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GROWTH STIMULATION OF *Lactococcus lactis* sps BY XYLOOLIGOSACCHARIDES PRODUCED USING SINGLE FERMENTATIVE STEP FROM LIGNOCELLULOSIC WASTE BY *Massilia Timonae* B2YR

Rajani Thanekar [1], *Dr. Yasmin Attar* [2*]

Address(es): *Dr. Yasmin Attar,*
[1] Research student: Rajaram College Kolhapur, Maharashtra, India 416004.
[2]*Head of Department of Microbiology, Rajaram College, Kolhapur, Maharashtra, India. 416004.

*Corresponding author: ycamicro@gmail.com; ycamicro@gmail.com

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ABSTRACT

As a source of xylan, lignocellulose waste such as wheat bran and aquatic weed *Pistia stratiotes* were employed, together with potassium nitrate as a nitrogen source, which were optimized using the Box- Behnken design to increase the production of xylooligosaccharides. The yield of xylooligosaccharides increased to 2.05- fold in the optimized medium than non-optimized medium. The produced xylooligosaccharides showed a peak at 890-900 cm-1 confirming the presence of β-glycosidic linkages when detected by Fourier Transform Infra-Red (FT IR). The prebiotic effect of produced xylooligosaccharides was studied on two strains named *Lactococcus lactis* Cl MN515342 and *Lactococcus lactis* Cp MN515343 isolated from curd samples. The growth of these *Lactococcus* strains was found to be stimulated. A log Colony Forming Unit/ml value of 7.813 and 8.146 for Cp and Cl was observed in a medium incorporated with XOS. The Short-Chain Fatty Acid (SCFA) production by *Lactococcus lactis* in a medium incorporated with xylooligosaccharides was detected by GCMS. The use of lignocellulosic waste for the production of xylooligosaccharides reduces the production cost. The xylooligosaccharides produced by *Massilia timonae* has shown promising result in stimulating the growth of *Lactococcus lactis*. Thus, XOS can be incorporated in curd or Yoghurt along with the *Lactococcus lactis* and used as synbiotic food.

Keywords: Fermentation, *Pistia stratiotes*, wheat bran, Box Behnken, prebiotics, Xylooligosaccharides, *Lactococcus lactis*
Abbreviation: FT IR- Fourier transform infra-red; XOS-xylooligosaccharides; SCFA-Short-chain fatty acid; CFU- Colony forming unit

INTRODUCTION

In the global food industry market, food or its ingredients with added functional properties that can improve consumer health are in higher demand. **(Manisseri and Gudipati 2012**). Functional food comprises food with natural bioactive, derived food e.g. prebiotics, and bioactive added to food as supplements e.g. probiotics, and antioxidants **(Singh, Banerjee, and Arora 2015)**. Prebiotics as defined by Professor Gibson is the non-digestible food ingredient by the host which selectively stimulates the growth or activity of one or the limited number of beneficial bacteria in the colon **(Gibson and Roberfroid 1995)**. With this perspective, xylooligosaccharides can selectively stimulate the growth of probiotic microorganisms such as *Lactobacillus* and *Bifidobacterium* species, thus exhibiting a prebiotic effect **(Aachary and Prapulla 2011)**. Prebiotic xylooligosaccharides (XOS) have a lot of potential as food components owing to their organoleptic properties, multifaceted health advantages for humans, and low cost compared to other prebiotics **(Amorim *et al* 2019)**. Thus, XOS may be used in the preparation of synbiotics which are defined as the combination of probiotics and prebiotics which beneficially affect the host by refining the survival and colonization of live microbial dietary supplements by selectively stimulating the growth of health-benefiting microorganisms**(Markowiak and Ślizewska 2017).** XOS also poses a beneficial effect in metabolic illnesses of diabetes as it can control body weight, glucose and lipid homeostasis, and insulin sensitivity which is mainly due to the production of SCFA in the colon, thereby increasing sodium and water absorption in the distal intestine improving polydipsia**(Freitas *et al* 2019)**. Generally, the production of xylooligosaccharides is carried out by the following methods a) Auto hydrolysis and b) Chemical extraction of xylan followed by enzymatic treatment**(Alonso, Vazquez, and Parajo 2001)**. Still, the production of xylooligosaccharides is expensive. Thus, extensive research has been carried out to reduce the production cost of xylooligosaccharides. Agriculture, forestry, and municipal lignocellulosic biomass (LCB) are all sources of potentially economical and renewable feedstock. Globally, 1.3 billion tonnes of lignocellulosic biomass are produced every year **(Dar, Pawar, Chintalchere, et al. 2019)**. Hence, the present study focuses to reduce the production cost by replacing the commercial xylan with lignocellulosic waste, like agro residue wheat bran and aquatic weed *Pistia stratiotes* which were used without any prior treatment for the extraction of xylan. Production of xylooligosaccharides is carried out in a single fermentative step in a statistically optimized medium since the microorganisms *Massilia timonae* produces an ample amount of endoxylanase with low levels of cellulase, amylase and protease enzyme. This multi-enzyme production ability of organisms is used for the production of xylooligosaccharides. Thus, this single fermentative mode of manufacturing xylooligosaccharides can reduce overall production costs.

MATERIAL AND METHODS

Chemicals used

The xylooligosaccharides standards xylobiose and xylotriose were purchased from Megazyme, Ireland. The silica gel 60 TLC sheet of Merck was used.

Microorganism

The microorganism *Massilia timona*e B2YR KY942185 was isolated from sawmill industry soil on a modified Horokoshi medium supplemented with xylan **(Thanekar and Attar 2017)**. The organism was maintained on a nutrient agar slant.

Production of Xylooligosaccharides

The production of xylooligosaccharides was carried out in medium containing Wheat bran 0.5% w/v and *Pistia stratiotes* 0.5% w/v (sun-dried and powdered) and KNO_3-0.25%w/v, NaCl -0.5 % w/v in 100 ml medium. Additional medium replacing the raw substrate with commercial xylan was also used for comparison. These media were inoculated with 0.3%v/v of suspension of *Massilia timonae* B2YR KY942185 corresponding to a cell density of 3×10^8 cells/ml and incubated at room temperature 28±2°C for 5 days. The produced xylooligosaccharides were quantified by measuring the reducing sugar formed by using the DNS method **(Miller 1959)** and expressed in mg/ml.

Statistical optimization and experimental design

The production medium was statistically optimized using Box Behnken design three factorial designs. The three independent variables wheat bran, *Pistia stratiotes*, and KNO_3 were selected to optimize them and study their effect and concentration for maximizing the production of xylooligosaccharides. The relation between actual and coded values is given as

$xi = \frac{Xi - xo}{\Delta Xi}$ ***Equation 1***

Where *xi* and *Xi* are coded and the actual values of the independent variable, *xo* is the centre value of the actual independent variable and ΔXi is the change of Xi. The relationship between A, B, and C to the response Y was calculated using the quadratic model equation.

$Y = \beta_0 + \beta_1 A + \beta_2 B_2 + \beta_3 C_3 + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$ ***Equation 2***

Where Y= Predicted response, β_0 = constant term, A= Wheat bran, B= *Pistia stratiotes* C = KNO_3, while $\beta_1, \beta_2, \beta_3$, were the linear coefficients, $\beta_{11}, \beta_{22}, \beta_{33}$ were square coefficients, and $\beta_{12}, \beta_{13}, \beta_{23}$ were interactive coefficients. (Irfan, Shakir, and Qazi 2017)

Data analysis

The responses obtained through the experiments were subjected to analysis by using the Design Expert Version 12 [Stat. Ease Inc, Minneapolis, Minneasota, USA] a statistical tool. The xylooligosaccharides yield was analyzed by applying analysis of variance (ANOVA) combined with F- Test to evaluate whether the given term has a significant effect ($P \leq 0.05$). Through the graphical and numerical analysis of the design expert program, optimum levels of significant variables were obtained.

Purification of xylooligosaccharides

Purification was carried out with slight modification of the method proposed by Digant Chapla et al. the fermented broth was filtered to remove the unfermented debris. The clear filtrate was subjected to centrifugation at 8000 rpm for 10 mins to obtain a cell free extract. The extract was passed through the activated charcoal to obtain colourless cell free extract. The extract was measured and a double volume of ice-cold isopropanol was added to it to precipitate unused xylan which was refrigerated overnight for precipitation. The precipitate of unused xylan formed was removed by centrifugation at 8000 rpm for 10 mins. The supernatant was then kept in a water bath for vaporization of isopropanol and concentration of broth. The concentration was carried out till the volume was reduced to 1/10. This was cooled and then subjected to crystallization of xylooligosaccharides. The broth was passed through activated charcoal followed by passing ethanol gradient of 80%, a whitish precipitate was observed which was then dried in freeze to form crystals**(Chapla, *et al* 2012).**

Detection of Formation of xylooligosaccharides

The freeze-dried crystals of xylooligosaccharides formed were dissolved in D/w at a concentration of 1mg/ml which were loaded on Silica gel 60 TLC. Similarly, standard xylobiose and xylotriose were also prepared in an amount of 1mg/ml concentration. The chromatography was carried out in a solvent system butanol: acetic acid: water (2:1:1). The chromatogram was developed with 0.2% w/v orcinol reagent prepared in 1% v/v sulphuric acid solution in methanol**(Chapla *et al* 2012)**.

Conformational study of XOS by FT IR

FT IR spectroscopy is a powerful tool for the study of the physicochemical and conformational properties of carbohydrates **(Kacurakova et al. 1998)**. The freeze-dried crystals were subjected to analysis by Fourier transform infrared using Shimadzu 8400S spectrophotometer at 4 cm^{-1} resolutions between 500 and 4000 cm^{-1} absorption mode in comparison to standard xylobiose and xylotriose.

Study of prebiotic effect of xylooligosaccharides

The freeze-dried crystals of xylooligosaccharides formed were subjected to study its prebiotic effect on lactic acid bacteria isolated from curd samples.
Isolation of probiotics: [lactic acid bacteria]
The isolation of Lactic acid bacteria was done on Neutral red chalk lactose agar. A loop full of curd samples purchased from the local market of Kolhapur district, Maharashtra was streaked on medium and incubated in the anaerobic jar at 28°±2° C for 24-48 hrs **(Manjunath and Pallavi 2017).** Pink-colored pinpoint catalase-negative colonies were selected for further study.
b) Identification of isolated lactic acid bacteria:
The isolated bacteria were subjected to morphological, biochemical, and cultural studies. The bacteria were also identified by the 16S rRNA technique. The obtained sequence was deposited at Gen bank NCBI and the accession number was acquired.

Study of prebiotic effect of isolated and identified Lactic acid bacteria

Measurement of growth as turbidity

Sterile peptone water broth was used as control, while peptone water incorporated with 1% w/v XOS was used to study the prebiotic effect. Similarly, 1% w/v lactose was used as a positive control. The broths were inoculated with *Lactococcus Lactis* Cp and *Lactococcus lactis* Cl in a cell density of $9x10^8$ cells /ml in 0.3 %v/v concentration. The absorbance was measured at 660 nm to record growth in form turbidity from o to 240 mins.

Standard plate count

A dilution from 10^{-4} to 10^{-8} was prepared from the above broth after 24 hrs of incubation. 0.1ml of dilution was spread on nutrient agar medium incorporated with 1% XOS w/v & and 0.5ml of neutral red. The method was performed for both Cp and Cl strains of *Lactococcus lactis.* The population density was noted as log CFU/ml**(Palaniappan, *et al* 2017).**

Detection of SCFA

The formation of short-chain fatty acid in a fermentation medium containing 1% XOS inoculated with a mixture of *Lactococcus lactic* CL and *Lactococcus lactic* Cp incubated at 28±2° C for 24 hrs anaerobically was verified by checking a drop in pH. The formation of short-chain fatty acid was confirmed by performing GC-MS analysis. The GC MS analysis was carried out in SHIMADZU 2010 Plus model with helium as carrier gas and the temperature programmed for the cycle run of 45 mins to an initial temperature of 40° C held for 1 min and then raise to 270° C. The sample was injected in split mode. The mass spectra were recorded in a range of 50-650 amu. The obtained mass spectrum of GC-MS was compared with the NIST library 17 databases of the National Institute of Technology.

RESULTS

Xylooligosaccharides Production

The production of xylooligosaccharides was monitored for 5 days in both commercial pure xylan and the raw substrate containing medium and produced xylooligosaccharides were quantified as reducing sugars which were expressed in mg/ml as presented in Table 1 showing the yield of xylooligosaccharides obtained was more about 7.3±0.028 mg/ml in raw substrate medium with xylanase activity of 283.18±0.01U/ml as compared to Commercial pure xylan which was 1.80±0.020 mg/ml at 72 hrs while xylanase activity observed was 236.44±03U/ml. The production of xylooligosaccharides began with 0.87±0.11mg/ml for pure xylan while for crude raw substrate5.52±0.019 mg/ml. On the fifth day decrease in yield in both sources as pure xylan showed 1.52±0.028mg/ml and that in crude substrate yield was 5.19±0.016 was noted.

Table 1 Time course for the production of Xylooligosaccharides(mg/ml)

Sr.No	Time in hrs	Pure Xylan	Raw crude substrate
1	24	0.87±0.011	5.52±0.019
2	48	0.92±0.020	6.04±0.03
3	72	1.82±0.020	7.3±0.028
4	96	1.56±0.013	5.69±0.019
5	120	1.520±0.028	5.19±0.016

Box-Behnken design and statistical analysis

The statistical approach of the Box-Behnken design was used to optimize medium components like the wheat bran, *Pistia stratiotes*, and KNO_3.A set of 15 experiments with 3 centre points aimed at three independent variables like wheat bran(A), *Pistia stratiotes* (B), KNO_3(C), and the effect of their varying concentration on *Massilia timonae* for production of xylooligosaccharides. The experimental design, for xylooligosaccharides yield and residual values, are shown in Table 2. The xylooligosaccharides production potential was the function of independent variables which was determined by the regression obtained after ANOVA, given by the equation.
6.94667 + 0.42 * A + 0.70625 * B + -3.29375 * C + -0.635 * AB + 0.445 * AC + 0.4825 * BC + -0.334583 * A^2 + 0.317917 * B^2 + 3.81792 * C^2

Table 2 Experimental design for Box Behnken with actual and predicted response of xylooligosaccharides yield by *Massilia timonae*

Run Order	Standard Order	Factor 1 Wheat bran(A)	Factor 2 Pistia stratiotes (B)	Factor KNO3 (C)	Actual Value	Predicted Value
1	15	1.5	1.5	0.25	6.95	6.95
2	5	1	1.5	0	13.21	13.75
3	14	1.5	1.5	0.25	8.07	6.95
4	4	2	2	0.25	6.95	7.42
5	8	2	1.5	0.5	8.54	8.00
6	11	1.5	1	0. 5	6.20	6.60
7	3	1	2	0.25	7.99	7.85
8	13	1.5	1.5	0.25	5.82	6.95
9	9	1.5	1	0	14.22	14.15
10	12	1.5	2	0.5	8.91	8.98
11	7	1	1.5	0. 5	6.20	6.27
12	2	2	1	0.25	7.14	7.28
13	6	2	1.5	0	13.77	13.70
14	10	1.5	2	0	15.00	14.60
15	1	1	1	0.25	5.64	5.17

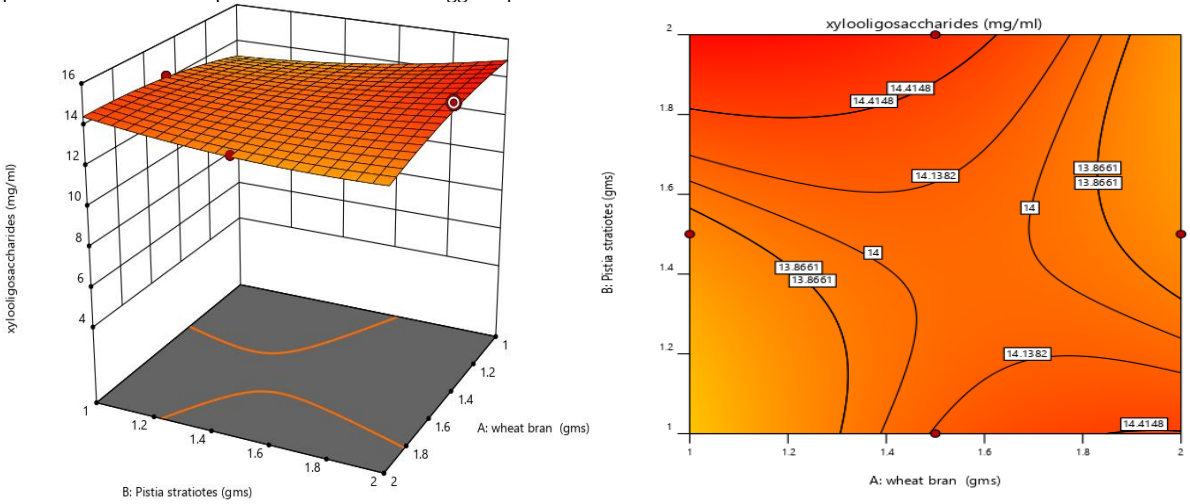
Linear coefficients with the negative sign-3.29375(C) i.e., nitrogen source KNO_3 has a negative effect on XOS yield, whereas wheat bran and *Pistia* have a positive effect. The negative effect means increasing the concentration of this variable will lead to a lowering of yield. The linear coefficients 0.42 and 0.70625 for wheat bran and *Pistia stratiotes* respectively suggest that *Pistia stratiotes* have a maximum effect on xylooligosaccharides since both are the source of carbon contributing to yield XOS **(Kocabas and Ozben 2014).**

ANOVA for the quadratic regression model obtained for xylooligosaccharides yield had a very low value of probability (0.0018) and very high F value of 21.30 indicating the model to be very significant. A lack of fit was found to be insignificant with a p-value of 0.7871 displayed in Table 3. The goodness of fit for the model was tested by the R^2 coefficient of determination value. The current study has shown an R^2 value of 0.9746, which is very close to 1, making the model accurate. An adjusted R^2 value of model 0.9288 is near to the value of R^2 in the present model **(Garai and Kumar 2013).** The difference between adjusted R^2(0.9288) and predicted R^2(0.8182) is less than 0.2 which is in reasonable agreement. Adequate precision measures signal-to-noise ratio. A ratio greater than 4 is desirable. This model has a ratio of 13.023 indicating an adequate signal.

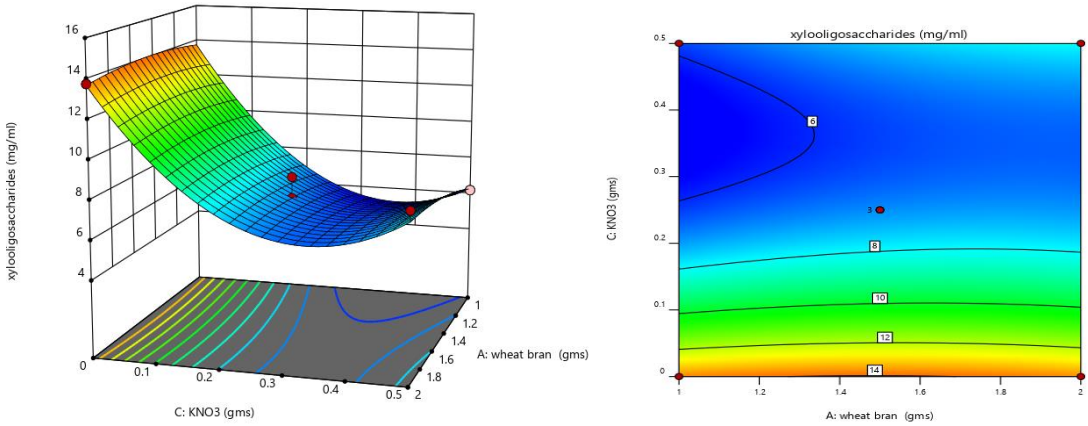
Table 3 ANOVA for regression analysis of xylooligosaccharides yield in the quadratic model of Box Behnken design of response surface methodology

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	150.83	9	16.76	21.30	0.0018	significant
A-wheat bran	1.41	1	1.41	1.79	0.2381	
B-Pistia stratiotes	3.99	1	3.99	5.07	0.0741	
C-KNO3	86.79	1	86.79	110.32	0.0001	
AB	1.61	1	1.61	2.05	0.2116	
AC	0.7921	1	0.7921	1.01	0.3617	
BC	0.9312	1	0.9312	1.18	0.3263	
A²	0.4133	1	0.4133	0.5254	0.5010	
B²	0.3732	1	0.3732	0.4743	0.5216	
C²	53.82	1	53.82	68.41	0.0004	
Residual	3.93	5	0.7867			
Lack of Fit	1.40	3	0.4675	0.3694	0.7871	not significant
Pure Error	2.53	2	1.27			
Cor Total	154.77	14				

Based on the model equation, the three-dimensional response plot and their corresponding contour plot were drawn to study the interaction among the variables and their concentration responsible for increasing yields of xylooligosaccharides. The smallest elliptical in the contour plot at the centre shows the maximum predicted value. The elliptical nature of contour suggests positive interaction between two independent variables as observed in Fig 1a interaction between wheat bran and *Pistia stratiotes* **(Kamble *et al.* 2018)**. Since both are carbon sources they act as a substrate for xylanase and ultimately increase the yield of xylooligosaccharides.



a



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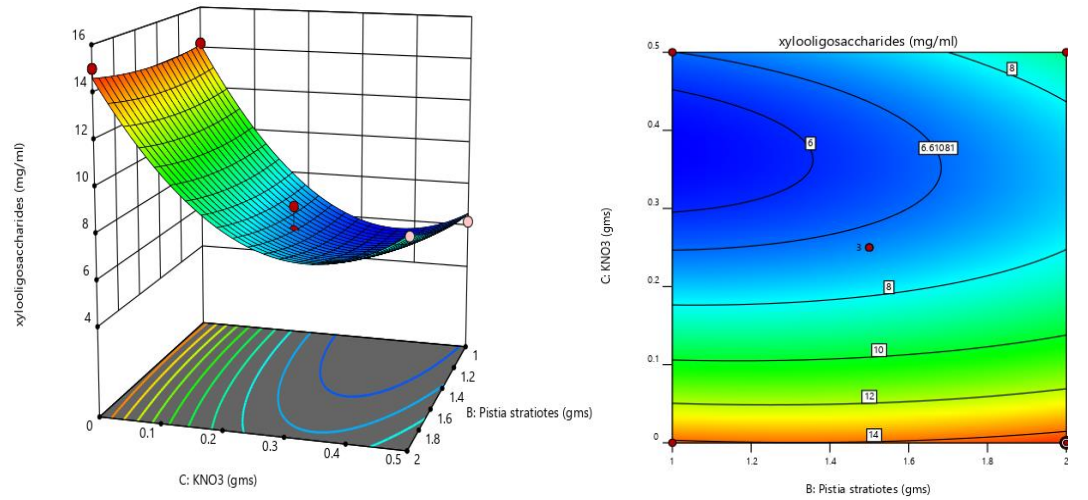

c

Figure 1a Response surface plot and contour plot showing the interaction between wheat bran and *Pistia stratiotes.* b. Response plot and contour plot depicting interaction between wheat bran and KNO3, c. response and contour plot between *Pistia* and KNO3

The circular contour plots show that there is no interaction between the independent variables and they both do not contribute to the yield of xylooligosaccharides **(Cao *et al.* 2008)**. Figure 1b demonstrates the interaction between KNO3 and wheat bran, which shows that wheat bran is independent of nitrogen source. The contour plot depicting the interaction between *Pistia* and KNO3 further shows that *Pistia stratiotes* behave independently of KNO3. Figs 1b and 1c show that both carbon sources are independent of KNO3 and it can be observed that the medium without nitrogen showed a maximum yield of xylooligosaccharides. The response surface plot was constructed by keeping a variable at centre level and the effect of two other variables studied on XOS yield. Wheat bran and *Pistia stratiotes* contribute to the production of XOS. The production of xylooligosaccharides has been found to increase from 7.29±0.028 mg/ml to 15.00 mg/ml which has shown a 2.05-fold increase in the product. Since this is the first approach for use of box-Behnken design for the production of xylooligosaccharides fermentative no comparative reports are available. But if compared with other reports of production of xylooligosaccharides as given by Kocabas et al showed the highest yield of XOS from sunflower stalk to 4.89 ± 0.02 mg/ mL**(Kocabas and Ozben 2014)**. While Samanta et al obtained a yield of 5.22mg/ml of XOS after optimizing pH, temperature, and enzyme dose**(Samanta *et al*. 2019).** Thus the optimal concentrations of medium component obtained after optimization are 2% w/v of *Pistia stratiotes* and 1.5 % w/v of wheat bran.

Detection of Formation of XOS

Purified XOS was freeze-dried to crystals which were weighed 0.120gms from 100 ml of medium incorporated with wheat bran and *Pistia stratiotes* in a concentration optimized and found to be 1.5% and 2% w/v. These crystals were dissolved in D/w and loaded and found that XOS showed a degree of polymerization of X2 and X3 as observed in Fig.2. The Rf values of standards xylose, xylobiose, xylotriose were 0.58, 0.47, 0.41. On the lane loaded with sample XOS, two spots were developed their Rf values were 0.50 and 0.41which are near to standard xylobiose and xylotriose

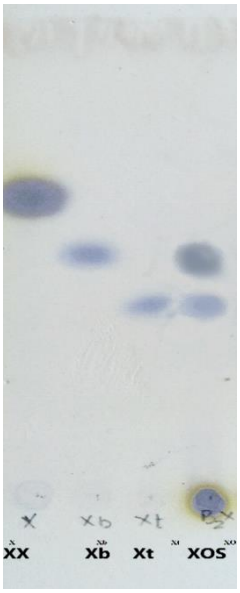

Figure 2 TLC image of purified crystals of Xylooligosaccharides Lane XX- Standard xylose, Lane Xb- Standard xylobiose, LaneXt- standard xylotriose, Lane XOS – purified crystals of xylooligosaccharides.

Conformational study of XOS by FT IR

FTIR analysis of xylooligosaccharides crystals produced and standard xylobiose and xylotriose are presented in Fig 3 a b c**.** The strong bands at 3500-3200 cm^{-1} are due to the O-H stretching vibrations of the hydrogen bonded to the hydroxyl group. The C-OH stretching vibrations are observed at 1050cm^{-1} **(Hofman and Witanowski 1959).** While bands at 3745.25-3853.12 cm^{-1} are designated for OH free stretching of the phenol group**(Bruice 2011).** The transmittance peak between 1200-1000 cm^{-1} is assigned to the stretching vibration of the C-C, C-O, and C-O-

C groups. The peak at 890-900 cm-1 confirms the presence of glycosidic linkage in the backbone of xylooligosaccharides**(Palaniappan, Balasubramaniam, and Antony 2017).**

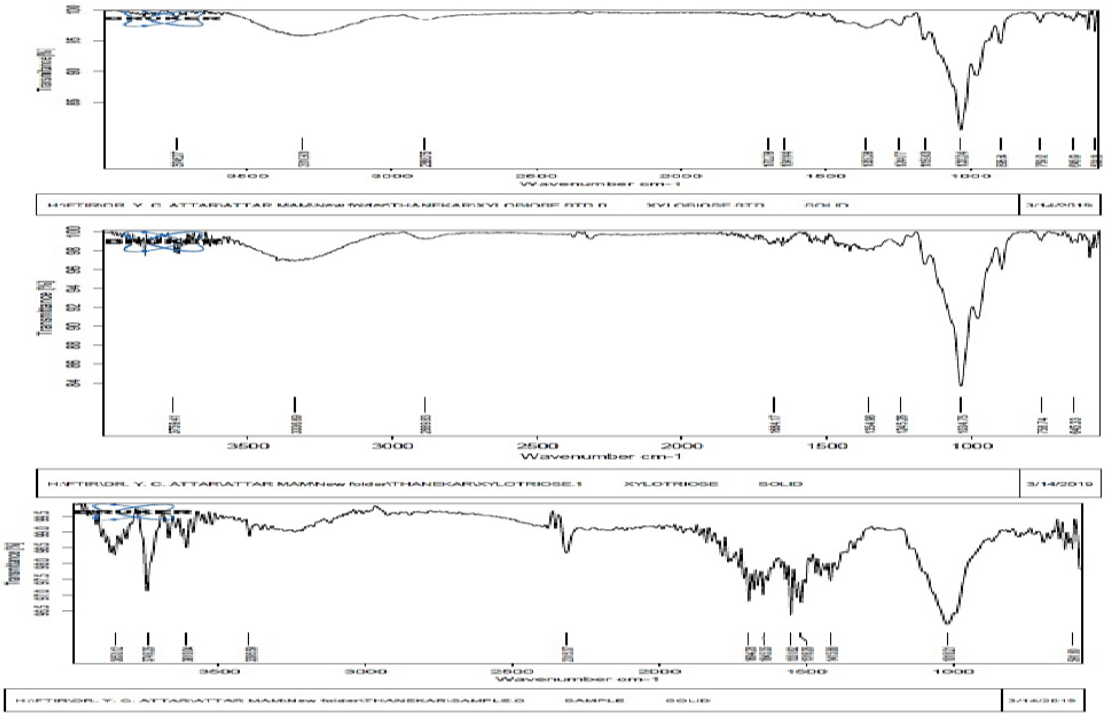

Figure 3FT IR analysis of a) xylobiose standard b) XOS sample c) Xylotriose standard

Study of prebiotic activity

Isolation of Lactic acid

The lactic acid bacteria strains Cp and Cl were chosen from the pink pinpoint catalase-negative two colonies. Cultural and morphological research was conducted on these specimens. Both the organisms were gram-positive cocci non-motile in nature and they were homofermentative since no gas was produced in glucose broth. Carbohydrate metabolism studied for both organisms showed that both can utilize glucose and lactose but not arabinose and mannitol while the Cl strain uses xylose but not sucrose while the Cp strain utilizes sucrose but not xylose.

Identification of lactic acid bacteria:

The cultures were identified by 16S rRNA sequence. The FASTA sequence was submitted to NCBI and accession no acquired and phylogenetic relationship was analyzed. These cultures were classified as *Lactococcus lactis* Cl MN515342 and *Lactococcus lactis* Cp MN515343. The phylogenetic tree for *Lactococcus lactis* Cl MN515342 and *Lactococcus lactis* Cp MN515343 was constructed using the Neighbor-Joining method**(Saitou and Nei 1987).** Evolutionary analyses were conducted in MEGA represented in Fig 4. The sequences for the top ten organisms were retrieved from NCBI and aligned using Clustal W for both organisms. Percent identity of 99.53% was observed for Cl with the topmost related organisms while Cp showed 96.18% relatedness **(Kumar *et al.* 2018)**.

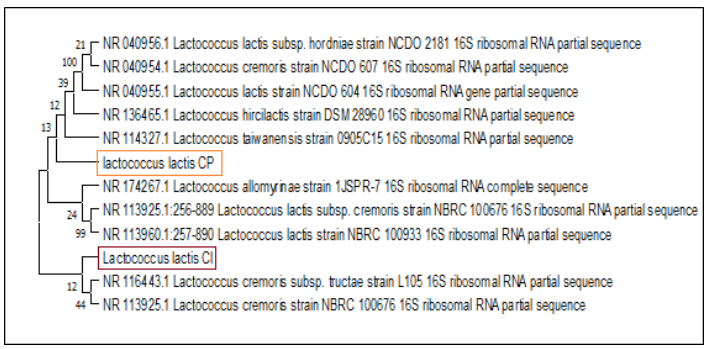

Figure 4 Phylogenetic analysis of *Lactococcus lactis* Cl and *Lactococcus lactis* Cp

Study of prebiotic effect of isolated and identified Lactic acid bacteria

Measuring absorbance of growth medium

The effect of XOS on *Lactococcus* growth stimulation was measured using optical density, and as shown in Fig 5, *Lactococcus lactis* Cl showed maximum growth than *Lactococcus lactis* Cp at 240 minutes. The optical density for Cl at 0 min in control,1% XOS, 1% Lactose containing broth was 0.11 in all the medium respectively which reached to 0.15, 0.35, and 0.43 at 240 mins while 0.23, 0.50, 0.42 after 24 hrs. The optical density observed for Cp strain in control, 1% XOS, and 1% lactose at 0 mins was 0.11 which raised to 0.17, 0.35, 0.18 at 240 mins, and then at 24 hrs, it was 0.20,0.43, 0.44 respectively. An average rise of 0.3 in the optical density was observed in Cp organisms in 24 hrs, while in Cl the rise of 0.4 is observed.

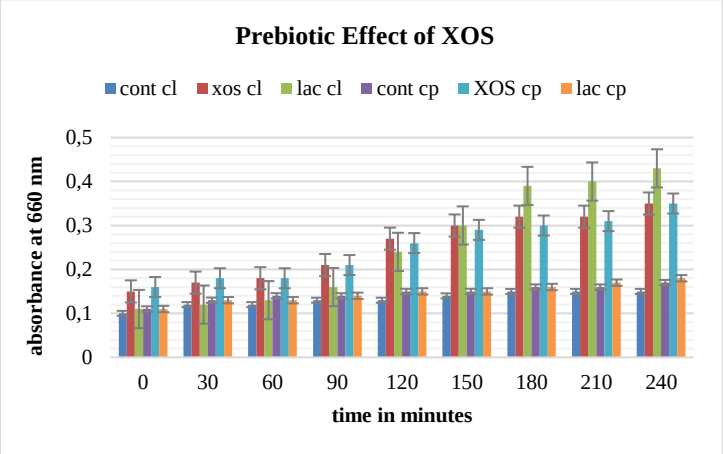

Figure 5 Prebiotic effect of xylooligosaccharides produced from wheat bran and *Pistia* on Cp and Cl strain

Standard plate count

The colony-forming unit of the above broth was recorded for both strains Cp and Cl. Growth of cl has shown more stimulation than Cp. The count of organisms is represented in the log value of CFU/ml. The count was found to increase in the broth supplemented with XOS. The count of control broth in log CFU/ml for Cp and Cl was 5.616 and 5.364 respectively, while the broth with XOS gave 7.813 and 8.146. The control broth showed the count to 1.65×10^6 and 1.7×10^6, count corresponding to $6.5 \times 10^7$and 5.6×10^8 CFU /ml for Cp and Cl strain in the medium

incorporated with XOS. Kaprelyant et al reported stimulation of *lactobacillus acidophilus* and *Bifidobacterium bifidum* with a count of 1.4×10^{10} CFU/cm^{3}and 9.2×10^{10} CFU/cm^3**(Kaprelyants *et al.* 2017)**.

Detection of SCFA

A drop in the pH of fermentation broth was noted from 7.0 to 3.5±0.4 after 24 hrs. A GC MS study of a cell free clear extract revealed the production of SCFA. The retention time and mass of ion (m/z) showed the production of acetic acid (C3) to heptanoic acid (C7) presented in Table 4 and Fig 6 **(Furuhashi and Ishihara 2018)**.

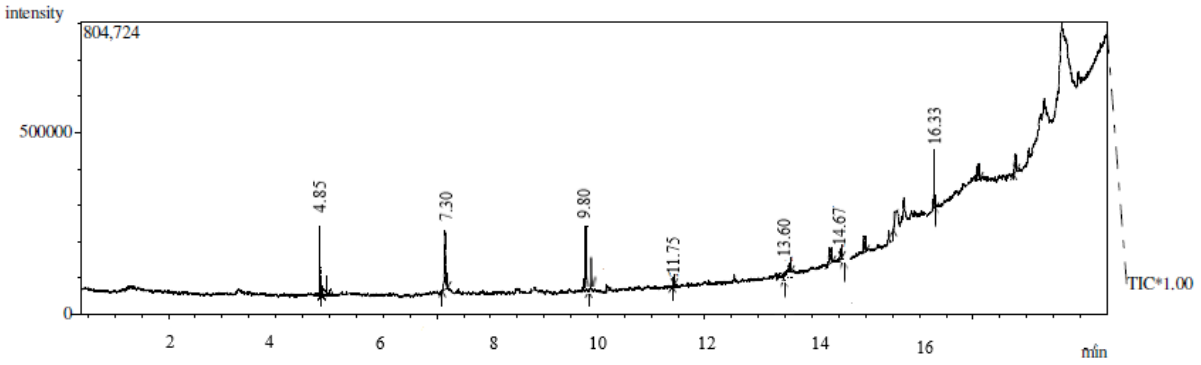

Figure 6 GC profile of SCFA detected in fermentation peak 1) acetic acid 2) propanoic acid 3) butanoic acid 4) isovaleric acid 5) Valeric acid 6) Caproic acid 7) Heptanoic acid

Table 4 List of SCFA produced during the fermentation of Xylooligosaccharides by a mixture of *Lactococcus lactis* Cl and *Lactococcus lactis* Cp

Sr.no	Compound name	Retention time	m/z	Retention index	Peak Area
1	Acetic acid	4.85	55	610	6.79
2	Propanoic acid	7.30	60	700	15.44
3	Butanoic acid	9.80	73	804	21.03
4	Isovaleric acid	11.75	88	860	11.05
5	Valeric acid	13.60	98	902	17.35
6	Caproic acid	14.67	116	990	5.64
7	Heptanoic acid	16.33	129	1078	6.86

DISCUSSION

To increase the yield of xylooligosaccharides, statistical approaches such as the design of experiments are used. The optimal concentrations of medium component obtained in the present study after optimization by response surface methodology's Box Behnken design are 2% w/v of *Pistia stratiotes* and 1.5 % w/v of wheat bran without any pre-treatment to both xylan sources. The use of the Box Behnken design has enabled to increase in the yield of XOS to 15.00 mg/ml a yield more than the other reports. Samanta et al after the RSM analysis obtained higher production of XOS to (1.91 mg/ml) through the alkali hydrolysis of corn cob to obtain xylan which was used in the concentration of 2% w/v in sodium citrate buffer at pH 5.8, temperature 44°C, with an incubation time of 17.5h and 5.73U of xylanase**(Samanta *et al.* 2019)**. The production of xylooligosaccharides as given by Kocabas et al showed the highest yield of XOS from sunflower stalk to 4.89 ± 0.02 mg/ mL**(Kocabas and Ozben 2014)**

The Xylooligosaccharides obtained in the present study show polymerization ranging from X2-X3 they exhibit maximum prebiotic activity as reported by Menzes et al **(da Silva Menezes *et al.* 2018).** The *Lactococcus lactis* cp *and Lactococcus lactis* cl showed good growth in medium incorporated with produced XOS which proves their prebiotic activity. Thus, this XOS can be used in preparing the dairy product with *Lactococcus lactis* as a starter culture. Such a synbiotic preparation may lead to modulation of intestinal flora providing health benefits to the host. The XOS of this polymerization shows a prebiotic effect and it can be incorporated into food. The commercial xylooligosaccharides (Qingdao Century Longlive International Trade Co., Ltd). derived from corn cob also showed the prebiotic effect on *Lactobacillus plantarum* with an average rise of 0.4 similar to the strain Cl in the present study **(Yu *et al.* 2015)**. Karuppasamy et al reported the production of prebiotic syrup from wheat bran which has shown growth stimulation of probiotics at 0.5% syrup concentration **(Geetha and Gunasekaran 2017).** The prebiotic effect of commercial XOS and finger millet derived XOS after enzymatic treatment was studied by Palaniappan et al and they also observed good growth of *Lactococcus lactis* in terms of absorbance. The 1% w/v concentration of XOS studied in the current research showed stimulation of growth in both *Lactococcus* strains. In vitro increase in growth of probiotic bacteria is encouraging with several studies stating XOS as emerging prebiotic biomolecules **(Joshi, Sharma, and Singh 2020).** The production of SCFA also confirms the prebiotic effect of xylooligosaccharides. The production of SCFA reduces the threat of colon cancer and normalizes the metabolic processes **(Rycroft *et al.* 2001)**.

An increase in population has led to exploring of more food providing additional functional properties one of them being prebiotic. But the high cost of such prebiotics prevents them to be added to the daily diet. Hence, an attempt to lower the production and purification cost of xylooligosaccharides was done in the current work by using a single-step fermentative mode for production. Followed by a simple purification technique without using specialized equipment required for ultrafiltration and costlier techniques of chromatography. This approach can successfully be replaced with the Auto hydrolysis technique used for producing XOS and also the frequently used method of chemical extraction of xylan from lignocellulosic biomass followed by the enzymatic process which is a long and costly method. The production was also made economical by replacing commercial xylan with crude substrates like wheat bran and *Pistia stratiotes* where *Pistia* is reported for the first time. Such an approach will not only reduce the production cost but also generate feedstock and a great amount of revenue from lignocellulosic biomass**(Dar, *et al.* 2019).**

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