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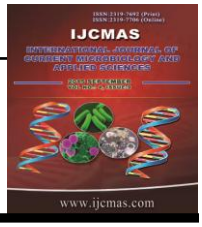

Original Research Article

Enzymatic Production of Xylooligosaccharides from Agricultural Byproducts

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Keywords

Enzymatic reactions, Xylanase activity, Xylooligo-saccharides, Agricultural Byproducts, *Bacillus*

A B S T R A C T

Enzymatic reactions especially in xylanase activity are taking place due to the degradation of proteins into amino acids or peptides by a variety of microorganisms. An investigation was taken up to find out the activity of proteolysis by *Bacillus* species under controlled condition. Attempts were made to isolate *Bacillus* species from three samples such as water, egg granules and flavour juice. A total of seven isolates were obtained from these samples and were designated as WB-1, WB-2, EB-1, EB-2, JMB, JLB and JGB. The isolated bacteria were characterized based on their morphological, culture characteristics and biochemical tests. Further, the results were compared with the Bergey's manual of Determinative Bacteriology, which showed *Bacillus cereus*. The activity of xylanase was studied by using two different media such as albumin agar and skim milk agar amended with agricultural products. The results revealed that it was found to be higher in term of the formation of halo zone around colonies than albumin agar medium.

Introduction

Prebiotics is a term coined by Professor Gibson and Roberfroid (1995) and are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth of one or a limited number of bacterial species in the colon, such as *Bifidobacteria* and *Lactobacilli*, which have the potential to improve host health." Prebiotics consist mainly of oligosaccharides, which are sugar molecules of three to six monomers in chains. Oligosaccharides are relatively new functional food ingredients that have great potential to improve the quality of many foods. These include non-carcinogenicity, a low calorific value and the ability to stimulate the growth of beneficial bacteria in the colon.

Prebiotics are not digested but go straight to the lower gut where they are fermented by specific bacteria, stimulating the bacteria's growth. They have antimicrobial activity, anticarcinogenic activity, hypo-lipidemic activity, anti-osteoporotic activity, blood glucose-modulatory activities, immune modulating activities, help in improving mineral absorption and balance, rid the gut of harmful microorganisms, and help in prevention of constipation and diarrhea. Xylooligo-saccharides (XOS) are sugar oligomers made up of xylose units. Xylan is a major component of plant hemicelluloses that could potentially be an appropriate starting material for production of a variety of chemicals and compounds (Saha, 2003).

Hemicelluloses are the second most abundant polysaccharide constituents of plant cell walls after cellulose (Harris and Ferguson, 1999). Depolymerization of xylan gives xylose and Xylooligosaccharides (XOS), which shows potential applications in pharmaceuticals, feed formulations, agricultural purposes and food applications. From nutritional point of view, *XOS behave as non-digestible oligosaccharides*. When consumed as a part of a diet, XOS have prebiotic properties due to their preferential utilization by *Bifidobacteria.* Additionally, they have various physiological importances such as reducing cholesterol, maintaining the gastrointestinal health, improving the biological availability of calcium, reducing risk of colon cancer and having a cytotoxic effect on human leukemia cells (Ando *et al.*, 2004).The growing commercial importance of non-digestible oligosaccharides is based on their beneficial health properties, particularly the prebiotic activity. XOS favor the selective growth of *Bifidobacterium spp,* which have important biological effects. XOS can be used as ingredients of functional foods, cosmetics, pharmaceuticals or agricultural products. In addition to the health effects, XOS present interesting physico-chemical properties; they are moderately sweet, stable over a wide range of pH and temperatures and have organoleptic characteristics suitable for incorporation into foods. XOS are advantageous over other nondigestible oligosaccharides in terms of both health and technological related properties.

However, the comparatively high production costs require further development of processing and purification technologies. Considering the economyical and environmental reasons, nowadays utilization of major agricultural wastes for industrial purposes has gained much importance. In this line, Rice bran and Finger millet seed coat can be employed for production of value-added products such as prebiotics xylooligosaccharides.

The scope of the present study deals with the enzymatic processing of agricultural by-products such as Rice bran and Finger millets seed coat (Ragi CO 9) using xylanase for the production of prebiotic XOS.To develop an efficient and environmentally friendly technique for hydrolysis and conversion of rice bran and finger millets seed coat (CO 9), a low-cost and abundant biomass, to valuable compounds (such as Prebiotic compounds (XOS), Phenolic compounds, Soluble sugars, Organic acids, and Amino acids) in methods followed by Enzymatic treatment.

Collection and proximate analysis of rice bran collected from different parts of Tamil Nadu and finger millets from TNAU. Extraction of xylan and their enzymatic conversion to XOS. Analytical characterization of Xylooligosaccharides by TLC.

Enzymatic method - Advantages

Both chemical and enzymatic methods are used for the isolation of xylooligosaccharides from heteroxylans (Reis *et al.*, 2005). But enzymatic method has several advantages over chemical methods such as their specificity, both to linkage type and substitution pattern, high reaction rates, control over the reaction etc.

Applications of prebiotics

Various foods - table food, baked food, condiments, desserts and snacks, various canned food, candy, pressed meat etc. Various drinks - especially yogurt, lactic acid bacterium beverages, carbonic acid beverages and fermented or non-fermented milks etc. In Drug delivery, cosmetics and mouth washes. Employed in feed, pharmaceutical, products for diabetics, and in cosmetics.

Source - Agricultural By-products

Rice bran

Cereals are grown over 73% of the total world harvested area providing dietary fiber, energy, proteins etc. required for human health (charalampopoulos *et al.,* 2002). Rice bran, an important by-product of cereal industry are rich in non-cellulosic polysaccharides such as arabinoxylans, 1, 3/1, 4-β-D-glucans and lignocelluloses complexes which represent a vast renewable energy resource which can be enzymatically converted into bioactive compounds such as oligosaccharides and phenolic acids. Recently the functional food research has moved progressively towards the development of dietary supplementation introducing the concept of prebiotics which considerably affect the gut microbial composition and activities.

Finger millet (Ragi seed coat)

Finger millet (*Eleusine coracana* L.) is important millet grown extensively in various regions of India and Africa, constitutes a staple food for a large segment of the population in these countries. It ranks sixth in production after wheat, rice, maize, sorghum and bajra in India.

Materials and Methods

Rice bran was procured from local modern rice mills Pvt Ltd., (Redhills, Chennai) and Finger millet white variety CO 9 (Ragi; *Eleusine oracana*) from Department of millets, TNAU, Coimbatore.

Chemicals

All chemicals used were of analytical grade and were obtained from HiMedia, Merck (India).

Enzymes

Glucoamylase (EC 3.2.1.3) from *Aspergillus niger*,
α- amylase (EC 3.2.1.1) from *Bacillus licheniformis*,
Xylanase from *Thermomyces lanuginosus*

Proximate analysis

Rice bran and Finger millet seed coat were taken and analyzed individually in triplicates on dry weight basis for crude protein, crude fat, crude fiber, ash, and total carbohydrate according to AACC methods.

Starch estimation

Samples were treated with 80% alcohol to remove sugars and then starch is extracted with 52% perchloric acid. In hot acidic medium, starch is hydrolyzed to glucose and dehydrated to hydroxymethyl furfural.

This compound forms a green coloured product with anthrone. Glucose (100 mg/ 100 ml) was taken as standard.

Defatting and isolation of free sugars

Bran and seed coat was defatted using petroleum ether in a soxhlet apparatus for 8 hr. The defatted bran (10 g) was extracted with aqueous ethanol (70%, 100 ml × 3), centrifuged and the resulting supernatant was analyzed for total carbohydrate (Phenol: sulphuric acid) and reducing sugars (DNS) (Suhasini *et al.,* 1997).

Isolation of dietary fiber

The defatted sample (1g) was suspended in sodium phosphate buffer (25 ml, pH 6.0, 0.1 M) followed by the addition to termamyl (α-amylase) 0.1ml and kept in a boiling water bath for 15 min to digest the starch. The

contents were cooled, added water (20 ml) and the pH was adjusted to 1.5 with HCl (4 N). Proteins were removed by digesting with pepsin (100 mg) at 40º C for 1 hr. Again water (20 ml) was added and incubated at 40º C for 1 hr. Finally the contents were cooled and the pH was adjusted to 4.5 with 1 N NaOH and filtered through a dried and weighted crucible containing celite.

Insoluble Fiber

The residue retained in the crucible was washed with ethanol (95%, 20 ml) followed by acetone (20 ml). The crucible was kept in an oven (105º C) till the weight became constant and the final weight was taken accurately (D1). The same was incinerated at 550º C for 5 hr and once again its weight (I1) was recorded.

Soluble Fiber

The volume of the filtrate was adjusted to 100 ml and the soluble fibers were precipitated by adding ethanol (95% warm ethanol 60º C, 4 volumes). The precipitate was filtered through celite dried and weighted after drying at 105º C (D2) followed by incineration at 550º C (I2) respectively.

Blank was prepared as above without the sample.

Soluble and insoluble fiber contents were calculated using the following formula;

% Insoluble fiber = D1- (I1 - B1)/ Weight × 100

% Soluble fiber = D2 – (I2 – B2)/ Weight × 100

Destarching

Defatted rice bran and ragi seed coat (10 g) was dissolved in sodium acetate buffer (100 ml, pH 4.8, 0.1 M) and incubated with termamyl (α-amylase, 1 ml ~1000 U) at 95º C for 1 hr. After cooling, glucoamylase (100 mg, ~7000 U) was added to the above solution and incubated at 55º C for 48 hr followed by centrifugation at 3000 rpm for 20 min to separate the supernatant (consisting of glucose emanated from starch) from the residue (dietary fiber).

Isolation of water soluble/insoluble polysaccharides

Defatted and Destarched rice bran (50 g) and ragi seed coat was taken separately and extracted with water (1:10 w/v) at room temperature (25º C) for 2 hr.

The supernatant obtained after centrifugation (3000 rpm, 20 min) was precipitated with 3 volumes of ethanol.

Precipitate was separated out, dialyzed and lyophilized to obtain water soluble polysaccharides.

The residue obtained after centrifugation was dried by solvent exchange and designated as water insoluble polysaccharides.

Enzymatic extraction of Xylooligosaccharides

Water soluble polysaccharides (both 10 mg) was dissolved in acetate buffer (10 ml, pH 4.8, 0.1 M) and incubated with xylanase (5 mg) in a constant shaking water bath at 50º C for 5 hr and subsequently the reaction was stopped by the addition of 3 volumes of ethanol and the precipitated material was removed by centrifugation (3000× g, 15 min).

The resultant supernatant consisting of mixture of monosaccharide and xylooligosaccharides was concentrated and used for further analysis.

Analytical characterization

Thin Layer Chromatography (TLC)

The concentrated crude XOS obtained from enzymatic hydrolysis is checked for the separation of oligosaccharides using Thin Layer Chromatography technique.

Two solvent systems were used.

Acetone: n-Butanol: Water (8: 1: 1)

2-Propanol: Ethyl acetate: Nitromethane: Water (6: 1: 1: 2)

The compounds in the TLC plate were identified by spraying the plates with the following mixture- Methanol: Sulphuric Acid (9: 1) + Orcinol (0.2 g).

Calculation of Rf value

$$\text{Rf Value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Results and Discussion

Starch estimation by Anthrone method

Starch, which is composed of several glucose molecules, is a mixture of two types of components namely amylose and amylopectin. Starch is hydrolysed into simple sugars by dilute acids and the quantity of simple sugars is measured calorimetrically at 600 nm.

Defatting and isolation of free sugar -.

Spectroscopic estimations

Estimation of total carbohydrates by phenol-sulphuric acid method

Total carbohydrate in the sample was estimated by phenol-sulphuric acid method. The sample (50 µL) was taken in a test tube and made up to 500 µL, phenol (5%, 0.3 ml) and concentrated sulphuric acid (1.8 ml) were added successively and mixed thoroughly, and the resultant solution was cooled to room temperature for 15 min. Absorbance was read at 490 nm. Sugar content was determined by referring to the standard graph, prepared by using D-xylose (5 µg/50 ml).

Estimation of reducing sugar by DNS method

To the sample (1 ml) in the test tube, DNS reagent (1 ml) was added and incubated in boiling water bath for 10 min. Content was then cooled and diluted with distilled water (2 ml). Absorbance was read at 550 nm. Reducing sugar content was determined by referring to the standard graph prepared by using D-xylose (50 mg in 50 ml)

Isolation of Dietary fiber

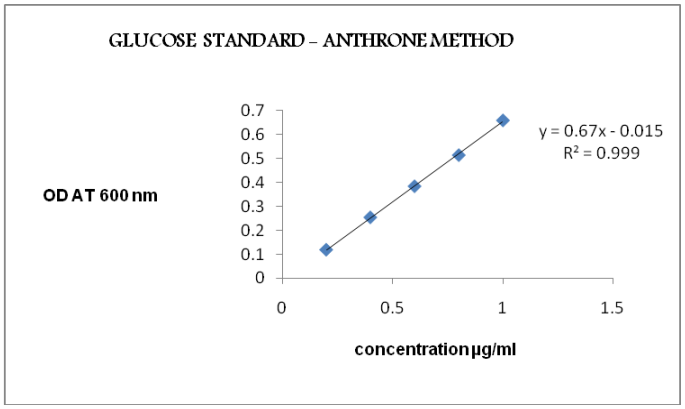
Finger Millet

Insoluble Dietary Fiber – 18.1%
Soluble Dietary Fiber – 1.7%

Rice Bran

Insoluble Dietary Fiber – 16.7%
Soluble Dietary Fiber – 2.0%

Graph.1 Starch Estimation by Anthrone Method

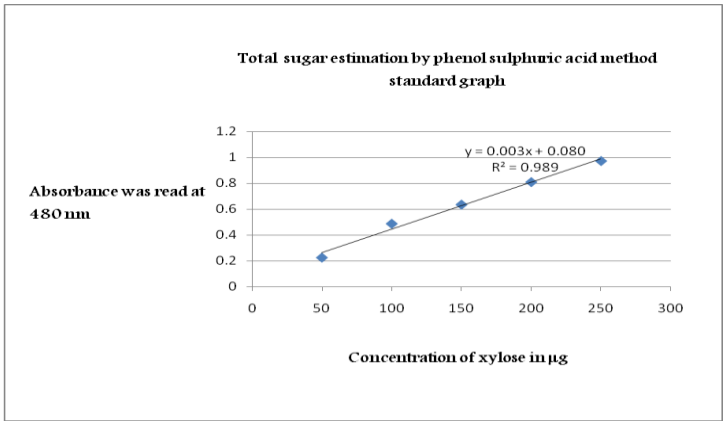


Starch content results were obtained by anthrone method and were found to be

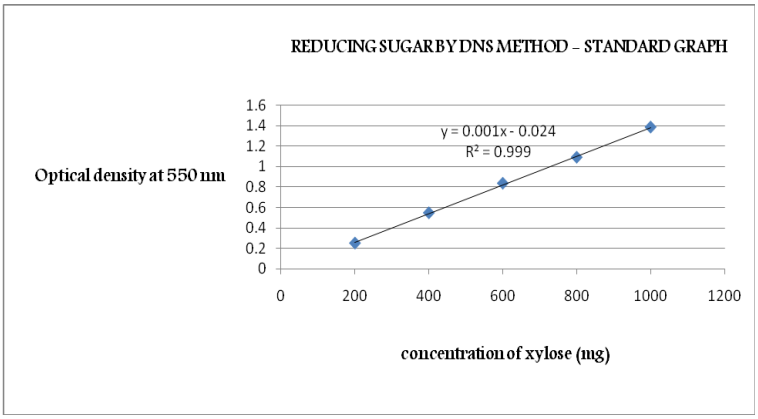
Rice bran - 0.04g/100 ml.

Finger millet's seed coat - 0.036g/100 ml

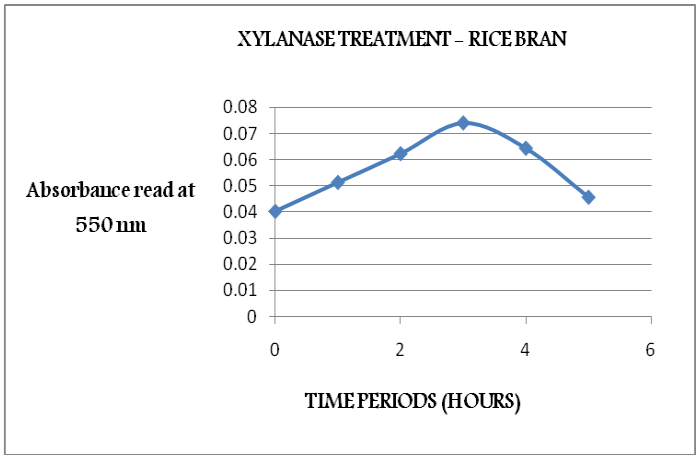
Graph.2 Estimation of total carbohydrates by phenol-sulphuric acid method



Graph.3 Estimation of reducing sugar by DNS method

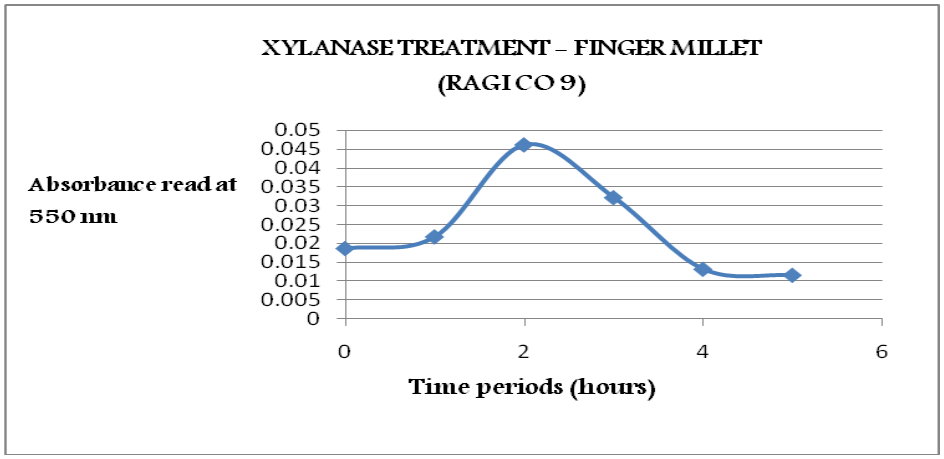


Graph.4 Enzymatic activity of rice bran



Enzyme activity/ molecular weight of xylose = 0.0025 µmoles/min/mg of protein
Specific activity = Enzyme activity/mg of protein
= 0.0025/0.58 = 0.004 µmoles/min/mg of protein

Graph.5 Enzymatic activity of Finger millet seed coat (Ragi CO 9)



Enzyme activity/ molecular weight of xylose = **0.0028 µmoles/min**
Specific activity = Enzyme activity/mg of protein
= 0.0028/0.58 = 0.0048 µmoles/min/mg of protein

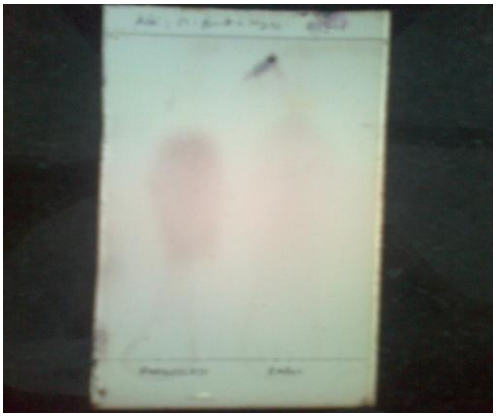
Table.1 Proximate Composite Data

Proximate analysis	Rice bran	Finger millet (*eleusine coracana*) seed coat
Moisture	11.9%	10.7%
Ash	7.9%	9.6%
Crude protein	13.0%	13.7%
Crude Fat	15.7%	1.3%
Crude Fiber	3.1%	2.0%
Total carbohydrate	48.4%	62.7%

Fig.1 2-Propanol: Ethyl acetate: Nitromethane: Water (6: 1: 1: 2)

Rf value:
Rice Bran – 0.71
Finger Millet – 0.77

Fig.2 Acetone: n-Butanol: Water (8: 1: 1)



Rf Value:
Rice Bran – 0.74
Finger Millet – 0.76

Determination of Total Sugar for WSP

Total sugar in the samples were estimated by phenol-sulphuric acid method and found to be as follows:

Rice Bran- 650 µg/ml
Finger Millet – 312 µg/ml

Liberation of oligosaccharides from rice bran and finger millet seed coat

Rice bran and seed coat when subjected to commercial xylanase hydrolysis resulted in crude XOS. Xylanase from *Thermomyces lanuginosus* is an endoxylanase, which cleaves arabinoxylans randomly (unbranched regions of both internal and external chains). After getting crude XOS the total sugar content was estimated.

Rice bran obtained after defatting and destarching was 73.5% and that of finger millet was 71.2% and the Water Solube Polysaccharide (WSP) was isolated. Total sugar content for WSP was found followed by Enzymatic treatment by xylanase enzyme for obtaining crude XOS. Xylanase showed optimum activity at pH 4.8 and temperature 40°C with rice bran and finger millet as substrates separately. The crude XOS was analysed for the presence of oligosaccharides by Thin Layer Chromatography technique with monosaccharides taken as standard and the retention factor values were interpreted. The research work will be continued with the determination of WSP by Gas-Liquid Chromatography technique using standard sugars. Also, standardisation of enzymatic method at various buffer concentrations (pH, Temperature, incubation time) is yet to be carried out to obtain the crude XOS. This will be followed by its purification and analysis using HPLC – ELSD. Finally, *in vitro* fermentation of crude XOS and purified XOS will be carried out for bringing out its functional characterisation as well.

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