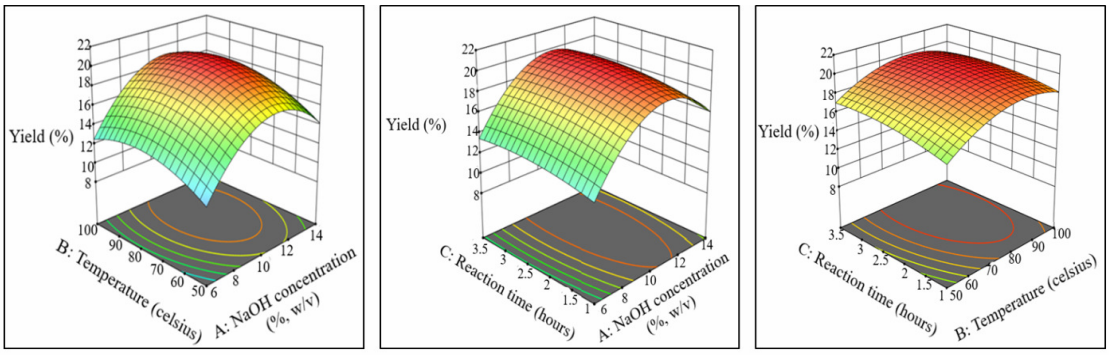
3D response plots, obtained from the central composite design, indicated variation of the response in simultaneous correlation with two independent variables, when the third variable is kept constant. The results of the interaction were interpreted below.

Combined effect of NaOH concentration and temperature

When the NaOH concentration was low (6%, w/v), the yield of hemicellulosic xylan increased with the temperature, even though the change was small. This was due to the fact that the increase in temperature decreased the activation energy barrier, so that the desired yield of hemicelluloses is obtained in a shorter time, but the smaller effect of temperature indicated that higher concentration of NaOH was still instrumental for breaking H bonds and covalent bonds, so that the hemicellulosic portion gets into the solution state. Also, when the concentration of NaOH was increased beyond the optimum level (11.04%, w/v), the temperature could not have a significant impact on the yield since degradation of the hemicellulosic xylan structure occurred at higher concentrations. This indicated that NaOH concentration was more influential, affecting the yield of hemicelluloses, than the reaction temperature (Fig. 4a).

Combined effect of NaOH concentration and reaction time

The effect of NaOH concentration and reaction time is not significant, as already analysed from ANOVA data (Table 3). It is also clear from Figure 4b that, when we move along the reaction time axis, parallel lines are encountered, indicating no significant change in the yield of hemicelluloses with changes in reaction time; however, when we move along the NaOH concentration axis, even a small change affects the yield of hemicellulosic xylan. This could be explained by the fact that when the concentration of NaOH is low, the contact time or the reaction time could not affect the yield or dissolution of hemicelluloses until NaOH concentration is increased for effective bond cleavages to occur. Also, at high NaOH concentrations (beyond 13%, w/v), the yield of hemicelluloses is not affected by time, as firstly, almost all of the hemicelluloses have been extracted and secondly, higher concentrations had resulted in structural decomposition of the hemicelluloses in solution.



(a) (b) (c)

Figure 4: 3D response surfaces indicating the combined effect of (a) NaOH concentration and temperature, (b) NaOH concentration and reaction time, and (c) temperature and reaction time when reaction time, temperature and NaOH concentration, respectively, were kept constant – generated in Design Expert Software

Combined effect of temperature and reaction time

It is clear from Figure 4c that, for a particular temperature, the increase in reaction time increased the yield of hemicelluloses (to a small extent). On the other hand, when we move along the temperature axis, a significant change is observed on the hemicellulose extraction yield for constant NaOH concentration, since the activation energy for the reaction significantly decreased with increasing temperature. However, at higher temperature, time didn't affect the hemicellulosic yield much, since evaporation of water decreases the interaction of straw with NaOH.

Validation of model

In the present study, the aim was to optimize the reaction conditions for obtaining maximum hemicellulosic xylan yield from rice straw. The optimum conditions for hemicellulosic xylan extraction obtained through the central composite design were: 11.04% NaOH, 3.126 hours and 80.146 °C. Under these conditions, 19.97% yield of hemicelluloses was obtained, with a desirability of 1.00, as predicted by the software. Further, the extraction of hemicelluloses was carried out under these predicted conditions for verifying the observed value, compared with the predicted yield. The hemicellulosic xylan yield under these conditions came around 19.4 ± 0.56 (for triplicates), which was found to be in close association with the predicted value. Therefore, the recovery (%) of hemicellulosic xylan from rice straw was observed to be 80.83%. García *et al*. (2013)[46] obtained 56.1% of total hemicelluloses from wheat straw through cold alkaline extraction, while 83.5% recovery of xylan was reported through alkaline extraction of corn cobs using 12% NaOH.[36] Lian *et al*. (2020)[47] reported the recovery of about 87.45% xylan by using the alkaline extraction method from biomass.

Structural characterization of alkali extracted xylan

Xylan forms hydrogen bonds with cellulose microfibrils, covalent bonds with lignin and ester linkages with various phenolic or hydroxycinnamic acids and acetyl groups, thus making its isolation difficult.[48] The major constituent of hemicelluloses extracted from rice straw is xylan.[49] The structural characterization of alkali extracted hemicelluloses is presented below.

FT-IR analysis

The stretching of the hydroxyl groups of xylan was observed at 3434 cm^{-1}; the C-H stretching vibrations gave a signal around 2926 cm^{-1}. The CH_2 bending vibration gave a signal at 1248 and 1210 cm^{-1}, while the band at 1339 cm^{-1} corresponds to O-H bending vibrations respectively.[50] The bands at 1562 cm^{-1} and 1413 cm^{-1} were attributed to asymmetric and symmetric stretching of uronic acid carboxylate.[51] The presence of arabinosyl side chains was indicated by the bands of low intensity at 1162 and 928 cm^{-1}.[52] A prominent band at 1046 cm^{-1} resulted from C-O-C stretching in the xylopyranose ring skeleton; the band at 1078 cm^{-1} is characteristic of C-OH stretching of alicyclic secondary alcohol in the 6-membered ring; a sharp band was observed at 898 cm^{-1}, which corresponds to β-configuration of the (1,4) glycosidic bond between the xylopyranose units forming the xylan backbone.[51] The band at 1640 cm^{-1} was attributed to absorbed water molecules, as hemicellulosic xylan has strong affinity for water. The absence of a band around 1550 cm^{-1} indicates the absence of lignin bound to xylan.[40] Also, the absence of a band around 1740-1715 cm^{-1} indicates the removal of the acetyl or carbonyl group during the xylan extraction procedure.

^{1}H-NMR analysis

The peaks between 4.3 to 3.1 ppm correspond to equatorial and axial protons of the xylose subunit in the xylan backbone.[53] A prominent peak observed around 4.2 ppm is characteristic of the β-glycosidic linkage in the D-xylose unit of the xylan backbone.[54] The anomeric proton of β-D-xylopyranose was observed at 4.8 ppm, while the signal for the anomeric proton of α-1,3 linked-L-arabinofuranose unit was observed at 5.1 ppm.[55] The signal around 5.3 ppm indicates the presence of α-1,2 linked glucuronic acid residue.[56] The absence of a signal at 7 ppm indicates no associated lignin. The solvent peak of DMSO was observed around 2.5 ppm.

^{13}C-NMR analysis

β-D-xylopyranose units show five strong signals at 102.45, 75.91, 75.10, 73.42 and 63.32 ppm, which are assigned to C1 to C5 position of ring carbons. The sharp signal at 40.6 corresponds to the solvent peak of DMSO. A weak signal observed at 174 ppm, which corresponds to the carboxylic group at C-6 of 4-*O*-methylglucuronic acid side unit.[46] Also, the absence of a signal around 125-150 ppm confirmed the removal of lignin during the extraction process.

Therefore, the structure can be defined as *L*-arabino (4-*O*-methylglucurono) xylan. Also, it can be concluded from the structural data obtained that the alkaline extraction method effectively cleaved the ester linkages between hemicelluloses and lignin, along with the acetyl group, resulting in the structure shown in Figure 5.

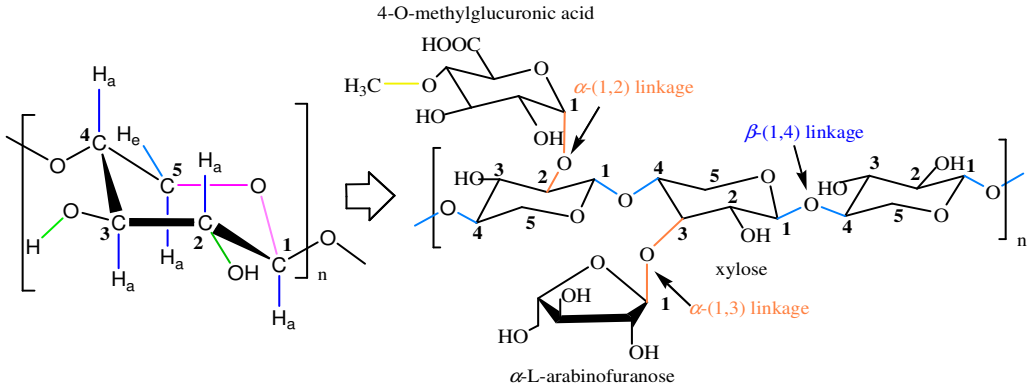


Figure 5: Predicted structure of hemicellulosic xylan – made in ChemDraw Ultra 8.0

Production of XOS via autohydrolysis of xylan

XOS have recently emerged as important substrates for the nutraceuticals industry, due to their stability over a wide range of temperature and pH, and their biological potential to serve as antioxidant, prebiotic, anticancer, anti-viral, anti-inflammatory agent *etc*.[57]

Autohydrolysis of alkali extracted xylan was carried out for production of XOS-rich autohydrolysate.

The production of XOS from rice straw was carried out in a two-step process. About 194 mg xylan was extracted from 1 g rice straw, of which 100 mg was autohydrolysed in 5 mL of distilled water at variable acid concentration and hydrolysis time. A total of 9 treatments (T1-T9) were studied to observe the effect of sulphuric acid concentration (0.1, 0.5 and 1.0 M) and time (10, 25 and 40 min) on autohydrolysate concentration (Table 4). T1 resulted in the dissolution of 34.5 mg xylan in the autohydrolysate, indicating that only 34.5% xylan was degraded into oligomers, monomers or by-products to obtain the autohydrolysate, so that the concentration of autohydrolysate was 6.9 mg/mL. When the reaction time for autohydrolysis was increased to 40 min at the acid concentration of 0.1M (T3), an increased dissolution of 39 mg xylan was achieved, *i.e.* 39% xylan was depolymerized and the obtained concentration was 7.8 mg/mL. The possible reason for the increase in the autohydrolysate concentration could be higher degradation and hence the dissolution of xylan in the autohydrolysate with hydrolysis time. Similarly, the concentration of autohydrolysate was found to increase with acid concentration, since it facilitated the cleavage of glycosidic bonds in xylan; thereby, bringing a portion of XOS into the autohydrolysate, so that a dissolution of 40% xylan was attained in the present study. Lian *et al*. (2020)[46] achieved a dissolution of about 48.37% of xylan in an autohydrolysate from biomass. The lower value of xylan degradation could be explained by the fact that the maximum amount of hemicellulosic xylan that could be degraded at 121 °C and 15 psi was attained.

Table 4
Concentration of autohydrolysate of xylan subjected to variable autohydrolysis conditions

Treatments		Concentration of autohydrolysate (mg/mL)
0.1M H_2SO_4	10 min (T1)	6.9
	25 min (T2)	7.3
	40 min (T3)	7.8
0.5M H_2SO_4	10 min (T4)	7.5
	25 min (T5)	7.9
	40 min (T6)	8.0
1.0M H_2SO_4	10 min (T7)	7.8
	25 min (T8)	8.0
	40 min (T9)	8.0

Further changes in severity conditions, *i.e.* acid concentration and hydrolysis time, could only affect the composition of the autohydrolysate (amount of monosaccharides, XOS, degradation products *etc*.). The autohydrolysate from each treatment was then subjected to qualitative and quantitative analyses for determining XOS, total reducing sugars, phenolic content and sugar degradation product (furfural).

Qualitative analysis of autohydrolysate

Determination of phenolic acids

The autohydrolysates from all the treatments showed no colour change with the addition of $FeCl_3$, indicating the absence of phenolic moiety. The structural characterization of xylan already proved the absence of the aromatic benzene ring, since no band or signal corresponding to it was observed.

Determination of XOS

Spots of hydrolysates (T3, T6 and T9), along with the mixture of xylose, xylobiose and xylotriose, were applied onto TLC plates to qualitatively analyse the presence of XOS. XOS with a degree of polymerization of up to 5 were observed on the TLC plate in T3 (Fig. 6). R_f values for xylose (X), xylobiose (X2), xylotriose (X3) and xylotetraose (X4), when calculated, were found to be 0.65, 0.55, 0.48 and 0.36, respectively. The results were in accordance with Wijaya *et al*. (2020),[58] whose calculated R_f values were 0.61, 0.51, 0.41, 0.33 and 0.26 for xylose, xylobiose, xylotriose, xylotetraose and xylopentaose, respectively, upon using a solvent system of n-butanol, acetic acid and water (2:1:1, v/v).

Quantitative analysis of autohydrolysate

Figure 7 presents the HPTLC plates showing the effect of reaction time and acid concentration on XOS composition for different autohydrolysate treatments (T1-T9). Table 5 gives the amount (mg/mL) of xylose, xylobiose and xylotriose, as quantified using HPTLC, furfural computed through the aniline acetate method, and total reducing sugars determined quantitatively through the dinitrosalicylic acid method. The total reducing sugars included not only the amount of monosaccharides (glucose, arabinose and xylose), but also oligosaccharides, since they have a free hydroxyl group at the anomeric carbon at one end, which makes them reducing in nature.

For T1 autohydrolysate, XOS with a degree of polymerization of 2 to 5 were visible on the HPTLC plate with amounts of xylose, xylobiose and xylotriose as 2.03, 1.57 and 0.89 mg/mL, respectively. When the autohydrolysis time was increased to 25 min (T2), increased amounts of xylose, xylobiose and xylotriose were produced, owing to the increased dissolution of xylan into the autohydrolysate at higher hydrolysis time. However, further increase of hydrolysis time to 40 min (T3) lowered the amount of xylobiose and xylotriose, with a usual increase in the xylose content. The decrease in the content of XOS with the increase in reaction time of hydrolysis beyond a certain limit could be attributed to the degradation of XOS into xylose.[36] This indicated that reaction time had a significant impact upon the composition of the autohydrolysate.

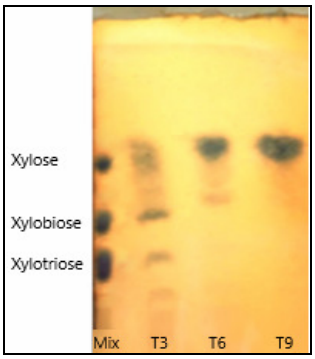


Figure 6: TLC plate with spots of a mixture of xylose, xylobiose and xylotriose and autohydrolysate treatments (T3, T6 and T9)

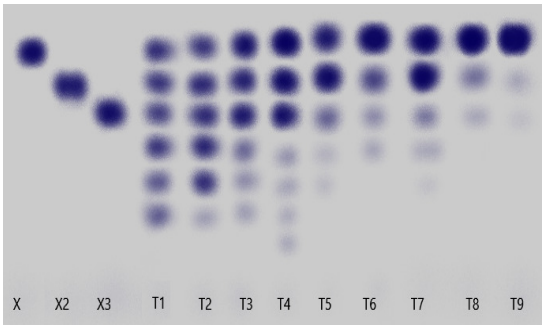


Figure 7: HPTLC plates with spots of standards xylose (X), xylobiose (X2) and xylotriose (X3), along with autohydrolysate treatments (T1-T9)

Table 5
Amount of xylose, xylobiose, xylotriose, furfural and total reducing sugars obtained through different autohydrolysis treatment of rice straw xylan

Treatments	Amount (mg/mL)				
	Xylose	Xylobiose	Xylotriose	Furfural	Total reducing sugars
T1	0.89 ± 0.03	2.03 ± 0.07	1.57 ± 0.02	0.01 ± 0.009	4.59 ± 0.09
T2	1.10 ± 0.04	2.37 ±0.05	1.82 ± 0.01	0.03 ± 0.009	5.19 ± 0.12
T3	1.36 ± 0.09	2.18 ± 0.03	1.55± 0.06	0.04 ± 0.01	5.56± 0.04
T4	1.79 ± 0.05	2.27 ± 0.02	1.19 ± 0.09	0.06 ± 0.02	5.62 ± 0.01
T5	2.11 ± 0.10	1.99 ± 0.08	0.98 ± 0.06	0.07 ± 0.01	5.99 ± 0.08
T6	2.53 ± 0.06	1.54 ± 0.01	0.80 ± 0.04	0.14 ± 0.03	6.21 ± 0.10
T7	2.76 ± 0.02	1.18 ± 0.03	0.61 ± 0.0 9	0.22 ± 0.06	6.13 ± 0.07
T8	2.29 ± 0.08	0.89 ± 0.05	0.49 ± 0.10	0.34 ± 0.05	5.56 ± 0.15
T9	1.99 ± 0.06	0.54 ± 0.01	0.17 ± 0.03	0.54 ± 0.09	5.23 ± 0.04

Presented values are means of triplicate values

Further, the effect of increasing acid concentration on the production of XOS was studied. The increase in the concentration of acid from 0.1M to 0.5M caused a decrease in the amount of higher oligosaccharides (even xylotriose), subsequently increasing the xylose amount (1.79 mg/mL) in the hydrolysate. When the hydrolysis time was increased from 10 min (T4) to 25 min (T5), even the amount of xylobiose started to diminish (from 2.27 to 1.99 mg/mL), resulting in increased production of xylose. The increase in reaction time to 40 min (T6) not only resulted in the decomposition of XOS, but also caused the dehydration of xylose or arabinose, so that the amount of furfural (0.14 mg/mL) doubled. From T1 to T6, an increase in the total reducing sugar content was observed, owing to increased hydrolysis of higher XOS, so that 6.21 mg/mL was obtained.

When autohydrolysis of xylan was carried out at still higher acid concentration, *i.e.* 1.0M, a decrease in the total reducing sugar content was observed, indicating the formation of sugar dehydration products (furan derivatives, *i.e.* furfural and 5-hydroxymethylfurfural or HMF). The standard aniline acetate method employed to quantify furan derivatives was found to be more sensitive for furfural than HMF, so that when both were present in liquor, relatively higher amounts of HMF were required for quantification. The maximum amount of furfural quantified in the autohydrolysate was 0.54 mg/mL. The percentage composition of xylose, xylobiose, xylotriose, along with the dehydration product (furfural) in the autohydrolysate obtained from nine treatments (T1-T9) is shown in Figure 8.

The autohydrolysate treatments (T1-T4) producing the maximum percentage of XOS (in terms of xylobiose and xylotriose) were selected for evaluating prebiotic potency. The amount of xylose, furfural and XOS produced in g per 100 g xylan taken for the four best autohydrolysate treatments is shown in Table 6.

T1 was found to be the best treatment, where 34.5% of 100 mg xylan was dissolved as autohydrolysate, of which 17.97 mg is the weight of xylobiose and xylotriose obtained per 515 mg of rice straw. This corresponded to 52.1% xylobiose and xylotriose in the autohydrolysate. Lian *et al*. (2020)[46] reported the degradation of 48.37% of xylan, constituting 57.83% XOS. Moniz *et al*. (2014) obtained 40.1 g of total XOS per 100 g xylan through hydrothermal processing of rice straw.[27]

Degree of polymerization of XOS

The MS-ESI spectrum of the autohydrolysate treatment (T1) showed the presence of neutral xylooligosaccharides with a degree of polymerization up to 7. A low abundance parent ion peak at the *m/z* value of 965 corresponds to xyloheptaose, while the base peak or the most abundant peak observed at the *m/z* value of 305 represents xylobiose. Other peaks of decreasing abundance at *m/z* values of 437, 569, 701 and 833 represent xylotriose, xylotetraose, xylopentaose and xylohexaose, respectively.[59]

It is important to note that, although the autohydrolysate contains XOS up to a degree of polymerization of 7, it is xyobiose, xylotriose and xylotetraose that are known to possess prebiotic potential.[18,34,36]

Evaluation of prebiotic potency of crude XOS

The prebiotic potency of crude XOS produced by autohydrolysis of alkali extracted xylan was

evaluated for *Lactobacillus casei* and *Lactobacillus rhamnosus*, serving as natural inhabitants of the human gut.[60] The effect of XOS rich autohydrolysates (T1-T4), xylose, xylobiose, xylotriose and standard FOS on the growth of these two probiotic bacteria was expressed in terms of logarithmic value of CFU/mL at 10^{-4} dilution, as shown in Table 7. Statistical analysis indicated significant growth of the two species, as clear from CD values (5%).

Xylobiose was found to exhibit the highest positive effect on the growth of the two species, followed by T1 and T2 hydrolysates, FOS, T3 and T4 hydrolysates and xylotriose (Fig. 9). Although xylose was found to show less impact on the growth of the two bacteria, the growth was higher in comparison with the control. In corroboration with these results, Kaur *et al*. (2019)[37] reported XOS to have better prebiotic potential than FOS, with xylobiose exhibiting the highest positive effect on the growth of all three probiotic strains, *i.e. Lactobacillus brevis*, *Lactobacillus acidophilus* and *Lactobacillus viridescens*. Similarly, Gullon *et al*. (2014)[61] studied the prebiotic effect of arabinoxylooligosaccharides from wheat bran on *Bifidobacterium* species; the arabinoxylooligosaccharides were found to increase the growth of bacteria more than standard FOS.

The sample T1 and T2 served as effective prebiotics in comparison with FOS, due to the presence of higher concentration of xylobiose and xylotriose. The result was supported by Gullon *et al*. (2008),[62] who reported that XOS up to three polymerization degree exhibit significant prebiotic potency, stimulating the growth of bifidobacteria (beneficial gut bacteria). On the other hand, the smaller log CFU/mL values of T3 and T4 than that of FOS might be due to the presence of increased percentage of monosaccharides, compared to oligosaccharides, in the autohydrolysate. It is important to note that the autohydrolysates (T1 to T4) did not contain any phenolic acid and acetic acid (deacetylated xylan), which serve as inhibitors of the fermentation process.

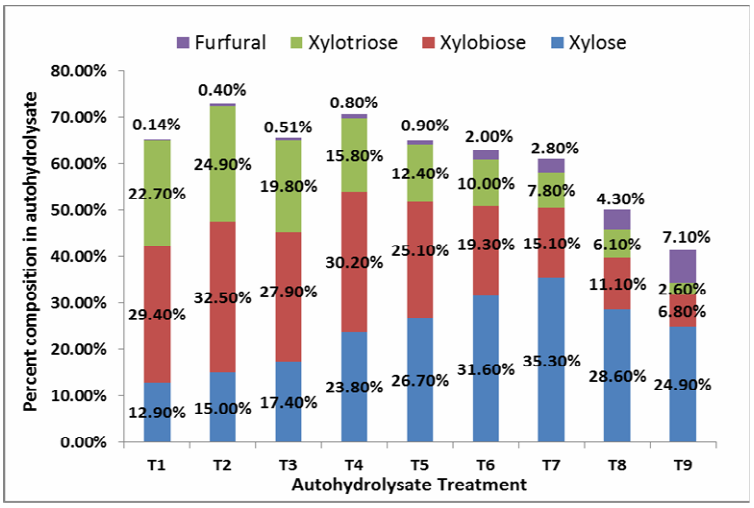


Figure 8: Percent composition of xylose, xylobiose, xylotriose and furfural in different autohydrolysate treatments (T1-T9) – made in Microsoft Excel

Table 6
Composition of autohydrolysate (mg) obtained by autohydrolysis of 100 mg xylan

	Amount (mg/100 mg xylan)		
	Xylose	Xylobiose and xylotriose	Furfural
T1	4.45	17.97	0.05
T2	5.48	20.95	0.15
T3	6.79	18.60	0.20
T4	8.93	17.25	0.30

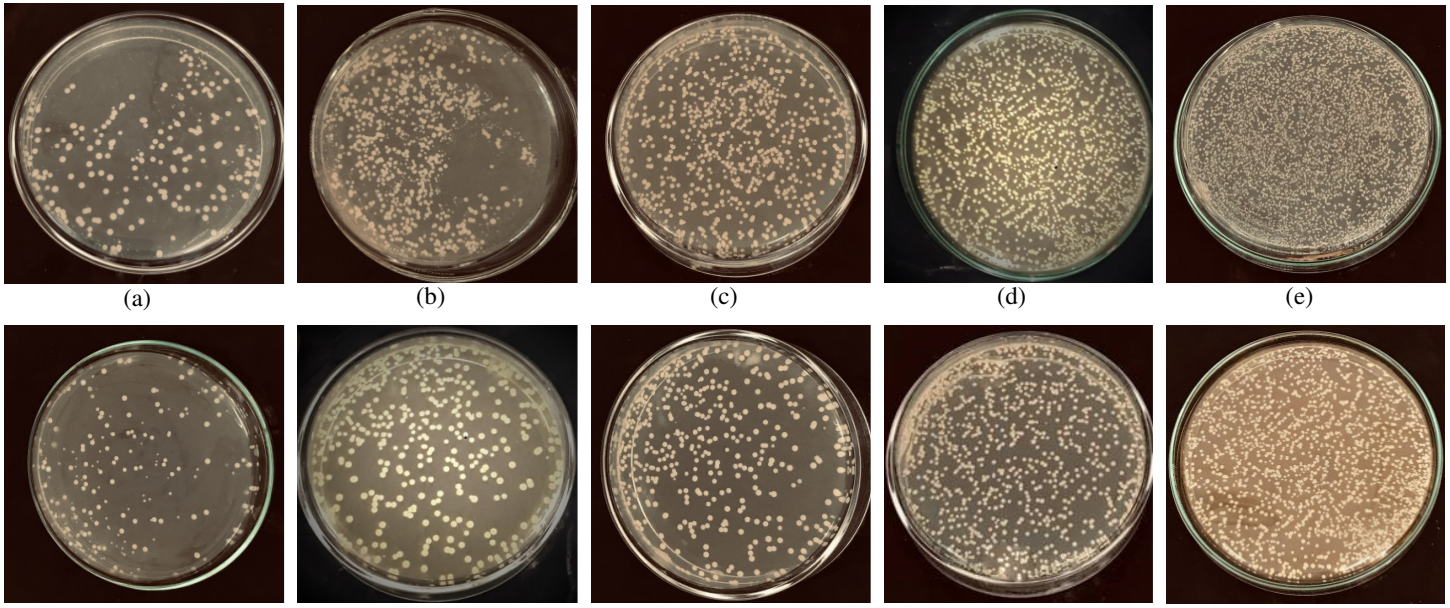


Figure 9: Growth of *Lactobacillus casei* (a, control) and effects on growth by xylose (b), FOS (c), XOS (d) and xylobiose (e); *Lactobacillus rhamnosus* (f, control) and effects on growth by xylose (g), FOS (h), XOS (i) and xylobiose (j)

Table 7
Effect of different treatments on growth of *L. casei* and *L. rhamnosus*

Treatments	log CFU/mL (10^{-4} dilution)*	
	L. casei	*L. rhamnosus*
T1	7.723	7.607
T2	7.647	7.467
T3	7.490	7.298
T4	7.308	7.170
Xylose	7.070	6.890
Xylobiose**	7.926	7.899
Xylotriose	7.283	7.004
FOS	7.573	7.327
Control	6.787	6.653
CD (5%)	0.334	0.368

*Values are means of triplicate values; **log CFU/mL for xylobiose was obtained from 10^{-5} dilution

Also, HMF and furfural, though present in the autohydrolysate, were in too low amounts (0.01 to 0.54 g/L) to cause any significant inhibitory effect on the growth of probiotics. Boguta *et al*. (2014)[63] studied the effect of fermentation inhibitors, such as acetic acid, HMF, phenolic acid and furfural, on the activity of a number of strains of lactic acid bacteria and found that all the strains grew well on plates containing up to 7 g/L furfural and HMF.

CONCLUSION

Rice straw containing 24% hemicelluloses and 9% lignin can serve as an effective raw material for selective extraction of hemicellulosic xylan. The alkaline extraction process was optimized using CCD of RSM, so that the maximum yield of hemicellulosic xylan (19.97%) was obtained by carrying out reaction with 11.04% (w/v) NaOH for 3.126 h at 80.146 °C. Structural characterization defined the structure as *L*-arabino (4-*O*-methylglucurono) xylan, with no acetyl groups and associated lignin. The best treatments for autohydrolysis of xylan were obtained using 0.1M sulphuric acid for hydrolysis time of 10, 25 and 40 min, along with the treatment employing 0.5M sulphuric acid with 10 min hydrolysis time. These autohydrolysate treatments (rich in xylobiose, xylotriose and neutral XOS with a degree of polymerization up to 7) were found to exhibit a positive effect on the growth of *Lactobacillus rhamnosus* and *Lactobacillus casei*, in comparison with existing FOS, pure xylotriose and xylose. Pure xylobiose was found to exhibit the highest positive effect on the growth of the two species.

Although efforts are being made to valorize rice straw, the value derived from it is not high enough for industries to approach farmers for its collection from fields. Therefore, the present study attempted to solve the disposal issue related to rice straw through the production of neutral XOS-rich hydrolysate, containing a limited amount of side products (monosaccharides and uronic acid) and inhibitors (furan derivatives), so that little purification would be necessary to achieve commercially important products, with great significance on the global nutraceuticals market.

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