ADDIS ABABA UNIVERSITY

INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

EXTRACTION AND OPTIMIZATION OF PROCESS PARAMETERS FOR XYLITOL PRODUCTION USING MICROBES FROM CORNCOBS

A Thesis Submitted to the School of Graduate Studies of of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Process Engineering

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Advisor

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Addis Aaba

Addis Ababa University

Institute of Technology (AAIT)
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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other University, and that all sources of materials used for the thesis have been duly acknowledged.

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ABSTRACT

This Research paper aimed mainly on the extraction of xylitol from corncob hydrolysate and optimization of fermentation parameters such as; fermentation period, yeast dose and initial pH. Raw corn cobs were collected from market in Addis Ababa, Ethiopia, and was hydrolyzed with 1% (w/w) dilute sulfuric acid. Hydrolysate was detoxified by activated carbon treatment and fermentation was carried out using the well-known xylitol producing Candida yeast, Candida mogii. Serial dilution method, Colony Forming Unit, was used to enumerate yeast cells present in a milliliter of inoculum. Box Behnken experimental design was employed to correlate the fermentation parameters to xylitol yield. Yeast dose was expressed in terms of percentage volume of fermentation medium on levels of 5%, 7.5% and 10% along with three levels of initial pH, 4.5, 5 and 5.5, and fermentation period of 24, 48 and 72 hours. HPLC with RI detector and Amine NH2 column was used to determine xylitol concentration. The highest xylitol yield of 23.56 gL^{-1} was obtained at 5% yeast dose, 45 pH and 45.68 hours. The results showed area of xylitol peak and the presence of sugar residuals. According to the experiment design, all three parameters have significant effect on xylitol yield. The effect of fermentation period and yeast dose showed dominant effect. The combined effect of yeast dose with pH, and yeast dose with fermentation period were also observed to be significant. The adequacy of the model was tested by analysis of variance. The value of R-squared for the developed correlation is 0.9821implying 98.21 percent variation in response is attributed to the experimental variables studied. In generally, this study has shown an alternative for the chemical production of xylitol can be carried out by candida mogii with economic and environmental friendly conditions from agricultural residue as lignocellulosic material. The yield of xylitol was promising with all parameters and down-stream process.

Keywords: Corncob, Candida mogii, Hydrolysate, Xylitol, Xylose

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LIST OF ACROMNYMS

ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
ANOVA	Analysis Of Variance
BMI	Body Mass Index
CFU	Colony Forming Unit
CSA	Central Statistical Agency
DP	Degree of polymerization
EBI	Ethiopia biodiversity Institute
ESSP	Ethiopia Strategy Support Program
FTIR	Fourier Transform Infrared spectrometry
GDP	Gross Domestic Product
HMF	Hydroxyl-Methyl-Furfural
HPLC	High Performance Liquid Chromatography
IDF	International Diabetes Federation
LCM	Lignocellulosic Materials
LDP	Lignin Degradation Products
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NDF	Neutral Detergent Fiber
NVC	Nonvolatile Components
PDA	Peptone Dextrose Agar

PDB	Potato Dextrose Broth
PPP	Pentose Phosphate Pathway
OD	Optical Density
RID	Refractive Index Detector
RSM	Response Surface Methodology
UV	Ultraviolet
XOS	Xylo-oligo-saccharides
XR	Xylose reductase
XRH	Xylose dehydrogenase

1. INTRODUCTION

1.1 Background

Growing up as a kid, sweets were the most exciting treats. Parents positively manipulate their children's behavior promising a reward. This trend keeps going until parents decide their kids should not have more sweets, not because they don't want to make them happy but to protect them from health problems that comes along with frequent consumption of sweets. Abnormal weight gain and tooth decay are the major threats. Uncontrolled abnormal weight gain, leads to Obesity.

Obesity is measured by one's Body Mass Index (BMI) which is calculated by taking a person's weight in kg and dividing it by the person's squared height in meter. A person is refereed as obese if the BMI is greater or equal to 30. In Africa, despite a high prevalence of under nutrition, the prevalence of obesity is increasing at an alarming rate. It is estimated that 25 to 60% of urban women are overweight. In Ethiopia, one study conducted in Addis Ababa in 2007 reported that the prevalence of overweight and obesity on elementary school students were 7.6% and 0.9% respectively. (Sub et al., 2014)

Overweight or obesity pose major risk for serious diet-related chronic diseases, including cardiovascular disease, hypertension and stroke, and Type 2 diabetes. As one gets older diabetes prevents the intake of certain food products because of their sugar contents. Ethiopia is one of the 32 countries of the IDF (International Diabetes Federation) in Africa region. 425 million people have diabetes in the world and more than 16 million people in the Africa region; by 2045 it will be round 41 million. Out of 49,483,000 adult population, there were 2,567,900 cases of diabetes in Ethiopia with 5.2% prevalence in 2015. According to World Health Organization, in 2016 3,980 deaths were recorded which were caused by diabetes between the age 30–69 and 4,870 deaths above the age 70. The same report shows 5,420 deaths were attributable to high blood glucose between the age 30-69 and 6,850 deaths above the age 70 (*Ethiopia*, 2016, Diabetes Country Profile). This is where the necessity of a sugar substitute with equal or approximate sweetening capacity and much lower calorie and health treat emerges.

Xylitol is a naturally occurring sugar alcohol having five carbon atoms and five hydroxyl groups, ($CH_2OH – CHOH – CHOH - CHOH-CH_2OH$) with molecular weight 152.15 g mol^{-1}. It is a polyol, with a sweetening power similar to sucrose, found in fruits and vegetables. It also appears as an intermediate in mammalian carbohydrate metabolism: for instance, human adults produce between

5 - 15 g xylitol/day. (Engineering, Ourense, Politocnico, & Lagoas, 1998). It's equivalent sweetening power with sucrose integrated with other favorable properties, such as anti-carcinogenicity, good gastrointestinal tolerance, possess only one-third caloric content than conventional sugar and near insulin-independent metabolism in humans, makes xylitol a perfect candidate as sugar substitute.(Nair & Zhao, 2010)

For many interesting characteristics, xylitol is a feed stock of great interest to food, odontological and pharmaceutical industries. For instance, with fructose, xylitol is the sugar recommended for diabetic patients (Ylikahri, 1979). The xylitol tolerance by diabetics lies in the fact that the two different pathways of its human metabolism (direct absorption, mainly in the liver, and indirect metabolism by intestinal bacteria) are not insulin-mediated. Its' *anti-ketogenic* effects, ability to inhibit the formation of ketone during metabolism of fats, makes xylitol preferable source of sugar in industries producing dietary food. Odonatological, the uniqueness lays in the fact that it is practically non-fermentable by oral bacteria. There is also a decrease in the amount of plaque, when there is habitual consumption of xylitol. (Press, 2014)Because of its negative heat of dissolution, xylitol produces a feeling of vaporization in the oral and nasal cavities and is used as a part of the coating of confectionery or pharmaceutical products (such as vitamins or expectorants, usually in combination with mannitol, sorbitol and citric acids) (Pepper & Olinger, 1988).

These benefits are held reasons for why currently xylitol market lies between 20,000 and 40,000 tons per year with an economic value ranging between 90 and 340 million dollars (Ur-rehman, Mushtaq, Zahoor, Jamil, & Anjum, 2015). Yet this seems limited market share as a sweetener which is likely to be caused by xylitol's comparatively high production cost, estimated about 10 times that of sucrose or sorbitol. Xylitol is currently manufactured by chemical hydrogenation of pure D-xylose in the presence of nickel catalyst at prominent temperature and pressure, which is an energy and cost demanding, yet yielding a product with a high purity (99.5 %) and of 50–60 %, with respect to the initial xylose. (Technology, 2011)

Alternatively, xylitol can be produced by biological process which shows certain advantages like milder conditions of pressure, temperature, pH, agitation, cell inhibitors, low xylose purification and downstream processing due to the production of lower amounts of by-products (C. Guo et al., 2006). Still, carbon source and operating cost must be economically competitive to ensure the feasibility of the process. Lignocellulosic materials (LCM) have been a promising carbon source.(Technology, 2011)

Corn cob, Lignocellulosic materials (LCM), is a major waste obtained in corn production and is a promising and attractive alternative for xylose rich hemi cellulosic hydrolysate stream (Yewale, Panchwagh, & Rajagopalan, 2016). Since it is a renewable source of fibrous lignocellulosic material that contains about 37% hemicelluloses, a carbohydrate polymer mainly constituted by xylose units, it could be hydrolyzed to yield a xylose solution and then used as fermentation media to obtain xylitol with the aid of micro-organisms. (Technology, 2011)

Ethiopia has considerably increased corn crop production in the last few years because of the government's policy of promoting alternative agricultural practice centering corn production. It is estimated that in the year 2015/2016, 33.87 Qt. per Ha of corn is cultivated in the country. (Cochrane & Bekele, 2018). This mass production thereby leaves an enormous supply of waste corn cobs. Due to their abundance in the country, corncob is very inexpensive and easily obtained to be put to use in many re-use studies such as Biomass fuel, activated carbon and xylitol Production.

This study is focused on the microbial production of xylitol using corncob, the country's richest agricultural waste. The effects of experimental parameters such as fermentation time, pH and yeast dese (inoculum/fermentation broth ratio) was examined in order to get optimum conditions of thesse parameters. A response surface methodology (RSM) was used to determine a statistical model for these parameters. Statistical designs have been employed in the evaluation of the influence of different factors on the xylitol production by yeasts.

1.2 Statement of problem

Now days cautions should be made on contents and calories of foods and drinks one consumes to lead a healthy and satisfying life. Which forces factories to produce a dietary and healthy products to stay competitive, if not preferable, in the market. This would call the need for substitutes to the most health disturbing yet very frequently used ingredient in product designs. Sucrose is crucially utilized as sweetener in many industries; foods, drinks and pharmaceuticals. However, it is medically known to cause health issues like, obesity, diabetes and tooth decay. In Ethiopia, only in 2016, 8,850 deaths were recorded which were caused by diabetes. In the same year, overweight and obesity on elementary school students were 7.6% and 0.9% respectively (Sub et al., 2014). As a remedy for these international problem, researchers has put up significant effort to explore a suitable substitute from renewable resources.

Xylitol, a naturally occurring sugar alcohol sweetener, has sweetness similar to sucrose but 40 % lower energy, negative heat of dissolution, low viscosity in solution, absence of maillard reaction, higher chemical stability, and several biomedical properties (Yewale et al., 2016). It has been produced both by catalytic chemical hydrogenation and microbial fermentation. Xylitol solution formed by catalytic reduction requires further chromatographic purification, concentration, and crystallization of the product to achieve pure xylitol. Therefore, the chemical process is expensive due to the extensive separation and purification steps.

Microbial production seems to be highly attractive and economically feasible. This process uses bacteria, fungi, and yeast for xylitol production from xylose in hemicellulos hydrolysate. The benefit of the microbial process over chemical procedures is its lower cost due to the non-necessity of extensive xylose purification.

Ethiopia is a country with a lot of agricultural resources with insufficient industrial exploitation of this resources especially when it comes to re-utilization of first hand agricultural wastes in product development. Corn production is very abundant in the country. It was quantified to be 8,116,787 tons in the year 2017, increasing from 2.34 million tons in 1998, growing at an average annual rate of 7.57% (Knoema, Ethiopia crop production, 2018). Even though annual corncob accumulation in the country has not been yet presented in studies, it is fortunate to undertake, from the annual corn production, it is plentiful. However, in many developing countries including Ethiopia, after the use of corn for wide variety of applications the corn cob is often disposed unusably in the fields

as very low economic value agricultural waste or at its best scenario, used as a burning biomass for household. To our knowledge, there is no currently commercial utilization of corn cobs in lignocellulosic composite production. This is due to lack of sufficient awareness, research and technology for the utilization of corncob for industrial purposes to produce much more advantageous products.

As per Ethiopia Minister of Revenue, there has not been a record on pure xylitol import. Yet candies and gums produced, made by using xylitol as a sweetener, are presented on the market with high prices. Hence, the unavailability of xylitol can be credited as why xylitol is not preferred from sucrose. If it can be produced locally, not only will it promote healthy consumption of sugar, but also can be source of income as an export product.

Corn cob, the countries rich agricultural waste, can be renewable lignocellulose raw material used for the production of xylitol using biotechnological procedures. The use of corncob as xylose source, fermentable sugar to xylitol, and investigating the process parameter in bioconversion of xylose to xylitol occurs to be the problem of this research. Special attention is paid to the biochemical fundamentals of xylitol production by yeast cultured in xylose-containing medium (hydrolysate) and the key factor affecting the feasibility of fermentative production of xylitol.

1.3 Objective

1.3.1 General objective

The main objective of this research was the microbial extraction and optimization of process variables for xylitol production using Candida mogii from local agricultural waste corncob.

1.3.2 Specific objective

Specific objectives include

- Composition characterization of corncob
- Investigate the effect of different operational conditions during microbial production of xylitol such as culture/broth ratio, pH and fermentation time in order to get optimum conditions.
- Determine and Investigate combined effects of selected parameters on xylitol yield.

1.4 Significance of the research

Xylitol has applications for at least three types of industries, these are;

1. *Food Industry*: for dietary especially in confectioneries and chewing gums,
2. *Odonatological Industry*: due to its anti-cariogenicity, tooth re-hardening, and remineralization properties, and
3. *Pharmaceutical Industry:* for its tooth friendly nature, capability of preventing otitis, ear and upper respiratory infections, and its possibility of being used as a sweetener in syrups, tonics, and vitamin formulations, (Prakasham et al., 2009).

In Ethiopia, where high mortality caused by high blood glucose is recorded, using healthier sugar substitute has not been an available choice both for industries and consumers. This is because of the combined expense of high xylitol production cost and import charge making it uneconomical to use xylitol. Globally, commercial production of this important sugar is based on hydrogenation of xylose in a nickel-catalyzed process which is an energy and cost demanding. This research is significant in a way it inspires local production of xylitol and studies the alternative biotechnological process of xylitol production using economical methodologies. It examines the main process factors in production to attain optimum conditions. Moreover, the countries' most abundant agricultural waste is used as a raw material contributing to the use of renewable and second hand resource.

1.5 Scope of the study

The scope of this research work covers; a collection and characterization of corncob, methods of breaking down hemicellulose to simple sugar (pentose), and bioconversion of xylose to xylitol. Three main process parameters affecting the fermentation process; yeast dose, pH and fermentation time are investigated and optimized. The research work does not emphasize on downstream process of xylitol such as broth de-colorization, desalination and crystallization as these operations render the xylitol yield obtained from fermentation which could corrupt the conclusion made about the optimum levels of studied parameters. For the sake of curiosity, a sample was processed through steps to crystallization yet in depth work on down-stream process is left as a room for growth for this research.

2. Literature Review

2.1 Overview on corncob

World widely, average yield for maize/corn is about 4.5 t/ha and that of developed countries is 6.2 t/ha, with a harvest of 10 t /ha. In Ethiopia, corn is leading cereal in terms of production, with 6 million tons produced in 2012 by 9 million farmers across 2 million hectares of land (CSA 2011/12). It continues to be a significant contributor to the economic and social development of the country. As it grows under a wide range of environmental conditions between 500 to 2,400 meters above sea level, it is a crop with the largest smallholder coverage at 8 million holders. Compared to Teff, with production of 3.0 million tons and sorghum at 2.7 million tons, corn is the main crop with the greatest production at 4.2 million tons in 2007/08, which makes up for 40% higher than Teff, 56% higher than sorghum, and 75%t higher than wheat production. With an average yield of 1.74 tons per hectare (equal to 3.2 million tons grown over 1.8 million hectares) from 1995 to 2008, Corn has been the leading cereal crop in Ethiopia since the mid-1990s in terms of both crop yield and production. Wheat and sorghum yields have averaged 1.39 and 1.36 tons per hectare, respectively.(*Maize Value Chain Potential in Ethiopia*, n.d.)

Moreover, corn plays a central role in Ethiopia's food security. It is the lowest cost source of cereal calories, providing one and half times and two times the calories per dollar compared to wheat and Teff respectively. In overall, five major cereals; Teff, Wheat, Maize, Sorghum, and Barly are the core of Ethiopia's agriculture and food economy, accounting for about three-quarters of total area cultivated and 29 percent of agricultural GDP in 2005/06 i.e. 14 percent of total GDP.

Crop	Production		Area Cultivated		Yield	
	Level * (quintal/ha)	Annual growth rate (%)	Level * (quintal/ha)	Annual growth rate (%)	Level * (quintal/ha)	Annual growth rate (%)
Cereals	120,629,724	12.2	8,230,211	4.8	14.0	6.2
Teff	24,077,480	15.9	2,337,850	6.7	10.2	7.7
Barley	13,264,217	0.7	1,024,30	3.4	133.0	4.5
Wheat	22,933,077	2.1	1,439,08	0.6	15.9	1.5
Maize	33,142,865	18.9	1,595,2338	.0	20.6	7.8
Sorghum	22,161,808	18.3	1,429,886	7.4	15.4	8.9

Table 2.1 Average annual growth rates of production, area cultivated and yield per cereal crop.

Table 2.1 shows analysis made by Ethiopia Strategy Support Program (ESSP II) on annual growth rates of production, area cultivated and yield per cereal crop based on data from the Central Statistical Agency (CSA). On this analysis, average annual growth was fast for maize and sorghum production, maize being the fastest.

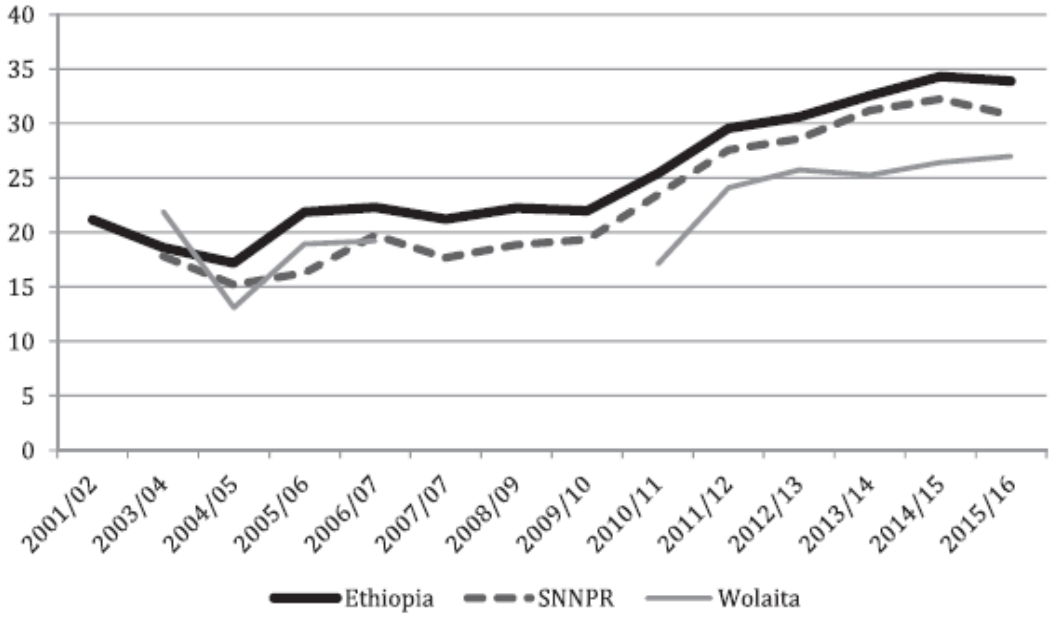
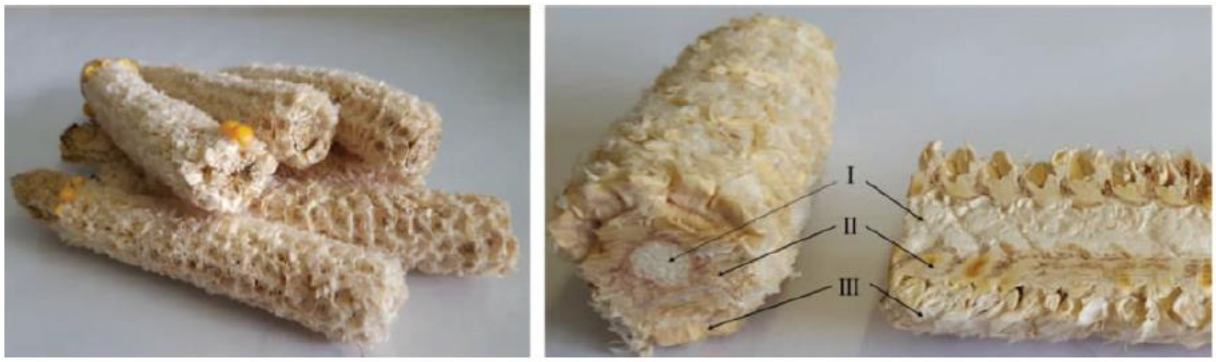


Fig 2.1 National, Regional and Zonal Average Maize Yield (million Qt per Ha).(Cochrane & Bekele, 2018)

Corncobs is an agricultural residue that is generated from maize and remains part of the ear on which the kernels grow. It is produced in large quantities every year. However, it has not been well re-utilized and causes environmental problems. The yield of corn cobs may range from 1.42 to 1.53 dry t/ha (Kaliyan & Morey, 2010). Locally, the best function of this agricultural waste is to be put to use for household purposes.

Corncob lignocellulosic residues can be used as substrates for the production of biofuels. On the other hand, it is a potential renewable source of fibrous lignocellulosic material that contains about >30% hemicelluloses, a carbohydrate polymer mainly constituted by xylose units, which could be hydrolyzed to yield a xylose solution and then used as fermentation media to obtain xylitol, ethanol and other useful products (Yuan, Zhang, Qian, & Yang, 2004) & (Wang, Wu, Tang, Fan, & Yuan, 2012).



a b

Fig 2.2 Corncob macrostructure: a) general view and b) longitudinal section (Jorge et al., 2016).

I - quite soft layer, layer
II - similar to solid softwood layer, and
III- very irregular layer.

Corncob analysis

An investigation was made by Pointner et al. to determine whether different corn varieties show varying compositions. The fiber compositions before the pre-treatment of ten different varieties of corncobs were determined. The contents of cellulose, hemicellulose and lignin were detected gravimetrically according to the modified neutral (Neutral Detergent Fibre = NDF) and acid detergent method (Acid Detergent Fibre = ADF), adapted from the method described by Van Soest et al. (1991). The principle of the detection of cellulose, hemicellulose and lignin is gravimetric analysis by hot filtration, extraction with organic solvents, and drying, followed by determination of the ash contents of the samples. Quantification of Acid Detergent Lignin (ADL) was replaced by direct determination of cellulose and was conducted by hot liquid extraction with acetic acid, nitric acid, and organic solvents followed by filtration according to the method of Horwitz & Latimer (2005).

The Hemicellulose content was calculated as the difference between NDF (cellulose, hemicellulose and lignin) and ADF. Lignin was detected indirectly by determination ADF (lignin and cellulose) and defined as the difference between ADF and cellulose. The contents of cellulose, hemicellulose and lignin of ten different varieties of corncobs were determined. For each of them, three repetitions of analyses were carried out. The following proportions of cellulose,

hemicellulose and lignin were detected: cellulose: 38.8% ± 2.5%; hemicellulose: 44.4% ± 5.2% and lignin: 11.9% ± 2.3%).

It was concluded there is no necessity to choose one specific plant variety for using as a renewable energy source, such as a substrate for the fermentation of bio-ethanol or fat by oleaginous yeasts since there were no significant differences, significance level $\alpha = 0.05$, amongst the corncobs tested.(Pointner, Kuttner, Obrlik, Jäger, & Kahr, 2014)

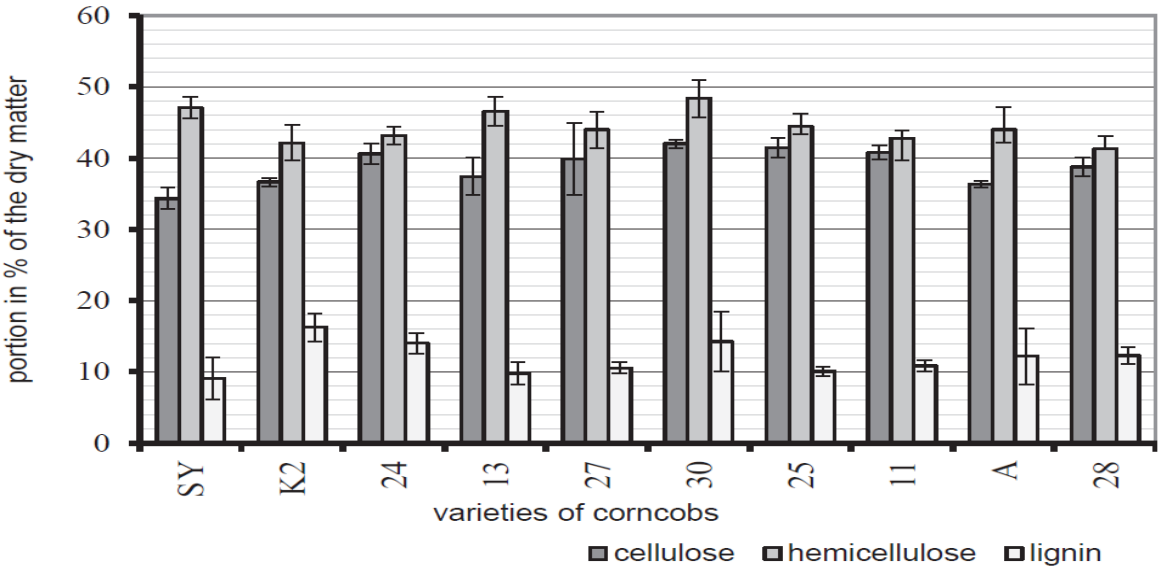


Fig. 2.3 Comparison of the fibers in different varieties of fresh corncob(Anukam, Goso, Okoh, & Mamphweli, 2017)

Another study found that the three major elemental components (C, H, and O) including its clearly exhibited fiber cells make corn cob a suitable feedstock for carbon source. FTIR analysis revealed the existence of –OH, C–O, C–H, and C=C as the major functional group of atoms in the structure of corn cob. (Anukam et al., 2017)

C	H	N	S	O	Reference
50.2	5.9	0.42	0.03	43.5	S. Danje, 2011
46.2	5.42	0.92	0.24	47.22	M. Danish, M. Naqvi, 2015
45.2	6.2	1.3	--	47.0	J. Wannapeera, N. Worasuwannarak, 2008

Table 2.2 Ultimate analysis of corn cob from previous studies (wt.%). (Anukam et al., 2017)

Elements	%
Carbon	43.5
Nitrogen	0.21
Oxygen	48.4
Hydrogen	7.9
Sulphur	0.013
Ash	1.2
Moisture	10 +/- 2
Lignin	6.8
Cellulose	47.1
Hemicellulose	37

Table 2.3 Elemental analysis of Corncob:(Shah & Stability, 2015)

2.2 Overview on Xylitol producing microbial

Microbial are of importance for xylitol production. Xylitol production involves complicated metabolic regulation including xylose transport, production of key enzymes and cofactor regeneration. Thus, screening of naturally occurring xylose-utilizing micro-organisms is a viable and effective mean to obtain xylitol producing organisms with industrial application. Ideal xylitol-producing micro-organisms should be easily cultivated, have high capacity of xylitol production and have excellent tolerance of stress and toxic compounds.

The microbial process of xylitol production uses bacteria, fungi, and yeast for xylitol production from xylose or hemicellulosic hydrolysate. The production of xylitol using yeast has been studied in much depth compared to bacteria and fungi. A few bacteria, such as *Enterobacter liquefaciens*, *Corynebacterium* sp., *Mycobacterium smegmatis*, and *Gluconobacter oxydans* have been reported to synthesize xylitol from pure D-xylose. The best xylitol producers among the microorganisms

are considered to be yeasts. For that reason, various researchers have been studying yeasts broadly in the last few decades (Rafiqul & Sakinah, 2013).

With the aim to discover naturally occurring xylitol producing yeast species with potential for industrial applications, C. Guo et al. cultivated 274 strains on both solid and liquid screening medium with xylose as the sole carbon resource. Five strains were selected on the basis of significant growth and high degree of xylose assimilation. Enzymatic analysis was conducted to compare xylose metabolism in each strain. A wide range of yeast species belonging to the genus Candida were identified for their potential industrial applications, some of them include Candida boidinii (Vandeska et al. 1995), Candida guilliermindii (Zagustina et al. 2001; Rodrigues et al. 2003), Candida parapsilosis (Oh et al. 1998), Candida peltata (Saha and Bothast 1999), Candida tropicalis (Kim et al. 2002; Lopez et al. 2004) and *Candida mogii* (Sirisansaneeyakul et al.).These yeasts showed high levels of xylitol production from xylose, signifying that these may have potential for industrial applications. (C. Guo et al., 2006)

It is known that several factors affect the biosynthesis of xylitol by yeasts such as initial xylose concentration, pH, inocula, culture media and aeration rate(C. Guo et al., 2006; Sirisansaneeyakul, Staniszewski, & Rizziz, 1995). Barbosa et al. screened Forty-four yeast strains from the five genera of *Candida, Kluyveromyces*, *Hansenula*, *Pachysolen*, and *Pichia*, for their ability to convert D-xylose to xylitol. *Candida guilliermondii* and *C. tropicalis* were found to be the highest xylitol producers. These yeasts produced 77.2 g/L xylitol from 104 g/L D-xylose using high cell densities and a defined medium under aerobic conditions. A volumetric productivity of 2.67 g/L·h xylitol with 172 g/L initial D-xylose as substrate was obtained by Horitsu et al. using *C. tropicalis*. The fermentation conditions were optimized by da Silva and Afschar during continuous cultivation of *Candida tropicalis* for xylitol production. *C. tropicalis* produced xylitol at a yield of 77–80% of the theoretical value (0.91 g/g) in a medium containing 100 g/L D-xylose. Among the tested yeasts, Vandeska et al. selected *Candida boidinii*, which gave a higher xylitol yield (0.47 g/g), corresponding to 52% of the theoretical value, with 150 g/L xylose after 14 days. *Candida mogii* gave the highest yield of 0.62 g/g in comparison with 11 other D-xylose-utilizing yeasts studied by Sirisansaneeyakul et al. (Rafiqul & Sakinah, 2013)

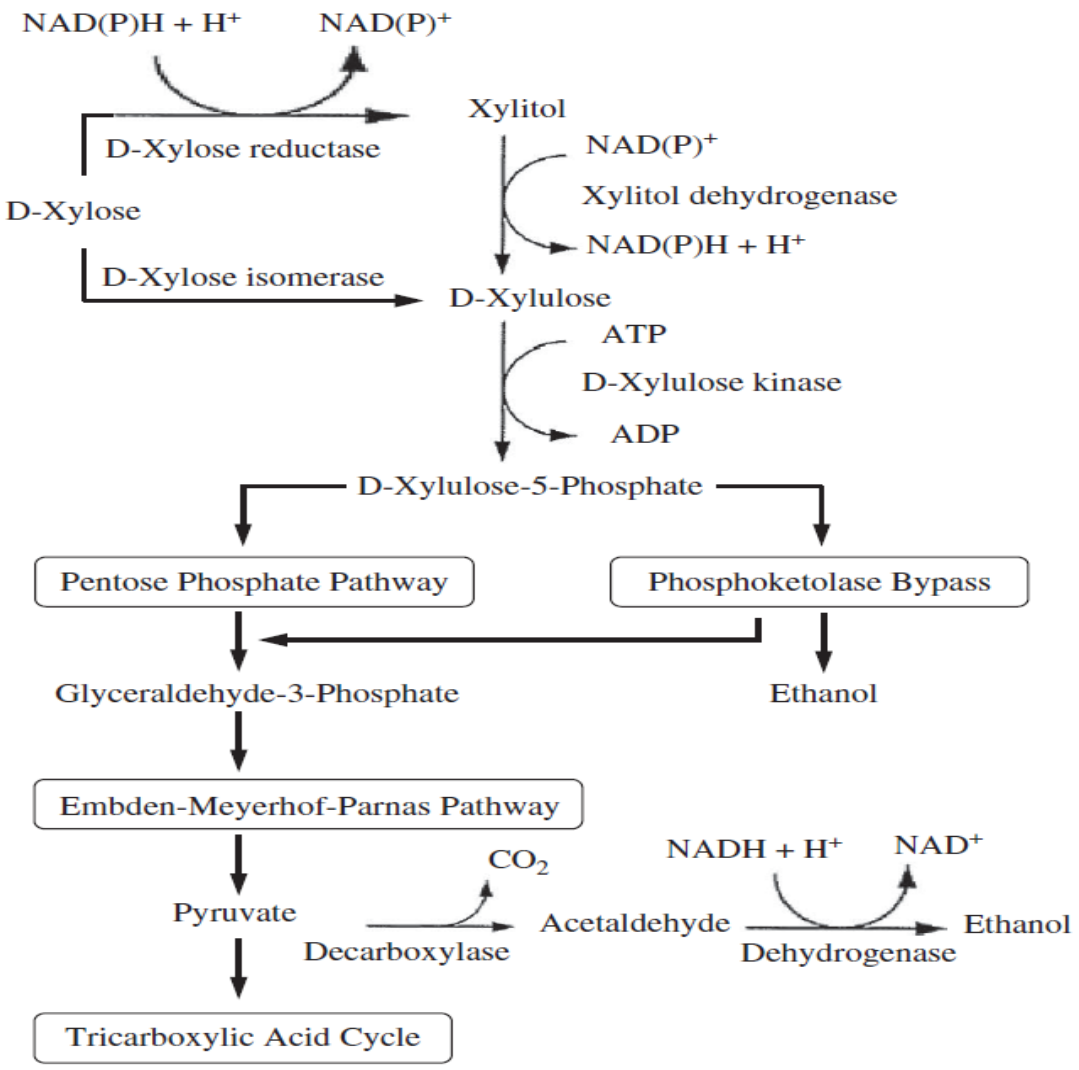


Fig 2.4 Xylose metabolism in microorganisms (Boskovic, 1998).

2.3 Overview on Xylitol

Xylitol ($C_5H_{12}O_5$) is a naturally occurring pentose sugar alcohol used as sweetener. It was first synthesized in 1891 by German and French scientists. During the next five decades, it got very little attention, however sugar shortage during World War II prompted the search for new sweetener (Ur-rehman et al., 2015). It has been then used as a food additive and sweetening agent since 1960s. It is a natural constituent of many fruits and vegetables. Although xylitol levels are usually less than 1%, it has been a natural component of the modern human's diet. The human body also produces 5-15 g of xylitol/day during normal carbohydrate metabolism in the liver.

There have been many studies conducted on the beneficial effects of usage of xylitol in prevention of caries and acute otitis media (State & State, 2010).

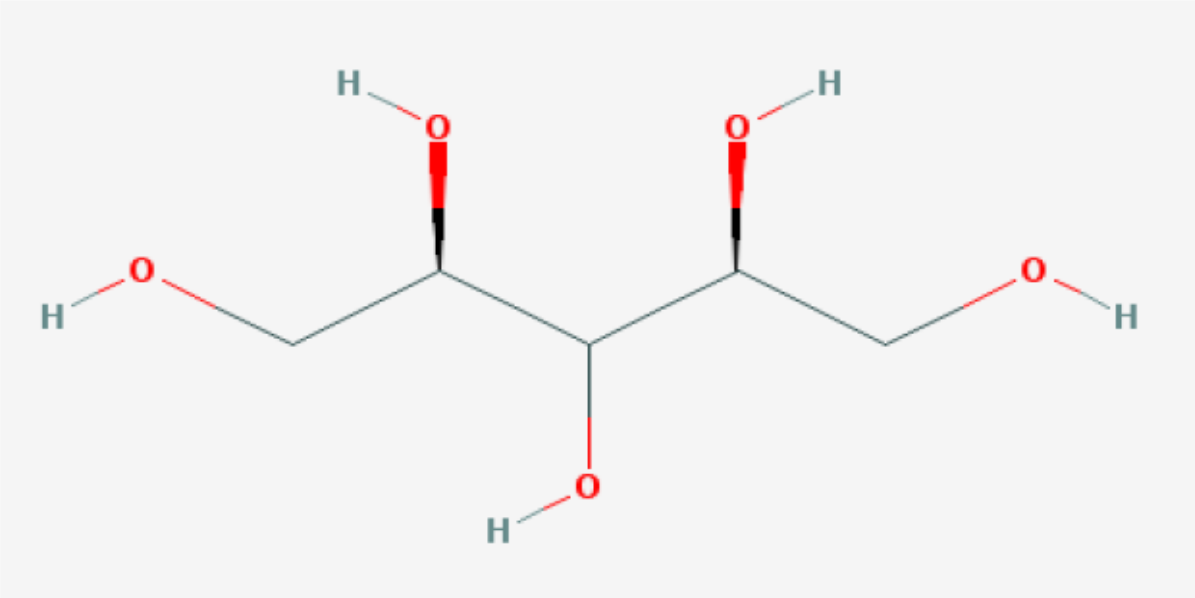


Fig. 2.4 Xylitol structure (Zea D V L Mayerhoff, Franco, & Roberto, 2008).

Xylitol is well-thought-out to be more suitable sucrose source for those lacking insulin to digest sugar. With fructose, xylitol is the sugar recommended for diabetic patients (Ylikahri, 1979). The xylitol tolerance by diabetics lies in the fact that the two different pathways of its human metabolism (direct absorption, mainly in the liver, and indirect metabolism by intestinal bacteria) are not insulin-mediated. Xylitol causes only limited increases in the glucose and insulin blood levels (Hassinger et al., 1981). Furthermore, it is also considered to be a functional food due to its prebiotic nature. These properties make xylitol useful for post-operative or post-traumatic states, when the excessive secretion of 'stress hormones' (cortisol, catecholamines, glucagon, growth hormone, etc.) causes insulin resistance and hinders the efficient utilization of glucose (Ylikahri, 1979 & Ritzel & Brubacher, 1976). In the same way, xylitol can be used to correct catabolic disorders (peripheral lipolysis, stimulation of gluconeogenesis, degradation of muscle proteins) and contribute to the anabolic effects.

Odonatological speaking, xylitol have important commercial implications related to their ability to inhibit the growth of oral bacteria (so reducing plaque formation). It reduces the adhesion of

microorganisms to the teeth surface and also reduces their acid production potential. In addition Xylitol promotes tooth enamel remineralization reversing small lesions and stimulates the flow of saliva without a pH decrease. Remineralization happens because, when in contact with xylitol, the saliva seems to be favorably influence, the chemical composition of xylitol induces the calcium ions and phosphates (Press, 2014; Shimizu et al., 2003).

2.3.1 Overview on Xylitol Application

Food and Confectionery

Food industry uses xylitol in the recipes of food products to improve shelf life, color, and taste of food products. It does not darken or reduce the nutritional value of proteins because it does not undergo Millard reaction. It is added in confectionery for infants and adults. It is used exclusively or in combination with other sugar substitutes in the production of sugar-free chocolate, chewing gum, hard candies, wafer fillings, chocolate, pastilles, and other sweets for diabetics.

The world's leading application of xylitol is in sugar-free chewing gum. It is used to sweeten both stick and pellet forms of chewing gum because it provides rapid sweetness, flavor and quick cooling effect. Due to its rapid drying and crystallization properties, it is often used to coat pellet forms of sugar-free chewing gum (Taylor et al., n.d.). Winkelhausen et al. (2007) studied the potential use of xylitol as a low-energy sweetener in baked goods. Cookies were prepared with 100% xylitol and their characteristics were compared with glucose and sucrose. The storage time of one to two weeks showed no significant effect on texture and flavor of cookies. The cookies prepared with sucrose showed statistically significant affect after three months storage on crispness and tenderness. However, the cookies containing 50% of xylitol got maximum sensory scores including taste, color, flavor, and texture (Taylor et al., n.d.& Mushtaq et al., 2010).

Pharmaceutical Industry

Xylitol can be used as an excipient or as a sweetener in many pharmaceutical preparations. As in foods, the advantages are suitability for diabetic patients, non-cariogenic properties, and non-fermentability. Cough syrups, tonics, and vitamin preparations made with xylitol can neither ferment nor mold. Because xylitol is chemically inert, it does not undergo Maillard reactions or

react with other excipients or active ingredients of pharmaceuticals. According to Feial et al. xylitol-sweetened medications can be given to children at night after tooth brushing without any harm to the teeth.

Chewing gums containing xylitol have been shown to protect from ear infection in children (Uhari et al., 1996). Xylitol-coated pharmaceutical, confectionery products, and dietary complement preparations cause a pleasant cooling in oral and nasal cavities similar to vaporization due to its negative heat of dissolution (Forester, 1988). It is utilized as a stabilizing agent in protein extractions to prevent denaturation of proteins. It has anti-cariogenic properties and reduces plaque formation because cariogenic bacteria cannot metabolize xylitol in its metabolism. Moreover, it has the capacity for moisture retention and hence is used in toothpastes. In tablets, xylitol can be used as a carrier and/or as a sweetener (Laakso et al., 1982). In addition to sweetness, non-reactivity, and microbial stability, it offers the advantages of high solubility at body temperature and a pleasing, cooling effect.

An inhibitory effect on enamel demineralization has been postulated as well. There is also evidence that use of a xylitol-containing dentifrice can result in a significant reduction of *Streptococcus mutans* in saliva. Because of its overall favorable effects on dental health, xylitol has also been applied in other oral care products, such as in mouth rinses and in artificial saliva (Featherstone et al., 1982).

Xylitol is considered to be an ideal alternate sweetener for diabetic patients, as the control of blood glucose, lipid level, and weight control are the three most important objectives of diabetes management. Suitability of xylitol for diabetics lies as it is not transported actively through the intestinal tract and does not require insulin for uptake by liver; therefore, it gives a low glycemic index. Slow and incomplete absorption by the upper gastrointestinal tract leads to very little effect on blood glucose.

Besides from minimum effect on blood glucose, it provides lower calories and a number of other health benefits. In a study conducted on diabetic patients, it was observed that the supplementation of xylitol in the diet up to the level of 30–60 g/day resulted in no harmful effects on diabetic patients, particularly in relation to fat and carbohydrate metabolism (Ur-rehman et al., 2015) ;Yamagata et al., 1969).

Xylitol dose (g)	Positive effect	Negative effect	Reference
4 – 4.5 g/d, 1 yr	Decrease dental caries made in human body	None	Wang and Van Eys, 1981
5-15 g/d		None	Wang and Van Eys, 1981
8.4 g/l	Decrease otitis media	None	Uhari et al., 1996
10-85 g/d, 50dys		Flatulence	Makinen, 2004
25 g/d	Improve glucose and lipid metabolism	None	Otto et al., 1993
30 g/d	Decrease yeast	None	Salminen et al., 1985
45g or >	Decrease decayed, missing or filled teeth	Diarrhea	Wang and Van Eys, 1981
1.6 kg/month		None	Wang and Van Eys, 1981
100 g/d		Transient diarrhea in half the subject	Wang and Van Eys, 1981
400 g/d		½ had diarrhea	Wang and Van Eys, 1981

Table 2.4 Health benefits of Xylitol (Ur-rehman et al., 2015)

2.4 Overview on Xylitol production

2.4.1 Chemical Process

Xylitol is industrially manufactured by reducing pure xylose achieved from hardwood hemi-cellulosic hydrolysate in the presence of a Raney nickel catalyst. The chemical synthesis of xylitol begins with the extraction of xylose from hemi-cellulosic material by acid-catalyzed hydrolysis. The amorphous and non-crystalline structure of hemicellulose allows easy diffusion of the hydronium ions in the polymer matrix, favoring the hydrolysis reaction. The hydrolysates generally contain a variety of sugars (xylose, arabinose, glucose, galactose, and mannose) in proportions that are dependent on the biomass type and experimental conditions.

After purification and color removal, xylose-containing hemi-cellulosic hydrolysate can be used for manufacturing xylitol by reduction of xylose at 80–140◦C and hydrogen pressures up to 50 atmospheres in the presence of metal catalysts (Raney nickel).

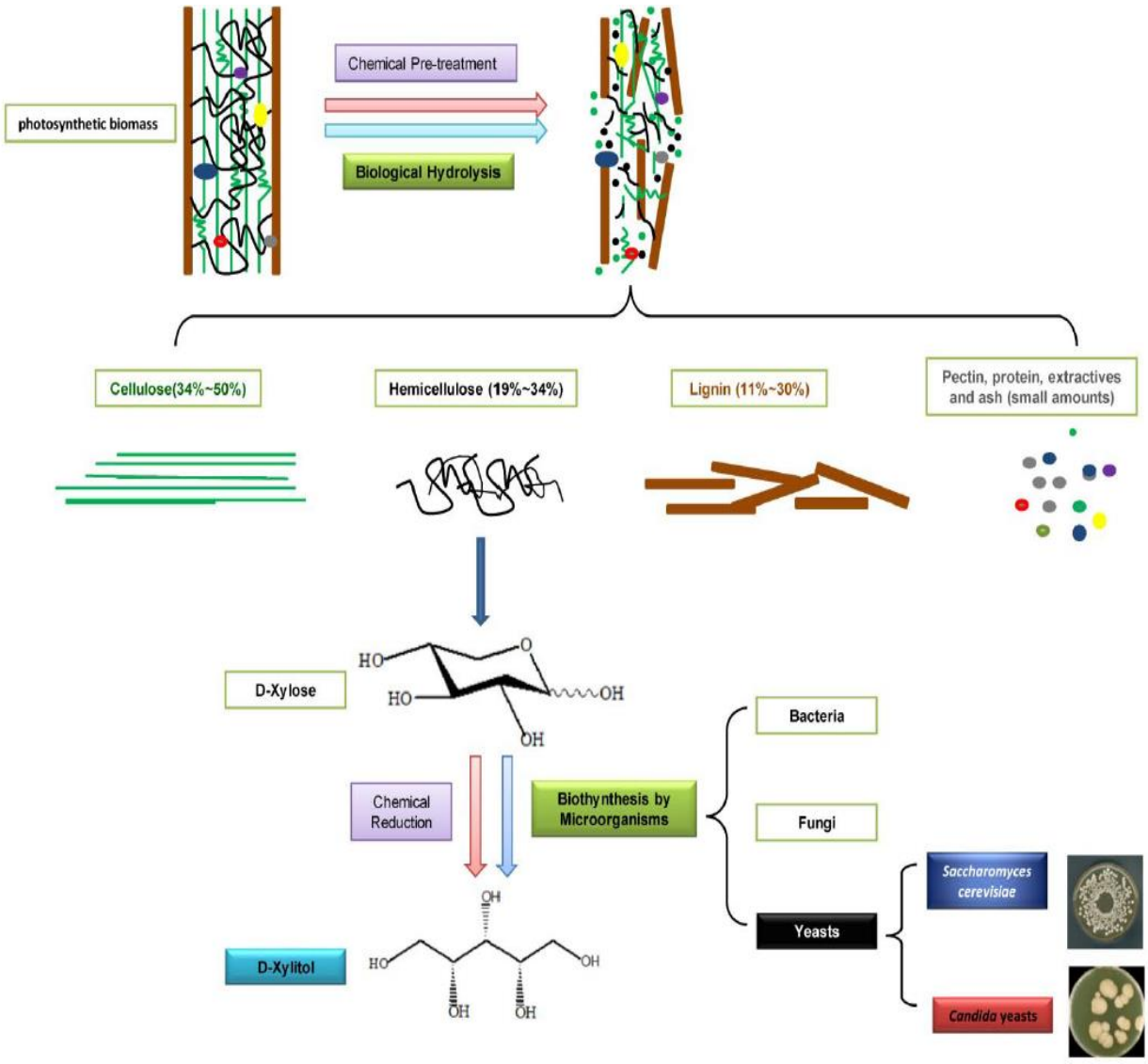


Fig 2.6 Production of D-xylose from Photosynthetic biomass (Chen, Jiang, Chen, & Qin, 2010).

The chemical process for manufacturing xylitol consists of four major steps:

i. Hydrolysis of lignocellulosic biomass by mineral acid;
ii. Purification and separation of the hydrolysate to obtain pure xylose as solution or in crystalline form;
iii. Catalytic reduction of the xylose to xylitol; and
iv. Crystallization and separation of the xylitol.

The xylitol solution formed by catalytic reduction requires further chromatographic purification, concentration, and crystallization of the product to achieve pure xylitol. Xylitol yield is only about 50–60% of the xylan fraction or 8–15% of the starting raw material. Therefore, the xylitol production process is expensive due to the extensive separation and purification steps. Chemical methods are technically complicated multistep processes that have relatively low efficiency. The greatest problem lies in achieving a complete and effective separation of xylose from other hydrolysis by-products. Thorough purification is essential because the catalysts employed in the hydrogenation of xylose are very sensitive to by-products. Despite a large number of drawbacks, the advantages of the chemical process are that

(i) It provides a xylose solution of sufficiently high purity that ensures specific hydrogenation to yield xylitol on a commercial scale;

(ii) Non-hydrogenated sugars are easily separated from the mixture of polyols by ion-exchange chromatographic techniques; and

(iii) It provides easy separation of desired polyols from the mixture after hydrogenation of the wood hydrolysates (containing 75% [*w/w*] xylose and 25% other sugars (such as glucose, mannose, galactose, and arabinose) (Rafiqul & Sakinah, 2013).

2.4.2 Biotechnological Processes

Biotechnological processes for the production of xylitol are based on the use of microorganisms or isolated enzymes. In view of alternatives to the chemical process, two biotechnological processes seem promising: the microbial process and the enzymatic approach employing isolated XR (xylose Reductase). The microbiological process uses bacteria, fungi, yeast, and recombinant strains to produce xylitol from pure xylose or a hemicellulosic hydrolysate.

Bacterial production of xylitol. It has been reported that a few bacteria, such as *Enterobacter liquefaciens*, *Corynebacterium* sp., *Mycobacterium smegmatis*, and *Gluconobacter oxydans*, produce xylitol in small amounts. Screening for xylose utilizing bacteria by Yoshitake et al. showed that an *Enterobacter* strain grew on D-xylose and produced xylitol extracellularly. Xylitol

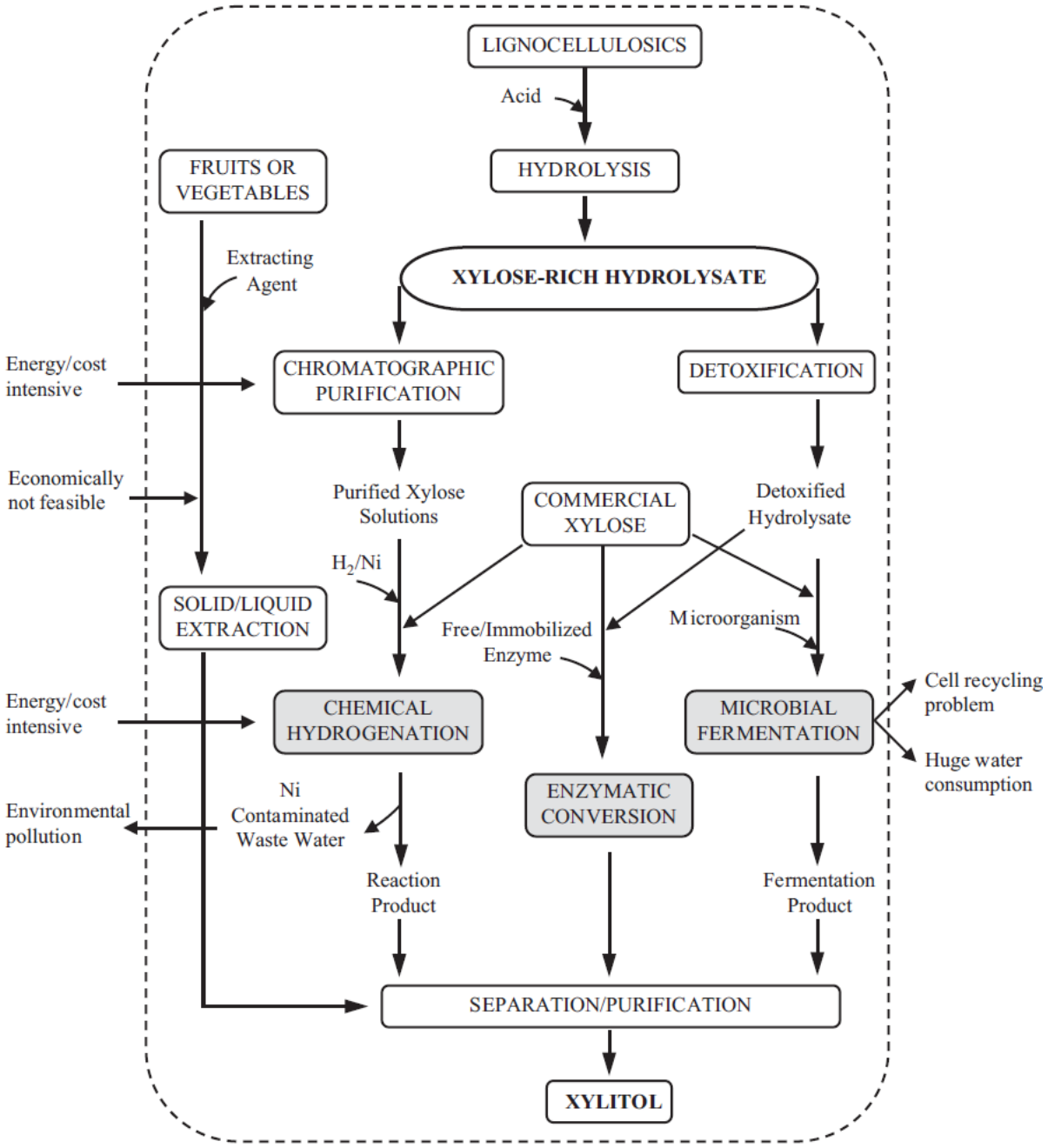


Fig 2.7 Xylitol Production Methods (Adapted from Parajo et. al)

production by the *Enterobacter* strain using D-xylose was by NADPH-dependent XR; this proved that enzymatic conversion was not confined to fungi and yeasts. This strain was reported to yield 33.3 g/L xylitol in a cultivation medium containing 100 g/L initial xylose for 4 days with a productivity of 0.35 g/L·h. The *Corynebacterium* species produced xylitol only when grown in

media having both D-xylose and gluconate. In another experiment, 17 cultures belonging to three genera of facultative bacteria were screened by Rangaswamy and Agblevor, and *Corynebacterium* sp. B-4247 produced the highest amount of xylitol among the screened cultures. The maximum xylitol yield produced in 24 hours was 0.57 g/g xylose using an initial xylose concentration of 75 g/L. About 80% xylitol production yield was reported by Izumori and Tuzaki with D-xylose as the substrate using immobilized D-xylose isomerase and *Mycobacterium smegmatis*. Suzuki et al. tested 420 bacterial strains based on their xylitol-producing capacity from D-arabinitol and observed that *Gluconobacter oxydans* was the best xylitol producer among the isolates, with a yield of 29.2 g/L xylitol from52.4 g/L D-arabinitol after 27 hours of incubation using intact cells as the enzyme source. However, xylose-fermenting bacteria do not currently attract researchers' interest due to the relatively low amount of xylitol produced.(Rafiqul & Sakinah, 2013)

Fungal production of xylitol. The production of xylitol using fungi has been studied to a lesser extent. In xylitol production experiments, the filamentous fungi *Aspergillus*, *Byssochlamys*, *Gliocladium*, *Myrothecium*, *Penicillium*, *Rhizopus*, and *Neurospora* sp. Have been shown to produce small quantities of xylitol in xylose-containing media. Ueng and Gong detected low amounts of xylitol in the fermentation of *Mucor* sp. on sugarcane bagasse hemicellulose hydrolysate. Suihko reported 1 g/L of xylitol production by *Fusarium oxysporum* when grown 2 days in a medium containing 50 g/L of initial Dxylose under aerobic conditions.

There is only one significant report regarding the capacity of the fungi *Petromyces albertensis* to produce xylitol. Dahiya studied xylitol production using *P. albertensis* and detected a yield of 0.4 g/g xylose after 10 days of incubation in a fermentation medium containing 100 g/L D-xylose.

Production of xylitol by yeast.

Xylitol is produced as a metabolic intermediate compound in all organisms whose xylose metabolism takes place in a sequential activity of XR and XDH enzymes. Keeping this in view, various researchers have been engaged in microbial screening studies to identify efficient strains for the production of xylitol(Rafiqul & Sakinah, 2013).

Candida species yeasts are employed for xylitol production as they have well-developed pentose phosphate pathway (PPP) and can grow on xylose only which is a single substrate and energy source. The oxidative phase of PPP produces pentose phosphates from hexose phosphates provides

NADPH which is required for its biosynthesis. The non-oxidative phase produces hexose phosphates and triglycerides from pentose phosphates (Ur-rehman et al., 2015).

Candida mogii was selected for a xylitol production ($Y_{P/S}$ = 0.62 g/g) from 11 strains of D-xylose utilizing yeasts. Systematic kinetic studies are presented for growth and xylitol formation in synthetic medium using D-xylose as the carbon and energy source. Xylitol is produced from D-xylose under aerobic as well as oxygen-limiting conditions, but not in the absence of oxygen (Sirisansaneeyakul et al., 1995)

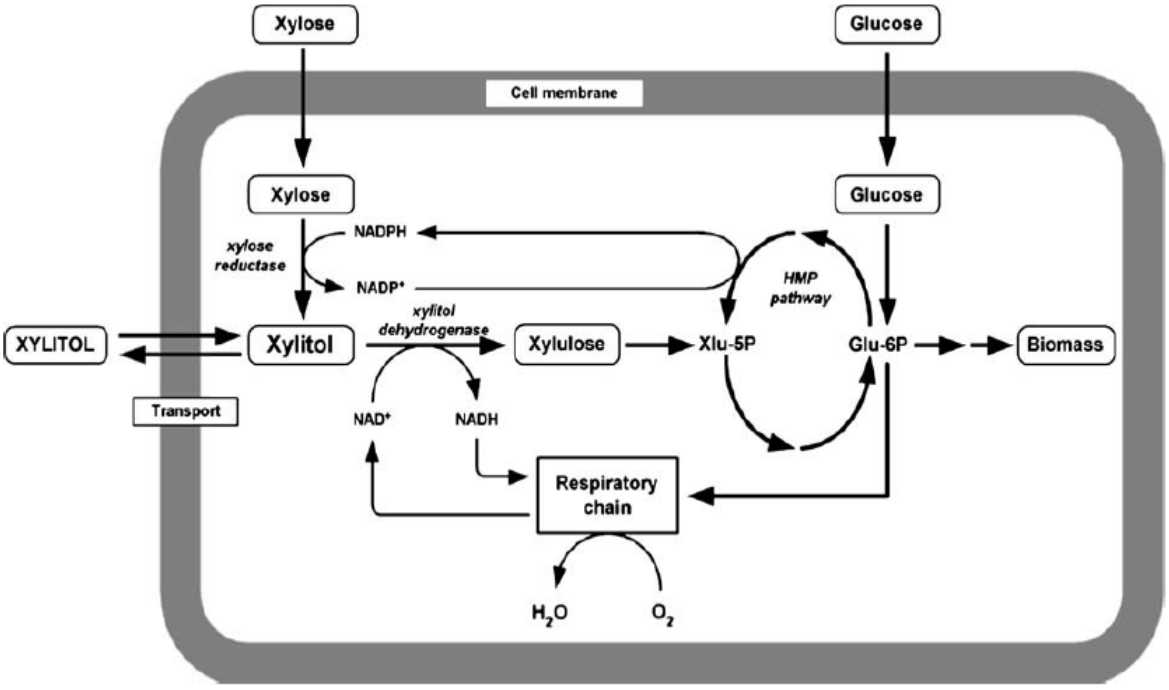


Fig.2.8 Simplified representation of xylose and glucose metabolism in candida mogii (Tochampa, Sirisansaneeyakul, Vanichsriratana, Huub, & Chisti, 2005)

Lignocellulose Material

Lignocellulose, the major component of biomass, makes up about half of the matter produced by photosynthesis. It consists of three types of polymers; cellulose, hemicellulose, and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages.

Cellulose, which is the major chemical components of LCM and is found in an organized fibrous structure. The size of a cellulose molecule is generally expressed by its degree of polymerization (DP) (i.e., the number of anhydro-glucose units that exist in a single chain). The cellulose chains in LCMs are linked together by hydrogen bonds. The long cellulose fibers are in turn held together with hemicellulose and lignin through hydrogen-bonding interaction and covalent interaction, respectively. In LCMs, about 50–90% of the cellulose chain is joined laterally by hydrogen bonds to form a crystalline (ordered) structure, whereas the remaining fraction is less ordered and is called amorphous cellulose.

Hemicellulose, a second major constituent of LCM, is a complex carbohydrate polymer whose chemical composition and structure vary from tissue to tissue within a single plant and from species to species. It is a polysaccharide with a lower molecular weight than cellulose which makes up 25–30% of total wood dry weight. It is composed of a variety of building blocks, including pentoses (e.g., xylose and arabinose), hexoses (e.g., glucose, galactose, and mannose), and uronic acids (e.g., 4-*O*-methylglucuronic and galacturonic acids) with acetyl side chains (Pe, 2002). Hemicellulose differs from cellulose in three main aspects. Firstly, it contains several different sugar units, whereas cellulose contains only β-(1,4)-D-gluco-pyranose units. Secondly, it exhibits a considerable degree of chain branching, whereas cellulose is strictly a linear polymer. Thirdly, the degree of polymerization (DP) of cellulose is 10–100 times higher than that of hemicellulose. In general, Hemicellulose is amorphous in structure and the variety of branching, linkages, and monomer units contribute to its complex architecture and thereby its variety of conformations and function.

Lignin is amorphous and hydrophobic in nature and is not hydrolyzed by acids, but is soluble in hot alkali, readily oxidized and easily condensable with phenol. Cellulose, hemicellulose, and lignin biopolymers are closely associated with each other; thus, biomasses can be considered as composite materials in which lignin acts as a protective agent that prevents plant cell destruction by fungi and bacteria for conversion to fuels and chemicals. (Ur-rehman et al., 2015).

Hemicellulose Hydrolysis Methods

Hydrolysis are generally enhanced by both acids and bases.

Acid Hydrolysis

Acid hydrolysis is a rapid and simple method for lignocellulosic biomass. The hydrolysis conditions vary with raw material type, acid type and concentration, and reaction temperature and

time. Xylose is the most abundant sugar released with a small amount of other sugars in the hydrolysate while fully grown or aged hardwoods or agricultural residues are utilized as feedstocks. Different acids, such as sulfuric, hydrochloric, phosphoric, nitric, and acetic acids, are commonly used as catalysts in the acid hydrolysis process. Acid hydrolysis is mainly of two types based on the concentration of the acid applied: *Concentrated acid* and *Dilute acid hydrolysis*.

Concentrated acid hydrolysis. is a relatively old method. It is conducted at low operating temperature (<50◦C) and atmospheric pressure with high acid concentration (50–70%, *v/v*). Approximately 100% cellulose is converted to glucose in this process. Normally, concentrated acids such as H_2SO_4 and HCl have been employed to treat LCMs. Although the concentrated acid is powerful agents for cellulose hydrolysis, its high acidity presents it toxic, corrosive, and hazardous and thus require special reactors that are resistant to corrosion, which makes the hydrolysis process very expensive. Furthermore, the concentrated acid must be recovered after hydrolysis in order to make the process economically viable.

Dilute acid hydrolysis. Dilute acid hydrolysis is one of the most studied and widely used method among all the hydrolysis methods because it is effective and inexpensive. This method can be applied either as a pretreatment preceding enzymatic hydrolysis or as the main method of hydrolysis. In general, dilute acid hydrolysis is carried out at temperatures as high as 120–230 C^0 and pressures ∼10 atm. The concentration of mineral acids such as H_2SO_4 or HCl used in this hydrolysis process is in the range of 2–5%. The dilute H_2SO_4 can effectively hydrolyze hemicellulose into monomeric sugars (xylose, arabinose, glucose, galactose, and mannose) and soluble oligomers. Compared with other hydrolysis methods, it is especially useful for the conversion of xylan in hemicellulose to xylose, which can be further fermented to xylitol or ethanol by many microbial strains. The major disadvantage of dilute acid hydrolysis over enzymatic treatment is that it generates a hydrolysate that contains not only the sugar needed for bioconversion but also the degradation products of sugar and lignin as well as acetic acid, which could slow down or inhibit the bioconversion of hydrolysate. Hence, it is important to choose less severe conditions that will maximize the yield of xylose while minimizing the formation of by-products such as furfural, hydroxyl-methyl-furfural (HMF), acetic acid, and lignin degradation products (LDPs).

Auto-hydrolysis is an alternative technology for the solubilization of hemicellulose, with various advantages over the dilute acid hydrolysis, namely a more limited delignification and reduced

quantities of sugar degradation products (furfural and HMF). Auto-hydrolysis is typically conducted at temperatures of 160–260◦C, which produces a high-molar mass of xylo-oligosaccharides without altering substantially the structure of cellulose and lignin, allowing improved recovery during further processing (including enzymatic hydrolysis of cellulose or chemical delignification). In auto-hydrolysis, water is the only reactive agent added to lignocellulosic biomass and the reaction includes two steps. In the first step, H_3O^+ coming from water auto-ionization leads to depolymerization of hemicellulose by selective hydrolysis of both acetyl groups and glycosidic bonds. In the second step, H_3O^+ coming from acetic acid also acts as a catalyst, increasing reaction kinetics.

Garrote et al. studied the production of xylooligosaccharides (XOS) from corncob by auto-hydrolysis and found that most of the cellulose was retained in the solid residue, whereas partial delignification (up to 26% lignin removal) was achieved (Parajo & Arbeit, 1999). However, the liquor of auto-hydrolysis is only partly fermentable by microorganisms because the sugars are mainly in the oligomeric form and thus a post hydrolysis step (with dilute acid) is needed to produce the corresponding monosaccharides. A two-step process (auto-hydrolysis followed by post-hydrolysis) is applied to obtain a fermentable hydrolysate. Furthermore, auto-hydrolysis is nonspecific, and other reactions than hemicellulose de-polymerization take place, leading to liquor with a complex composition. Therefore, auto-hydrolysis is not feasible for the production of xylose to a satisfactory level.

Enzymatic Hydrolysis

Hydrolysis of LCM by enzyme technology has been justified as an alternative hydrolysis approach. In enzymatic hydrolysis, the utility cost is low compared with chemical hydrolysis because enzymatic hydrolysis is usually performed at mild conditions (pH ∼5 and temperature 45–50◦C) and does not have a corrosion problem. Compared with acid hydrolysis, enzyme hydrolysis is milder, environmental friendly, and more specific, but it requires pretreatment to enhance the enzymatic digestibility. The yield and rate of enzymatic hydrolysis of LCM are dependent on various parameters such as catalytic properties of enzymes, their loading concentrations, incubation time, raw material type, process variables, pretreatment process, and compounds released during pretreatment method.

Even with a number of advantages, the major drawback of enzymatic hydrolysis is that the presence of solid residuals (mainly lignin) and dissolution of enzymes in the hydrolyzate make it

difficult to separate and recycle the enzymes in order to reduce the cost. In addition, enzymes cannot freely penetrate the lignocellulosic matrix without pretreatment because of the lignin and, thus, the rate of enzyme hydrolysis is slower than acid hydrolysis. Therefore, the feasibility and applicability of enzymatic hydrolysis of hemicellulose requires further study. Dilute acid hydrolysis is still preferable to enzymatic hydrolysis because it is a low-cost, simple, and faster method. (Ur-rehman et al., 2015).

Detoxification Methods

The common and major problem associated with efficient bioconversion of xylose to xylitol is that the hemicellulosic hydrolysate contains a wide range of toxic compounds that are inhibitory to xylose-fermenting microorganisms. These compounds result from hydrolysis of the lignocellulosic biomass and their concentration in the hydrolysate depends on the type of raw material and hydrolysis conditions employed. Microbial growth inhibitors are usually divided into four main groups based on their origin: weak acids, furan derivatives, phenolic compounds, and heavy metals such ass copper, chromium, iron, and nickel released from the hydrolysis equipment. (Ur-rehman et al., 2015).

Duarte et al. reported that phenolic and furfural compounds had strongly detrimental effect on specific growth rate of microorganism and biomass productivity. Because of these negative impacts of inhibitors on microbial metabolism, it is necessary to exploit a detoxification process of acid hydrolysate prior to microbial fermentation for xylitol production. (Parajo & Arbeit, 1999)

A variety of detoxification methods, including physical, chemical, and biological treatments, have been developed to reduce the concentration of inhibitors or to transform them into inactive compounds. Physical detoxification method include vacuum evaporation which has limited scope and helps to minimize only volatile toxic compounds such as acetic acid, furfural, HMF, and vanillin. By employing a vacuum evaporation method, more than 90% of these compounds are removed from wood, rice straw, and sugarcane bagasse hemicellolosic hydrolysates (Mussatto and Roberto, 2004). However, this process enhances concentration of nonvolatile fermentation inhibitors and reduces volume of hydrolysate (Larsson et al., 1999).

Biological detoxification can be done either by using specific enzymes or microorganism. Laccases and peroxides are generally employed for detoxification (Mussatto and Roberto, 2004). Microbial detoxification of hydrolysate involves utilization of toxic compounds for microbial

growth or adaptation of specific microbes for hemicellulosic hydrolysate (Sreenivas et al., 2006) (Ur-rehman et al., 2015)..

Chemical detoxification methods include over-liming, solvent extraction, the use of ion-exchange resins and charcoal adsorption. The effectiveness of a detoxification process depends both on the composition of hemi-cellulosic hydrolysate and on the species of microbes used. Four different approaches have been reported by Taherzadeh et al. for minimizing the presence of inhibitors in hemi-cellulosic hydrolysates:

1. Use bioconversion friendly hydrolysis methods in order to avoid formation of inhibitors;
2. Detoxify the hydrolysate before use for fermentation;
3. Develop and/or use inhibitor resistant microorganisms; and
4. Convert toxic compounds into nontoxic products that do not interfere with microbial metabolism.

When detoxification enhances the production costs, it is necessary either to bypass the detoxification steps or to develop efficient and cheap methods. To bypass detoxification, approach one and three can be considered yet with simultaneous disadvantage of inefficient hydrolysis and cost enhancing for respective approaches.

To study economical detoxification methods; Series of experiments were made by Guo et al. 2013, ten different detoxification treatments were performed. The results show that activated charcoal treatment did not affect the sugar concentration very much, but instead removed most of the furan aldehydes and the phenols. The acids also decreased, but about two thirds remained. Among the different treatments, the activated charcoal treatment was most efficient in removing furans (94% of 1.0 g/L), acetic acid (28% of 1.72 g/L), formic acid (39% of 0.18 g/L), and total phenolic (88% of 1.3 g/L). In this study, the removal of furan aldehydes decreased in the order: activated charcoal (94%) > over liming (35-45%) > anion exchanger at pH 10 (22-26%) > cation exchanger at pH 10 (12-15%) > NH_4OH (10-15%) > anion exchanger at pH 5.5 (6-9%), NaOH (8%) > cation exchanger at pH 5.5 (4-6%). For aliphatic acids, the efficiency decreased in the order: activated charcoal (28-39%) > anion exchanger at pH 10 (22-33%) > anion exchanger at pH 5.5 (20-28%) > cation exchanger at pH 10 (10%) > cation exchanger at pH 5.5 (X. Guo, Cavka, Jönsson, & Hong, 2013).

Activated charcoal detoxification method was applied by Parajó *et al.* 1996 with concentrated rice straw hemi-cellulosic hydrolysate which improved the conversion of xylose to xylitol by the yeast

Candida guilliermondii by 22% as it removed the phenolic compounds, at least partly, from the hydrolysate (Mussatto & Roberto, 2001). The application of activated carbon treatment is also aimed to determine the best conditions to clarify the fermented broth prior to crystallization.(Mussatto & Roberto, 2001). Sreenivas et al. (2006) has worked on xylitol production from sugarcane bagasse and corn cob hydrolysate and reported that combined detoxification methodologies, i.e., chemical and biological are more effective as compared to single treatment process (Ur-rehman et al., 2015).

Regulatory factors in microbial xylitol production.

The microbial production of xylitol has been examined as an alternative to the chemical process, but its viability is dependent on the optimization of the various fermentation parameters. All research papers reporting the conversion of xylose to xylitol utilizing microorganisms have represented that the microbial production of xylitol is influenced by several operating variables. Investigating the effects of these variables is of particular interest as a prerequisite for achieving higher yield and productivity of xylitol.

Xylitol bio production by yeasts is usually influenced by the process and culture (pH, temperature, aeration, reactor time, and inoculum concentration) and the nutritional composition (carbon source, nitrogen source, and micronutrients and their concentrations) (Rafiqul & Sakinah, 2013).

Nutrient composition and inoculum concentrations for yeasts have been investigated by researchers for different xylitol producing yeasts. A research paper was adopted on statistical experimental designs to optimize the culture medium in xylitol production by Candida tropicalis HDY-02 with corncob hemicellulose hydrolysate as substrate (Technology, 2011). Primarily a design was used for identifying the most significant nutrient variables. KH_2PO_4, yeast extract, $(NH_4)_2SO_4$ and $MgSO_4\_7H_2O$ were found to significantly affect xylitol yield by the specific yeast. On the second design, the optimum level of each significant variables were determined. The interactive effects of yeast extract and $MgSO_4\_7H_2O$ on xylitol yield of C. tropicalis HDY-02 were determined to be significant. The optimum combinations of these nutrients for xylitol yield were 5 g /l $(NH_4)_2SO_4$, 1.3 g/l KH_2PO_4, 4.6 g /l yeast extract and 0.6 g /l $MgSO_4\_7H_2O$. Under these optimal conditions, the continuous fed-batch experiments could produce xylitol of 58 g /l with a yield of 0.73 g /g xylose. This experiment was carried out using corncob hemicellulose, sulfuric acid hydrolysis, activated carbon detoxification and fermentation conditions of; 5% (v/v)

inoculation of fermentation medium, at 35C^0 and 200 rpm for 48 hours in 250 ml flask containing 100 ml medium.

Effect of Nitrogenous Sources on Bioproduction of Xylitol

Type and concentration of nitrogen source in the medium influence the xylitol production by microorganism. Palnitkar and Lachke (1992) found that xylose consumption enhanced when an organic nitrogen source was present in the media. Among nitrogen sources, the yeast extract and the urea are the nutrients preferred by the yeasts producing xylitol (Ghindea et al., 2010).

Effect of Temperature on the Bioproduction of Xylitol

The most appropriate temperature for xylitol production by *C. tropicalis* is 30- 35 C^0. However, the yield for xylitol production is temperature independent, if the yeast is cultured at a temperature of between 30 and 37 C^o while temperature above 37◦C, the yield decreases dramatically (Silva and Afshar, 1994; Ghindea et al., 2010). However, the conversion to xylitol by *Candida* sp has been observed constant over the temperature range of 35–40◦C while at 45◦C and higher, the yield declined greatly (Cao et al., 1994 ; (Barathikannan & Agastian, 2016). This may be due to the loss of activities of NADPH and NADH dependent xylose reductase linked with temperature increase (Slininger et al., 1987)

Effect of pH on bioproduction of Xylitol

The optimum pH for xylitol production for *Candida* species is 4.5–7 (Ghindea et al., 2010 (Barathikannan & Agastian, 2016). It has been observed by Cheng et al. (2009) that ascending in the pH from 4.5 to 6.0 leads to dramatic increase in xylitol and productivity. However, the highest yield of xylitol is found at pH 6.0. The findings of El-Batal and Khalaf (2004) show that xylitol production is observed low at pH 3.0 as compared to pH 6.0. Pfeifer et al. (1996) and Rodrigues et al. (1998) have observed that the toxic effect of acetic acid increased due to low pH of the medium because of the entry of acid into the cell in its un-dissociated form. Acetic acid leads to cytoplasmic acidification inside the cells (Barathikannan & Agastian, 2016).

Effect of Oxygen and Inoculum on bioproduction of Xylitol

Oxygen plays an important role on xylose uptake rate by pentose fermenting yeasts. Sreenath *et al* showed that the yeast *Candida shehatae* fermented xylose under strictly anaerobic conditions and

in the presence of oxygen, both cellular growth and xylose consumption were favored. The increase in the initial inoculum concentration also negatively affected both biomass formation and xylitol production. When the medium containing hydrolysate was inoculated with an initial cell concentration of 0.67 g L^{-1} the values of xylitol concentration and cell mass were 39.46 g L^{-1} and 6.74 g L^{-1}, respectively, after 72 h of fermentation. These results decreased to 21.74 g L^{-1} and 4.79 g L^{-1}, respectively, when the initial inoculum concentration was increased to 2.41 g L^{-1}. Wood and Millis, working with *Pachysolen tannophilus,* also showed that xylitol production was reduced when the initial cell density of the medium was increased. This behavior can possibly be attributed to low oxygen availability since a higher inoculum concentration tends to reduce the oxygen level in the medium (Pe, 2002).

Xylitol Recovery from Fermentation Broth

The ideal treatment of the hydrolysate and the downstream processing for xylitol recovery are still a bottleneck on which there is only a few data in the literature. The impurities present in fermentation broth contain nutrients remaining from fermentation that are yeast extract, polypeptides, amino acids, pigments, proteins and inorganic salts. The recovery of dilute concentrations of xylitol from such a complex mixture is a major challenge.

Proposed methods recovering xylitol are ion exchange resins, activated charcoal and chromatography. A loss of xylitol was also observed as a consequence of the treatment with activated charcoal and resins. Gurgel et al. (1995) used both anion and cation exchange resins to purify xylitol from sugar cane bagasse hydrolysate fermentation broth. It has strong affinity for both cation exchange resin and anion exchange, which resulted in 40–55% loss of product (Quimica & Janeiro, 1995).

The loss of xylitol after activated charcoal treatment was proportional to the increase in the activated charcoal concentration. The maximal xylitol loss occurred when 25% of activated charcoal was used. The adsorption property of the activated charcoal in adsorbing substances such as sugars, reduced compounds and also xylitol is well known (G urgel, 1993; Frazer, 1989; R odrigues *et al.,* 1995). Al-polychloride and 10% of activated charcoal were used to efficiently clarify the sugar cane bagasse hydrolysate and reduce the phenoliccom pounds with an insignificant loss of xylitol. As a result, a 93.5% reduction in the phenolic com pound was observed

and xylitol was recovered from the broth with 9.7% of loss of this com pound (Quimica & Janeiro, 1995).

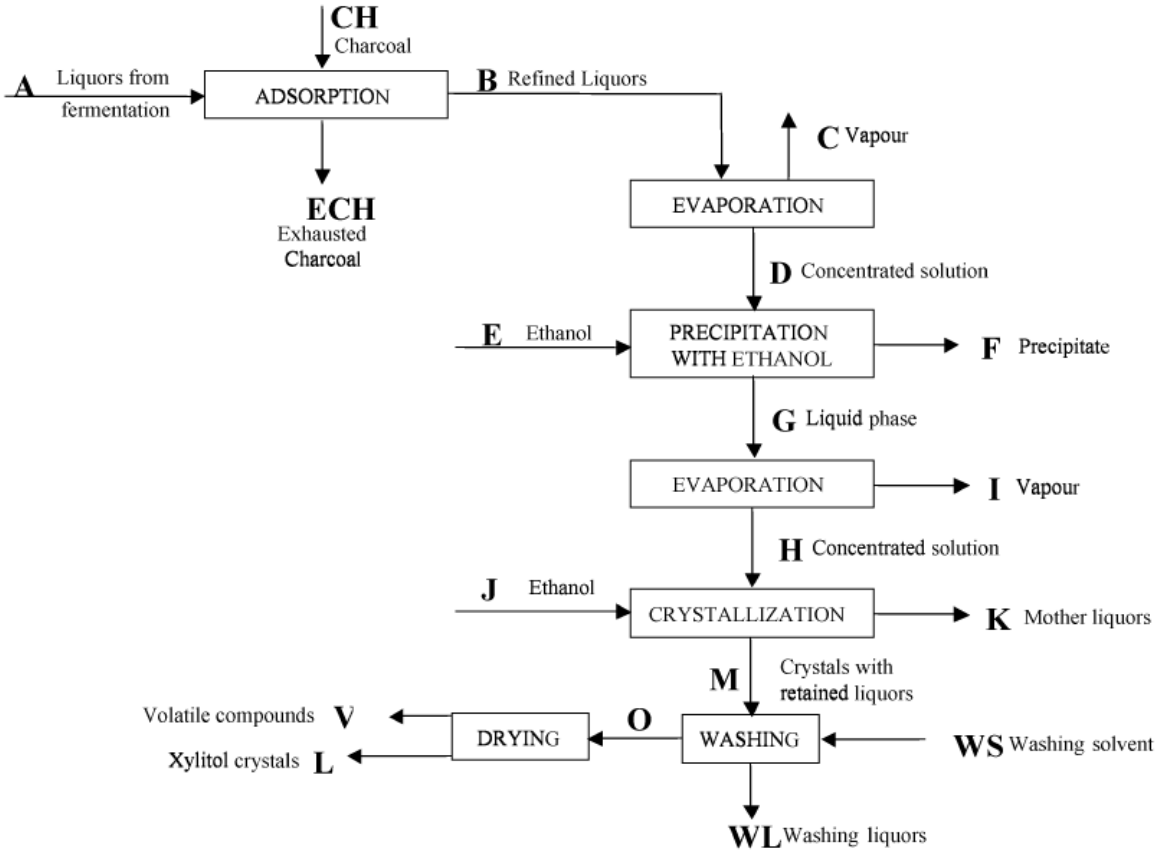


Fig 2.9 Scheme of the process considered in this work for xylitol purification (Wei, Yuan, Wang, & Wang, 2010).

On another research; initial xylitol fermentation broth was decolored with activated carbon (1% M^{-1}, 60°C, 165 rpm), desalted with a combination of two ion-exchange resins, and residual sugars were separated with UBK-555(Ca2+). Then the solution was vacuum-concentrated up to supersaturation (750 g/L xylitol). After adding 1% xylitol crystal seeds, the supersaturated solution was cooled to – 20°C for 48 h. The crystalline xylitol of a regular tetrahedral shape with purity 95% and crystallization yield 60.2% was obtained from the clarified xylitol fermentation broth. Results on this research demonstrate that powdered types of activated carbon (M1, GH-95L, Lh) were better than granular types of activated carbon (LH, WL, W-2) by the high de-colorization ratio and low xylitol loss ratio, less than 5%.

The foremost factor affecting the yield and purity of xylitol in crystallization, are residual sugars. As the concentration of residual sugars increasing, the xylitol purity and yield were decreased. The possible reason is that the residual sugar was crystallized together with xylitol. The existence of the residual sugar does not only affect the purity and yield, but also affects the crystalline form (Wei et al., 2010).

After the removal of crystallization inhibitors, addition of ethanol to the clarified liquor since the solubility of xylitol in water is decreased by the presence of ethanol (Wei et al., 2010) and to precipitate a part of the NVC (mainly proteins, but also uronic acids, ashes, and other nonvolatile compounds) (Omi, 2006). Ethanol – water ratio also affect the degree of crystallization and purity of xylitol crystals. It was possible to increase the crystallization yield in the ethanol water mixture, with the purity degree lowering.

Test	Ethanol : Water (V∶V)	Y24%	Y48%	PD/%
1	1∶3	87.33	95.35	63.48
2	1∶2	83.44	90.66	66.52
3	1∶1	52.67	56.67	69.82
4	0∶1	23.53	37.78	97.57

Table 2.5 Effects of the ethanol/water volume ratio on the xylitol crystallization (Wei et al., 2010).

a) Y_{24}: crystallization ratio after 24 h; b) Y_{48}: crystallization ratio after 48 h; c) PD: purity degree

3. Material and Methods

3.1 Materials

The leading raw materials to be used in this research are: corncob, mainly found in most part of Ethiopia, and Candida genus yeast, which will be obtained from Ethiopia biodiversity Institute.

3.1.1 Equipment

- Jaw crusher
- Screen sieves (Retsch, AS200),
- Electronic balance (OHAS, Ranger), Crucibles,
- Drying Oven (Memmert 100-800),
- Heating mantel,
- Water bath (Grant TXF 200),
- UV-Spectrophotometer (UV-vis double beam, Laomed UVD-3200)
- Colony Counter
- Laminar flow Hood (FlowFast V)
- Magnetic stirrer with hot plate,
- Centrifuge (Hettich universal 320 R)
- pH Meter (Jenway, 3505),
- Incubator (Memmert, model 100-800)
- Vertical-Steam Sterilizer (Lx- B901 (digital))
- Autoclave (Sano-clav),
- Furnace (Vecstar Ltd)
- HPLC, UV and RI detectors

3.1.2 Chemicals

- Distilled Water
- Sulfuric Acid (H_2So_4)

- Activated Carbon
- D-Xylose
- Phenol Crystals
- Potato Dextrose Broth (PDB)
- Peptone Dextrose Agar (PDA)
- Sodium Hydroxide (NaOH)
- Yeast Extract, Magnesium Sulfate 7-hydrate
- Peptone
- Potassium dihydrogen Phosphate
- Ethanol

3.2 Methods

3.2.1 Raw material collection, transportation, sample preparation and storage

Corn, basis of the main raw material corncob, was purchase from a market found in Addis Ababa, locally called Autobus Tera. It was brought to a household for primary separation of the peel, corn seeds and the corncob. Size of the corn cob was reduced as small as possible manually with cocking axe, and placed on an open air exposed to the sunlight for drying. After two days and half drying, the corncob was placed in plastic bag and transported to the chemical engineering laboratory of Addis Ababa Institute of Technology.

The dried corncob was grinded in a Jaw crusher repeatedly in a decreasing grinder mesh size until 1-1.5 mm particle (Technology, 2011) size was obtained. The grinded corncob was sieve analyzed with 1mm and 2mm mesh sieves to ensure particle size range. Afterwards, it was contained in a paper bag for the subsequent operation, Hydrolysis.

From the desired Candida genus yeasts, Candida Mogii was requested from Ethiopia biodiversity Institute (EBI), Addis Ababa. 24 hours incubated, on a petri dish containing PDA and yeast extract, colony of candida mogii was placed in sterilized plastic container and covered with aluminum foil paper. The prepared yeast cells collected and transported to the laboratory and stored in refrigerator at $4C^{o}$.

3.2.2 Corncob hydrolysis

Hydrolysis is used since polysaccharide sugar need to be reduced to simple sugar so it can be consumed by microbes. For dilute acid hydrolysis, sulfur acid was diluted at 0.1% (w/w) with distilled water in a round bottom flask. Equal amount of prepared corncob was weighed in four different conical flasks and diluted Sulphur acid was added to each flask at a solid loading of 10% (w/w) (Liaw, Chen, Chang, & Chen, 2008; Technology, 2011).

The flasks were manually vortexed to promote entire soaking of the corncob and were sealed with aluminum paper to prevent leaking during hydrolysis. An autoclave was set at a temperature of 120 C^0, pressure of 1 bar for one hour and a flasks was loaded in. Each flask was hydrolyzed for an hour one at a time. After an hour of hydrolysis, the flasks were removed an allowed to cool down. A vacuum filtration set was used to separate the liquid fraction (hydrolysate) and solid residue. Filtration was carried out in two steps. First step, without filter paper for simple filtration, and with the use of filter paper to remove fine solid residue. The hydrolysates from all filtrations were pooled together and ready for detoxification.

3.2.3 Detoxification

Hemicellulose hydrolysates with acids are prone to wide range of toxic compounds that could inhibit the activity of microorganisms in bioconversion of xylose to xylitol. Hydrolysate was heated to 100C^0 for 15 min on a hot plate to reduce volatile components. (Technology, 2011). It was then cooled down for activated carbon treatment. A hot plate with digital mixer was set and used to mix hydrolysate and activated carbon at 205 g/g. Temperature was fixed at 30C^0 and agitated at 300 rpm for an hour. (Mussatto & Roberto, 2001; Paraj, Domfnguez, & Dominguez, 1996). The activated carbon, along with the absorbed impurities, in the hydrolysate was separated with vacuum filtration with the aid of a filter paper. Absorbance of hydrolysate before and after detoxification was measured at 420 nm to quantify de-colorizattion ratio. The detoxified hydrolysate was then concentrated under vacuum at 50 C^o for an hour and absorbance was measured to determine xylose concentration for later usage.

3.2.4 Inoculum Development

A yeast media was prepared containing 2.4g Potato Dextrose Broth (PDB), 1g Yeast Extract in a 250 ml conical flask and sealed with cotton. The media was sterilized in an autoclave at 121 C^0, 1 bar for 15 min. it was then allowed to cool in a bio hood protected from microbial contaminations. From preserved at 4 C^o, 100 microliter of Candida mogii was inoculated to the 250 ml conical flask containing 100 ml sterilized media and was cultured in an incubator endangered on a shaker at 200 rpm and 30 C^0 for 48 hrs. (Z D V L Mayerhoff, Roberto, & Silva, 1998).

3.2.5 Fermentation

Pretreated and detoxified corncob hydrolysate was supplemented with nutrients of 1.13 g/L of $MgSO_4$. $7H_2O$, 18.755 g/L KH_2PO_4, 5 g/L peptone and 3 g/l yeast extract, and mixed intensively (Sirisansaneeyakul et al., 1995; Tochampa et al., 2005). Hydrolysate was then placed in three 1000 ml cylindrical flasks each containing 500 ml. 3M Sodium hydroxide solution was prepared to adjust pH according the preset levels (Technology, 2011). It was added drop wise until pH reached 4.5, 5 and 5.5 in the respective three cylinders (Boskovic, 1998; Sirisansaneeyakul et al., 1995; Tochampa et al., 2005).

Fourteen 250 ml conical flasks were washed with tap water and rinsed with distilled water and dried to be filled with fermentation media. The flasks were engaged with 100 ml of different pH hydrolysate and openings were sealed with cotton ready to be sterilized in an autoclave at 121 C^0 and 1 bar for 15 min. Sterilized flasks containing fermentation media was set to cool in a laminar flow hood which was then inoculate with three different dose, 5%, 7.5% and 10% v/v (Sirisansaneeyakul et al., 1995; Wannawilai & Sirisansaneeyakul, 2015).

With 100 ml basis, 5ml, 7.5ml and 10 ml inoculum was injected to fermentation mediums. The viable yeast cell quantification in terms of CFU is presented in table 4.3. Aerobic fermentation was carried out in an incubator integrated with rotary shaker at 200 rpm and 35 C^o for three fermentation time levels, 24, 48 and 72 hrs. (Quimica & Janeiro, 1995; Technology, 2011).

3.2.5 Xylitol Recovery Process

Fermented broth according to their time arrangements were taken out of incubator. Cell biomass was separated by centrifuge at 6000 rpm for 15 minutes (Zea D V L Mayerhoff et al., 2008). Supernatant was collected to be treated with 4g activated carbon per 100 ml biomass free fermentation broth (4% w/v) so colored substances, proteins, uronic acid, and other nonvolatile components (NVCs) are removed.

Decolorized fermented broth was concentrated by vacuum evaporation at 60 C^0 until about 60% of the volume was vaporized to increase its NVC level. The concentrated liquid was mixed with laboratory grade ethanol at 1:3 ratio. 1% (w/v) commercial xylitol was added to precipitate the NVCs other than xylitol (mainly proteins, but also uronic acids, and inorganic compounds) and facilitate crystallization. The remaining solution was placed in the freezer at approximately – 20 C^0 for 48 hours (Wei et al., 2010). Finally, xylitol crystals were separated by vacuum filtration

3.2.6 Analytical methods

Corncob characterization

Moisture Content: Two crucibles were cleaned and oven dried in an oven at 550 C^o over night to eradicate any moisture. They were then maintained in a desiccator until 5 grams of corncob samples were weighed and placed in each. Combined weight of sample and crucible was recorded and the placed in an oven at 105 C^0. After four hours of drying, both samples were taken out of the oven to be weighed and recorded for both combined weight and weight of corncob. For second step recording, samples were placed back to the oven for an hour. Four successive recordings were made until constant weight as obtained on the fifth and sixth trials. The moisture content of corncob sample was calculated as: (Anukam et al., 2017)

$$\%\text{Total Solid} = (W_{c+ds} - W_c)*100/W_{ws} \quad \text{Eq. 3.1}$$

$$\%\text{Moisture content} = 100 - \%\text{ Total solid} \quad \text{Eq. 3.2}$$

Where: c: dried crucibles, ds: dried sample and ws: wet sample.

Ash Content and organic contents: The moisture free corncob samples endured from moisture content examination were maintained in a desiccator to inspect the ash content. These samples

were first tarred on a heating mantel until all organic matters were smoked out and then burned in a furnace at 600 C^o. After three hours of combustion, both combined weight of crucible plus ash and lone ashes left in the crucible were weighed. These recordings were used to compute the ash content or the inorganic residue as:

$$\%Ash = (W_{a+c} - W_c)*100/W_{ods} \quad \text{.....Eq. 3.3}$$

$$\%Organic\ Matter = 100 - \%\ ash \text{.....Eq. 3.4}$$

Where: c: crucible, a: ash, od: oven dried sample

Determination of percentage Extractives: Soxhlet extractor set up was adjusted at 70 C^o on a water bath. 5 g of dry corncob sample was weighed and 300 ml acetone as measured for Soxhlet extraction. After 4 hours of extraction, solid sample was placed on drying tray to cool at room temperature in an open air. Once it riches room temperature, the extractive free sample was placed in a drying oven at 105 C^0 for an hour and weighed. Persistent drying and balancing was carried out until constant weight was obtained. The percentage of extractives (waxes, fatty acids, resin acids and terpenes) content was evaluated as:

$$\%W_E = (W_{rb} - W_{efb}) *100\% / W_{rb} \text{.....Eq.3.5}$$

Where: W_{rb} = raw extractive-laden biomass

$W_{efb.}$ = extractive-free biomass

Determination of percentage Hemicellulose: Sodium hydroxide solution was prepared in a cylindrical flask with concentration of 20 g/L. 300 ml of the solution was taken and mixed with 2 g of extracted dried corncob. The mixture was boiled on a heating mantle for 3 and half hours. It was then cooled and vacuum filtered. The solid residue was washed with distilled water until neutral pH was obtained. It was then oven dried at 105 C^o. The % w/w hemicellulose content was calculated according to equation 3.6. (Foyle et al., 2007).

$$\%\ W/W_H = W_{sbt} - W_{sat} \text{.....Eq. 3.6}$$

Where: W_{sbt} = Weight before treatment

W_{sat} = Weight after treatment

Determination of percentage Lignin: A clean test tube and 72% sulfuric acid was carefully prepared. 0.3 g of dried extractive free corncob sample and 3 ml of sulfuric acid solution were

mixed in the test tube. The test tube was subjected to initial hydrolysis as it was kept at room temperature for 2 hours with cautious manual shaking every 30 minutes to promote efficient hydrolysis. For the final hydrolysis, 84 ml of distilled water was poured in the test tube and was autoclaved at 121 C^o for an hour. The resulting mixture was cooled at room temperature and engaged to vacuum filter. Lignin was present in both and liquid hydrolysate. Acid insoluble lignin present in solid residue was determined by drying the reside in an oven at 105 C^o with continuous weighing until constant weight was achieved and accounting the dry hydrolyzed sample for ash by incinerating it in furnace at 550 C^O. The acid insoluble lignin is the calculated as the weight difference between dry hydrolyzed sample and the ash obtained. Acid soluble lignin fraction determined as liquid hydrolysate sample was analyzed by UV-spectrophotometer for absorbance at 320 wavelength. Total lignin content was calculated as total sum of acid soluble and acid insoluble lignin. (Foyle et al., 2007)

Determination of percentage Cellulose: With the assumption of the total biomass is composed of only ash, extractives, hemicellulose, lining and cellulose, the %w/w of cellulose was obtained by difference from total of the rest of the components. i.e.

$$\%W/W \text{ cellulose} = 100\% - (\%W/W_{ash} + \%W/W_{hemicelllose} + \%W/W_{lignin} + \% W/W_{extractives})$$

Eq. 3.7

Hydrolysate analysis

Concentration of hydrolysate was analyzed with respect to Xylose concentration using UV-spectrophotometer. Primarily, to determine the peak wavelength, 0.1 g of commercial xylose as suspended in 100 milliliter distilled water in a 250 ml conical flask and mixed well producing 1g/L stock solution. 200 microliter of xylose solution was taken into a test tube followed by 200 microliter of 5% phenol solution. One milliliter of concentrated Sulfuric acid was added to the test tube from top. The test tube was vortexed for through mixing and left at room temperature to cool. Two cuvettes were washed and dried, one was filled with distilled water while the other was filled with solution in test tube. The two cuvettes were places in the UV-spectrophotometer machine, distilled water as a blank. Adjusting the spectrometer at “Spectrum” measurement, selecting minimum and maximum wavelength range of measurement 400nm and 800 nm respectively, rang

to be 0 – 1.5 and samples reading of only two cuvettes, wavelength vs range graph was plotted and peak wavelength was read at 566 nm.

Phenol Sulfuric acid method was utilized for the process of peak wavelength determination and xylose standard curve plotting. For the stand curve 8 test tubes were washed and labeled 1 – 8 placed on a test tube rack. The following standard solution per 200 µL were prepare in each test tube from the 1g/L xylose stock solution.

µL Stock solution µg/200 µL	0	20	40	60	80	100	120	140
µL DI water, µL	200	180	160	140	120	100	80	60

Table 3.3 Xylose standard solution dilution

Starting from test tube two, 200 µL of 5% phenol was added in each test tube followed by immediate addition of 1 milliliter concentrate Sulfuric acid. The tubes were vortex for mixing and allowed to cool at room temperature. Samples from each test tube were poured to eight cuvettes to be analyzed in spectrophotometer distilled water as a blank. Absorbance was read for each sample. The absorbance vs concentration graph as plotted on Microsoft excel revealing linear relation equation with coefficient of determination R- square of 99.7. This equation was used to determine the xylose concentration in detoxified hydrolysate. The absorbance of the hydrolysate was read according to phenol sulfuric acid method procedure mention above.

Inoculum analysis

For the purpose of postulating yeast growth in lieu of dose determination, colony forming unit (CFU) was determined with serial dilution. For serial dilution method, 6 petri dishes and 16 test tubes filled with 9ml distilled water were washed and sterilized in a steam sterilizer and cooled in the bio hood at room temperature. 9.75g of Potato Dextrose Agar (PDA) was suspended in 250 ml distilled water and well mixed as moderately heated on hot plate to facilitate mixing and prevent solidification. The solution was then sterilized and poured on the sterilized petri dishes immediately where it was left to cool in hood. A sample from 48 hours cultured was taken. At the same time 6 sterilized test tubes, with sterilized 9ml water in each, were taken from the prepared set and labeled 1 to 6. One milliliter of the sample cultured was poured in to test tube one and

vortexed vigorously. One milliliter of test tube one dilution was poured in to test tube two and this dilution was continued up to test tube six. Clean pipette tips were used for each transfer. 10 microliter of dilute was taken from test tube four, five and six to be dropped and spread on three separate petri dishes fixed with PDA. The petri dishes were placed in an incubator at 35 C^0 to grow. After 48 hrs of growth on the PDA, the colony formed on the petri dish were counted with aid of colony counter. Counting was carried on dilution containing 30 – 300 colonies. The count was used to calculate the colony count unit which implies the number of cells present in a milliliter of inoculum. CFU was calculated as: (Campbell et al., 2015).

CFU/ml = Number of colony/ (Dilution factor * inoculated volume) ….Eq. 3.8

1 OD unit = 1.55 g/L ……..… …Eq. 3.9

Fermented broth analysis

The liquid samples (fermented broth) was transported to Ethiopia Conformity Assessment Enterprise in a test tube to be analyzed by high performance liquid chromatography (HPLC), equipped with RI detectors. The concentrations of xylitol in each sample was determined with refractive index detector and Amine Zorbax NH2 column (size 4.6 * 250 mm) at 30^0C with 70% acetonitrile as mobile phase at 1.3 ml/ min. Single injection of 10 µL was used per each sample. 3 % pure xylitol solution was prepared to calibrate standard, concentration vs. area, curve. A linear equation relating these two parameters was obtained (R^2 = 0.998) to further equate concentration of fermented broth sample from area reading from chromatographic plot.

As a trial for downstream processing, supernatant from centrifugal separation of broth and biomass, a sample was taken to be detoxified for crystallization. Absorbance of supernatant was recited on UV-spectrophotometer pre and post to activated carbon de-coloration at 420 nm.

De-colorization ratio and percentage xylitol lost due to carbon treatment was determine using equation 3.10 and 3.11.

$$\eta\% = (A_o - A) * 100\% / A_o \quad \text{…….Eq. 3.10}$$

$$\alpha\% = (C_0V_0 - C_1V_1) *100\% / C_0V_0 \quad \text{……Eq. 3.11}$$

Where; η% = de-coloration ratio

α% = loss ratio of xylitol

A_o = Absorbance before treatment

A = Absorbance after treatment

C_0 = Concentration of the fermentation broth before decoloration (g/L)

C_1=, Concentration of the fermentation broth after decoloration (g//L)

V_0= Volume of fermentation broth before decoloration (mL)

V_1= Volume of the fermentation broth after decoloration(mL)

3.2.7 Experimental Design and statistical analysis

In order to determine the maximum xylitol yield with a model, Yeast dose (A), pH (B) and fermentation period (C) were chosen as independent variables to model and optimize according Box Behnken design where these fermentation factors were analyzed for different combination of their test levels. Using response surface method, the optimum combination of the operational factors was determined. Seventeen fermentation media samples were prepared. Randomization of experimental runs as well as appropriate analysis techniques were ensured through proper utilization of software package Design-Expert 6.0.8. Results from the analysis are used to make conclusion about the optimal conditions.

Factor	Levels		
	Low [-1]	Medium [0]	High [+1]
Fermentation Time (hours)	24	48	72
Inoculum ratio (%v/v)	5%	7.5%	10%
Ph	4.5	5	5.5

Table 3.4 Experimental Factors and Levels.

The design matrix for the three variables were varied at three levels (+1, 0 and -1). The experiments were performed in random order to avoid systematic error. The analysis of variance (ANOVA) reveals that quadratic terms is effective on correlating xylitol yield from the examined factors.

4. Result and Discussions

4.1 Corncob analysis

Before being used as a substrate hydrolysate in the research, characterization on corn cob was performed. The characterization performed included the compositional analysis of cellulose, hemicellulose, and lignin. The results are summarized in table 4.1.

No.	Composition	% w/w
1	Moisture content	9.575
2	Ash content	1.34
3	Extractives	6.6
4	Hemicellulose	39.8
5	Lignin	13
6	Cellulose	39.26

Table 4.1 Result summary; compositional analysis of corncob

The extractive and hemicellulose content of the corn cob were recorded as 6.6 and 38.9%, respectively. High hemicellulose percentage is the main interest since it is the carbohydrate polymer mainly constituted by xylose, used as a carbon source for xylitol fermentation. The current results were comparable to the results described by Shah & Stability, (2015) and Efri et. al. (2018) in the dry matter bases and was found to be in agreement. However, a variation of results were observed comparing to Wang et al. (2011) which indicated contents of cellulose of 40–44%, hemicellulose of 31–33%, and lignin of 16–18% (Wang et al., 2011). Such difference in hemicellulose content might be accounted to different harvesting and storing conditions of corncob, among others.

4.2 Hydrolysate Analysis

Before subjected to fermentation process, the amount of xylose in hydrolysate was determined. But first the spectrum analysis to determine the absorbance reading wavelength needed to be carried out. 200 µL stock solution was used to analyze spectrum and a peak wavelength at 566 nm was displayed as shown on figure 4.1 this implies the maximum absorbance of xylose in the

standard solution are achieve at this wavelength. Standard solution was then prepared as tabulated in Table 3.3, phenol solution and sulfuric acid was added and absorbance on peak wavelength was read as shown in figure 4.2.

Conc. g L^{-1}	0	0.01	0.02	0.03	0.04	0.05	0.06	0.07
Abs_{566}	0	0.338	0.509	0.732	0.984	1.193	1.423	1.67

Table 4.2. Absorbance reading of standard solution

Concentration vs absorbance graph was plotted on Microsoft excel as shown below with linear equation and R^2 value of 0.9972.

For absorbance A and Concentration C, the linear equation was obtained as:

$$A = 0.0828C - 0.1842 \quad \text{.....Eq. 4.1}$$

With the same procedure the absorbance of hydrolysate sample was read before and after vacuum concentration as 2.407 and 3.128. Hence, concentration was calculated using Eq. 4.1.

$$C_o = (2.407 + 0.1842) / 0.0828 = 31.29 \text{ g } L^{-1}$$
$$C = (3.128 + 0.1842) / 0.0828 = 40.002 \text{ g } L^{-1}$$

Initially, 31.29 g L^{-1} of xylose was present in the hydrolysate. The concentration of xylose was this high from the original 39.8% hemicellulose since first wash hydrolysate was not combined with the second wash of hydrolysis solid residue. Meanwhile xylitol yield is higher at high initial xylose concentration (Mussatto & Roberto, 2001) as yeasts get enough substrate to convert to xylitol. Hydrolysate, therefore, needed to be vacuum concentrated at low temperature as a caution not to degrade fermentable sugar. At the laboratory scale it was possible to vacuum concentrate hydrolysate at 50 C^0 to achieve an increased xylose concentration of 40.002 g/L. Known xylose concentration is compulsory to compute fermentation efficiency by determining yield facto i.e. gram xylitol production / gram xylose consumed.

4.3 Inoculum analysis

To quantify the number of viable yeast cells in inoculum levels used in the experiment, colony counting unit was performed after 48 hours of cell activation. With 10 µL of inoculation volume from the 10^{-5} dilution, 100 colonies were counted. Simultaneously, absorbance of inoculum was measured at 230 nm to be 2.407. Number of cells in a milliliter of inoculum was determined using colony forming unit equation, Eq. 3.8, to give12 in log 10 basis. This means there are 10^{12} number of cells in a milliliter of inoculum. Results are summarized in table 4.3 for the three level of yeast doses.

Level	Dose (inoculum : fermentation media)	100 ml basis	CFU (log_{10})
1	5%	5 ml	60
2	7.5%	7.5 ml	90
3	10%	10 ml	120

Table 4.3 CFU in yeast dose levels

4.4 Fermented broth Analysis

According to the scope of this study, xylitol yield from fermented broth was determined. HPLC is the ideal method for determining the concentration of constituent in a liquid solution. Once again xylitol concentration in each sample ware analyzed using HPLC equipped with refractive index detector. Amine NH2, Zorbax column with 70% acetonitrile as mobile phase at 1.3 ml/ min and 30^0C. Five samples of known xylitol concentration were used to read area on a chromatographic scheme and concentration vs. area was plotted as standard curve. The standard curve was simulated with linear equation, Eq. 4. 2, of $R^2 = 0.998$ as shown in figure 4.4

Conc. (g/L)	1	5	10	15	30
Area	74749.2	344754.8	578983	878508.1	1785872

Table 4.4 Concentration and area reading of standard.

$Y = 58515X + 18696$ ……Eq. 4.2

Where Y= Area

X = Concentration

Accordingly the area at peak refractive index signals of each sample were read and used to compute concentration of xylitol present fermented samples. Table 4.5 shows area recorded and thereafter computed concentration sing equation 4.2. The index signals of each sample are shown in Appendix A. The chromatographic diagram of refractive index signal of the sample with the highest concentration is shown in figure 4.5. A single peak of xylitol was observed. Disturbances from the presence of sugars such as fructose and galactose were observed. Wei et al., 2010 pointed out the presence of other residual while explaining the difficulty of separation between residual sugars (xylose and arabinose) and xylitol due to their similar structures.

With the curiosity and intention of minimizing these noises, chromatographic analysis of a random sample pre and post activated carbon treatment of fermented broth was compared. There was no significant change on visible disturbance reduction but reduction of xylitol area. This observation agrees with literatures where activated carbon treatment was found functional only for decolorizing broth but not for removal of residual sugars present. Strong cation-exchange resins chelated Ca2+ for the separation of xylitol as it has been discovered that it adsorbs xylitol more strongly than residual sugars (Wei et al., 2010).

Sample code	Area	Concentration (g/L)
01	948153.8	15.88
02	784851.6	13.09
03	800409.6	13.36
04	595062.8	9.85
05	1123578.4	18.8
06	1088355.9	18.28
07	1170203.5	19.68
08	1162003.3	19.54
09	1397227.1	23.56
10	915641.0	15.33
11	1074544.0	18.04
12	1080610.3	18.15
13	1039737.6	17.45

Table 4.5 Area reading of samples with corresponding concentration.

4.5 Statistical Result

Demonstrated in Table 4.6 is the result of analysis of variance for response surface quadratic model for xylitol yield. The P values were used as a tool for checking the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The higher the magnitude of F-test value and the lesser the magnitude of P-values, the higher the significance of corresponding coefficient. Here Model F-value of 52.36 implies the model is significant. There is only a 0.0001% chance that a "Model F-Value" this large could occur due to personal error or disturbance. Values of P less than 0.05 indicate that the model

terms are significant. In the current case all three factors, yeast dose (A), pH (B) and fermentation period (C) along with their second order, were found to be significant. Furthermore, two interaction effects, yeast dose with pH (AB) and yeast dose with fermentation period (AC) similarly showed significant with P-value less than 0.05. The fitness of the model equation was also expressed by the coefficient of determination, R^2. The adequacy of the model was tested by analysis of variance. The value of R-squared for the developed correlation is 0.9821. This implies that 98.21% of the total variation in the percentage xylitol yield is attributed to the experimental variables studied. The graph of the predicted values obtained using the developed correlation versus actual values, normal probability and residuals vs. predicted plot are shown in Appendix A.

The normal probability plot indicates the residuals following a normal distribution, in the case of this experiment the points in the plots shows fit to a straight line in the figure, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal.

Response: Xylitol yield

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares]

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**
Model	150.37	9	16.71	42.69	< 0.0001
A	*5.17*	*1*	*5.17*	*13.21*	*0.0084*
B	*3.30*	*1*	*3.30*	*8.44*	*0.0228*
C	*35.24*	*1*	*35.24*	*90.05*	*< 0.0001*
A^2	*2.22*	*1*	*2.22*	*5.68*	*0.0487*
B^2	*8.36*	*1*	*8.36*	*21.35*	*0.0024*
C^2	*74.41*	*1*	*74.41*	*190.14*	*< 0.0001*
AB	*5.15*	*1*	*5.15*	*13.17*	*0.0084*
AC	*19.58*	*1*	*19.58*	*50.04*	*0.0002*
BC	*0.18*	*1*	*0.18*	*0.47*	*0.5140*
Residual	2.74	7	0.39		
Lack of Fit	*2.74*	*3*	*0.91*		
Pure Error	*0.000*	*4*	*0.000*		
Cor Total	153.11	16			

Std. Dev.	0.63	R-Squared	0.9821
Mean	17.31	Adj R-Squared	0.9591
C.V.	3.61	Pred R-Squared	0.7137
PRESS	43.83	Adeq Precision	27.739

Table 4.6 Design expert output (ANOVA) for xylitol yield

From residuals vs. predicted plot, if the model is correct and the assumptions are satisfied the residuals should be structure less. In particular, they should be unrelated to any other variable including the predicted response. A simple check is to plot the residuals versus the fitted (predicted) values. A plot of the residuals versus the rising predicted response values tests the assumption of constant variance. The plot shows random scatter which justifying no need for an alteration to minimize personal error.

Development of regression model equation

Table 4.8 shows sequential model sum of squares for percentage yield of xylitol. The models were chosen because of their high correlation coefficient (actual and adjusted) value as shown in Appendix A. The equations generated from the quadratic models for the xylitol yield operation is shown in Equations 4.3. These equation is mathematical representation of the experimental results and can be used to determine the fermentation parameters required to achieve a particular xylitol concentration prior the experiment and the vice-verse. *Kumar et al. (2008)* stated that when regression coefficient has a positive sign, the increase of the associated factor causes an increase in response and a negative sign would cause a decrease in the optimization parameter.

Final Equation in Terms of Coded Factors:

$$\text{Xylitol yield} = +18.28 - 0.80*A - 0.64*B + 2.10*C + 0.73*A^2 + 1.41*B^2 - 4.20*C^2 + 1.13*A*B + 2.21*A*C \quad \text{Eq. 4.3}$$

The coefficient of Variation, the standard deviation expressed as a percentage of the mean; Predicted Residual Error Sum of Squares, which is a measure of how the model fits each point in the design; the R-Squared, measure of the amount of variation around the mean described by the model; Pred R-Squared, a measure of the amount of variation in new data explained by the model, and Adequate Precision, a signal to disturbance ratio due to random error, presented in the table 4.7, below, are used to decide whether the model is adequate or not.

Std. Dev	0.63	R-Squared	0.9821
Mean	17.31	Adj R-Squared	0.9591
C.V.	3.61	Pred R-Squared	0.7137
PRESS	43.83	Adeq Precision	27.739

Table 4.7 Model adequacy measures

Response: Xylitol yield
***** WARNING: The Cubic Model is Aliased! *****

Sequential Model Sum of Squares

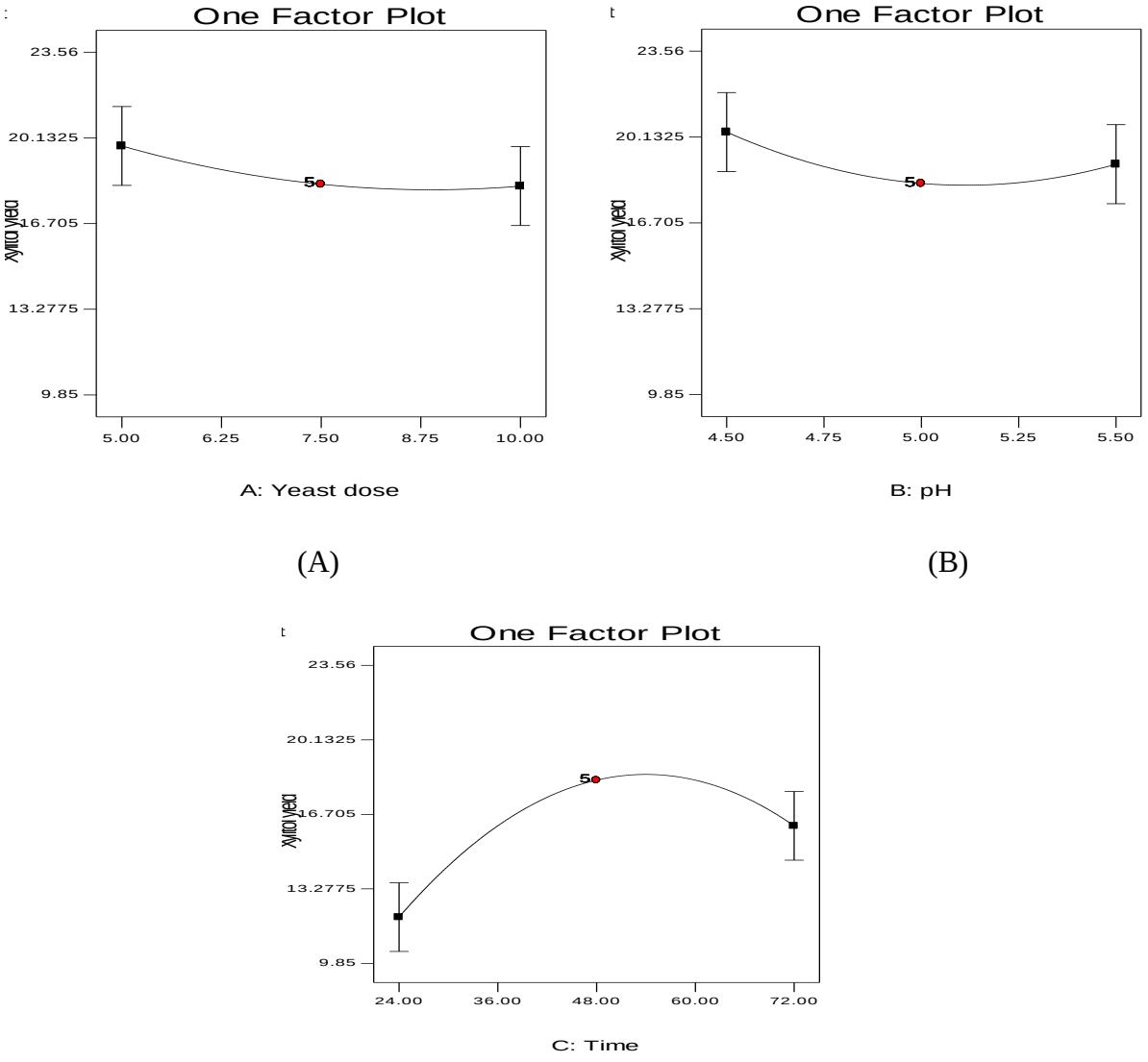
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Mean	5091.74	1	5091.74			
Linear	43.71	3	14.57	1.73	0.2098	
2FI	24.92	3	8.31	0.98	0.4392	
Quadratic	81.74	3	27.25	69.63	< 0.0001	Suggested
Cubic	2.74	3	0.91	6.366E+007	< 0.0001	Aliased
Residual	0.000	4	0.000			
Total	5244.84	17	308.52			

Table 4.8 Sequential Model Sum of Squares for xylitol yield

4.5.1 Effect of individual parameters on xylitol yield

Figure 4.5 A, B and C demonstrates the effect of yeast dose, pH and fermentation period on xylitol yield respectively. A decreased xylitol concentration is observed with increased in yeast dose. Opposite effect of pH and fermentation period are noticed on xylitol yield. At first, increases in pH decreases xylitol yield and later, xylitol yield increases slightly. On the contrary, with the increment of fermentation period, xylitol yield increases attainment peak and then decreases.

As it can be seen from figure 4.5 (A), increase in yeast dose causes a decrease in xylitol yield. This result is in agreement with a foundation by Roberto, Sato, & Mancilha, 1996, which reasoned out this behavior can be attributed to low oxygen availability since the metabolic pathway of xylitol formation is aerobic and higher inoculum concentration tends to reduce the oxygen availability in the medium.



(A) (B)

(C)

Fig. 4.5 Effect of individual parameters on xylitol yield; (A) Yeast Dose, (B) pH, (C) fermentation time

The xylitol yield is negatively affected by increase in pH initially as shown in fig 4.5 (B). Yet a slight increase in yield (not greater than the starting yield) was noticed as pH further increased. According to a study by Mayerhoff et al., 1998, interaction effect of pH and aeration has been reported to possess significant effect on yield factor of xylitol. Even though aeration has not been considered as a factor in the present study, its' interaction effect with pH could be taken into consideration account.

The individual effect of fermentation period is almost stronger than that of yeast dose and pH. Xylitol yield increased in the first range of fermentation time increment and eventually displayed a decrease. This can be attributed to numerous motives such as; low nutrients and oxygen availability since, unless refreshed, increased in fermentation period would exhaust essential nutrients in fermentation media for yeasts. Another attribute can be the change in pH of fermentation media caused by metabolic byproduct from yeasts. This change can create unfavorable condition for yeasts to further convert xylose into xylitol.

4.5.2 Interaction effects on xylitol yield

According to design expert results, two combined effects were found significant. These are interaction effect of yeast dose with pH (AB) and yeast dose with fermentation period (AC). The interaction, contour and 3D response surface plots of these interactions, at midpoint of third factor, are demonstrated below in figure 4.6 and 4.7 respectively.

The interaction effect of pH and yeast dose is shown in Figure 4.6 at constant fermentation period of 48 hours. It can be seen from figure 4.6 (C), at low pH, xylitol yield is higher at low yeast dose and at high pH, yield is high at high yeast dose. In other word, at low pH, xylitol yield decreases significantly as yeast dose increases. On the other hand, at higher pH, xylitol yield increases slightly as yeast dose increases. This can be attributed to the activity of candida mogii in pH variation. Xylitol production is observed low at pH 3.0 as toxic effect of acetic acid increased because of the entry of acid into the cell in its un-dissociated form (Pfeifer et al. (1996) and Rodrigues et al. (1998)). As yeast dose increases, the metabolic wastes accumulate causing pH variation in fermentation medium. The higher the yeast dose the higher its effect on pH alteration.

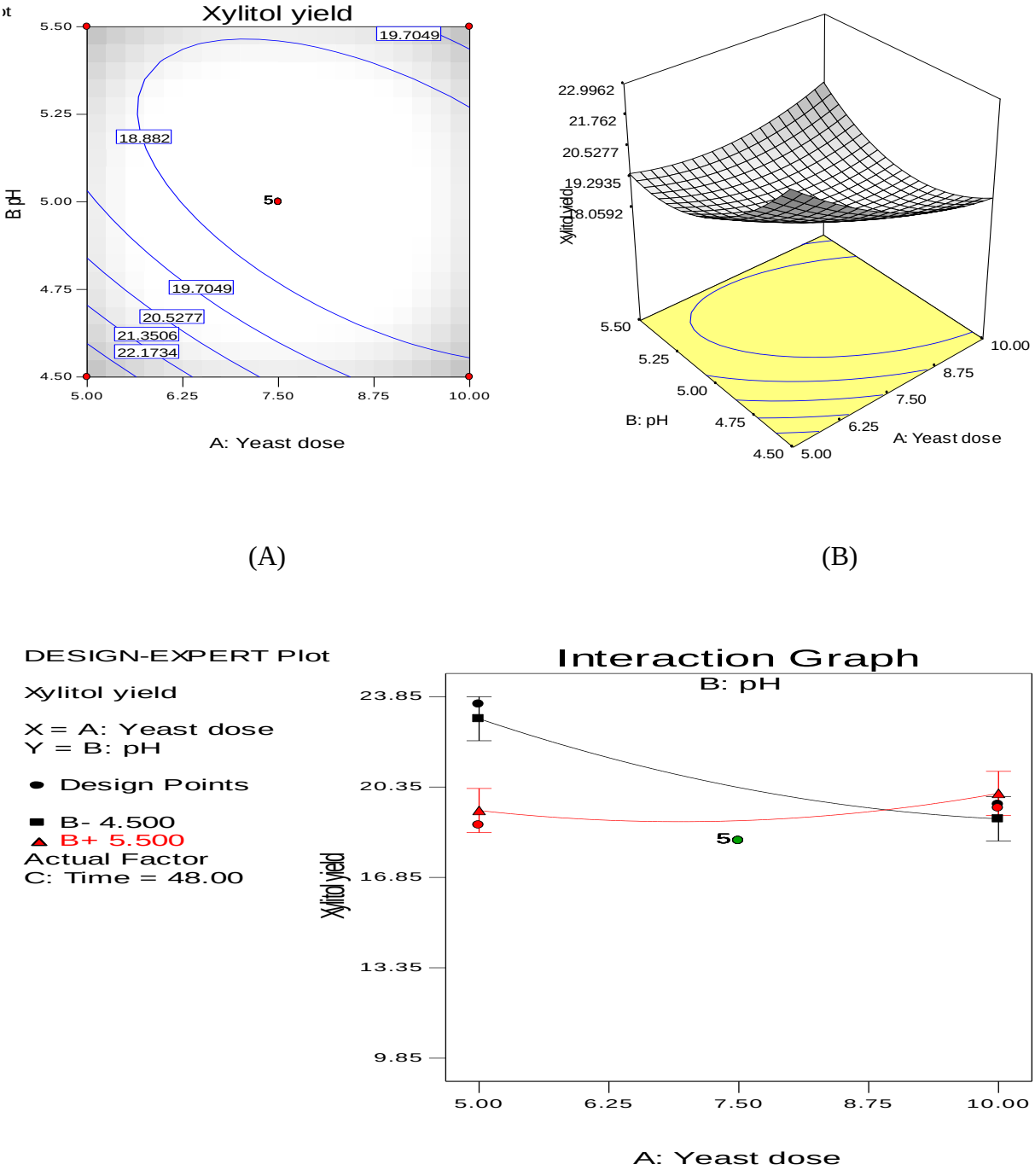
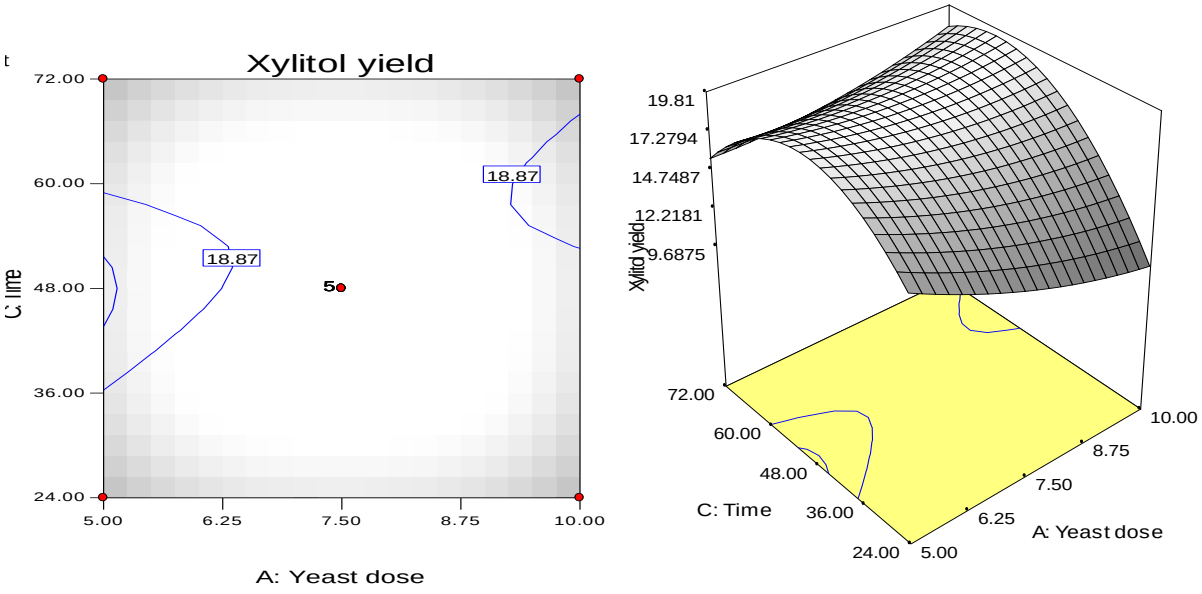


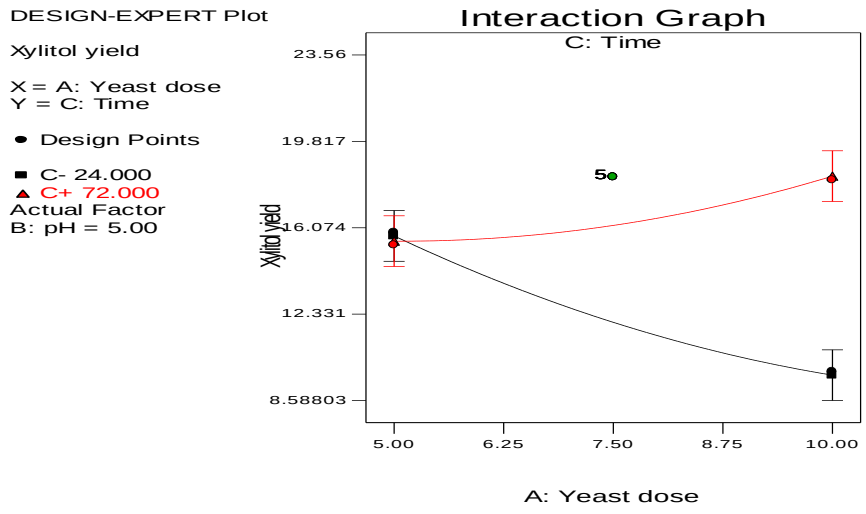
Fig. 4.6 Combined effect of pH and yeast dose on xylitol; (A) Contour plot, (B) 3D plot and (C) interaction graph

To validate this relation, pH of fermented broth was measured and compared to the initial fermentation medium pH. The result showed a decrease in pH after fermentation. This explains,

at lower level of initial pH of 4.5, as fermentation proceeds the pH decreases too low creating unfavorable environment for yeast. Yet at higher level of initial pH of 5.5, decrease in pH as fermentation proceeds, slightly favors yeasts since highest conversion of candida mogii was recorded at 4.5 – 5 by (Sirisansaneeyakul et al., 1995 and Z D V L Mayerhoff et al., 1998)



(A) (B)



(C)

Fig. 4.7 Combined effect of fermentation period and yeast dose on xylitol; (A) Contour plot, (B) 3D plot and (C) interaction graph

Combined effect of fermentation period and yeast dose at constant pH of middle value 5 is shown in figure 4.7. It was understood that at shorter fermentation period, xylitol yield is higher at the low percentage of yeast dose and at longer period yield is higher at high percentage of yeast dose. That means at lower fermentation period, xylitol yield decreased significantly as yeast dose increased. This shows the increase in the initial inoculum concentration at short fermentation period negatively affected and xylitol production. Yet at longer fermentation period, xylitol yield increases slightly as yeast dose increased. Increased yeast dose presents large amount of yeasts to convert xylose for prolonged period yet for short period.

4.5.3 Optimal parameters to obtain maximum xylitol yield

Preliminary observations show that xylitol yield varies significantly, according to the experiment parameters, ranging values of 9.85-23.56 g L^{-1} under certain operating conditions. A clear evidence for the careful choice of the parameters range is that the highest yield is obtained around 4.5 pH, 5% yeast dose and in 48 hours of fermentation period. Considering the other response, highest percent yield is obtained at minimum pH and yeast dose. But the main objective of this research is to find the optimum fermentation parameters for the highest xylitol yield. The fermentation parameters were optimized subject to constraints shown in Table 4.9. Ten solutions were found and the solution with the highest desirability was chosen.

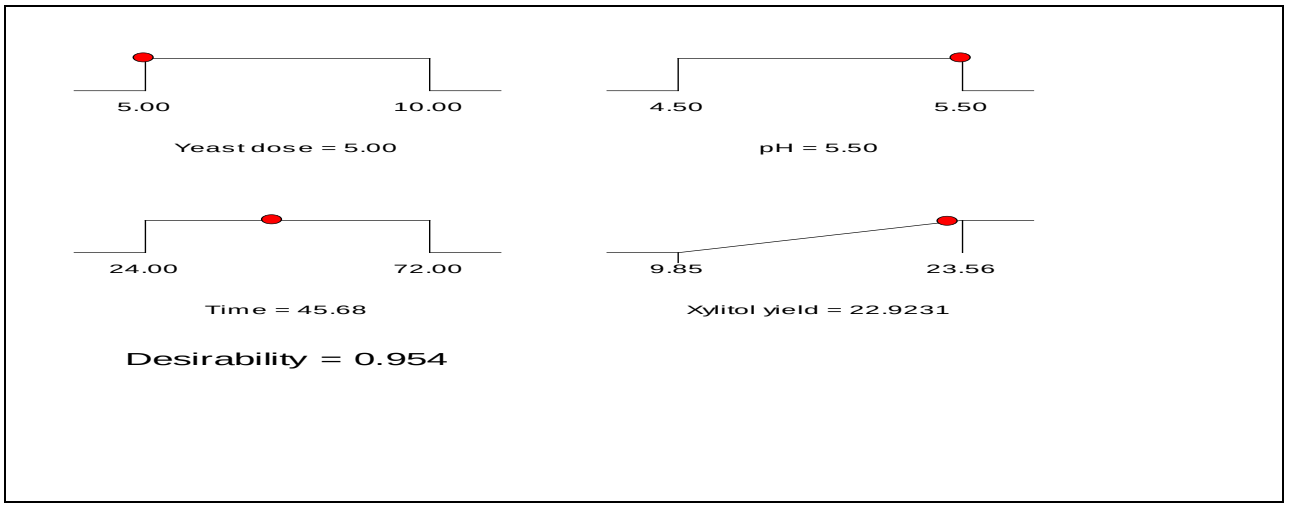


Fig. 4.8 Optimum solution for maximum xylitol yield

As can be seen on figuer 4.8, 22.921 g L^{-1} of xylitol was obtained at optimal conditions of 4.5 pH, 5% yeast dose and in 45.68 hours of fermentation period.

This iptimal conditions agree with a study by Sirisansaneeyakul et al., 1995, however a difference in optimum pH is noticed caompared to Mayerhoff et al., 1998, which suggested optimum initial pH of 6.2. Nevertheless, the same study rephrases that influence of the initial pH on this bioconversion seems to be dependent on the yeast strain employed, and on the composition of the fermentation medium. The latter has been associated with the presence of acetic acid, which occurs in hydrolysate-based media. Actually, the xylitol production using *Candida* sp B-22 in synthetic medium without acetic acid was not influenced by the initial pH.

To comparing the optimum yield with that of Sirisansaneeyakul et al., 199, the fermentation efficiency of xylose to xylitol would be calculated as the ratio of the net amount of xylitol produced (g/L) to the initial xylose (g/L) of the medium. With respect to initial xylose in fermentation medium, 40.002 gL^{-1}, this result can be interprated as yield factor of 0.5748 g/g. This yield is fairly accurate with xylitol yield of 0.62 g/g, by candida mogii, reported by (Sirisansaneeyakul et al., 1995) from initial xylose concentration and controled aeration of 53 g/L and 0.5 mmol O_2/g/h, respectively on a fed-batch experiments with 4.5 initial pH. Additional data is from Gong ***et al.***, who found that C. *mogii* produced xylitol with a yield of 0.59 g/g in synthetic medium during aerobic growth on D-xylose.

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Yeast dose	**is in range**	**5**	**10**	**1**	**1**	
pH	**is in range**	**4.5**	**5.5**	**1**	**1**	
Time	**is in range**	**24**	**72**	**1**	**1**	
Xylitol yield	**maximize**	**9.85**	**23.56**	**1**	**1**	

Solutions

Number	Yeast dose	pH	Time	Xylitol yield	Desirability	
1	5.00	5.50	45.68	22.9231	0.954	Selected
2	5.05	5.50	48.63	22.8751	0.950	
3	5.00	5.48	48.13	22.7884	0.944	
4	5.00	5.47	48.02	22.6499	0.934	
5	5.42	5.50	52.10	22.3099	0.909	
6	5.00	5.34	49.31	21.6144	0.858	
7	10.00	4.50	60.32	21.3912	0.842	
8	10.00	5.50	60.29	20.0389	0.743	
9	5.00	4.50	47.71	19.487	0.703	
10	5.00	4.76	49.01	19.2886	0.688	

Table 4.9 Optimization constraints and solutions for fermentation parameter

5. Conclusion and Recommendation

5.1 Conclusion

Xylitol, as an alternative low calorie sweetener is well accepted in formulations of various confectioneries and healthcare products. Worldwide it is industrially produced by catalytic hydrogenation of pure D-xylose solution under high temperature and pressure. Biotechnological xylitol production is a potentially attractive replacement for chemical process, as it occurs under much milder process conditions and can be based on sugar mixtures derived from low-cost industrial and agro-waste.

Corncob, abundant agricultural residue was found a promising carbon source as it contains about 37% hemicellulose, a carbohydrate polymer mainly constituted by xylose units. Corncob LCM was hydrolyzed to yield a xylose solution and used as fermentation media to obtain xylitol with the aid of micro-organisms. The most acknowledge microorganism in xylitol production Candida sp. yeast, particularly, candida mogii was found to perform adequate microbial activity with optimized fermentation parameters. These parameters were investigated and models were developed by utilizing Box-Benhken experimental design in order to determine optimal conditions. The maximum xylitol yield was determined with respect to yeast dose, pH and fermentation period. According to the developed model, maximum yield of 22.997 g L^{-1} was achieved at optimum conditions of 5% yeast dose, pH of 4.5 and 48 hours fermentation period.

The individual effect of these parameters were also inspected. Increase in yeast dose at constant pH and fermentation period decreased xylitol yield which leads to a reasoning of nutrient insufficiency and since aerobic fermentation, oxygen demand increases with increased yeast dose. pH also showed a negative relationship with xylitol yield. Candida mogii yeasts have been reported to function at best at pH of 4.5 and 5. The effect of fermentation period exhibited increase at first and decrease eventually. The activity of yeasts at log and exponential phase contribute to the increase in xylitol yield. Ultimately, xylitol yield decrease attributing to decreasing of necessary nutrients in fermentation medium and the change in medium pH due to metabolic byproducts of yeasts.

5.2 Recommendation

If further research work is to be done, recommended focus point of studies are listed below:

- The effect of other fermentation parameters such as initial xylose concentration and aeration rate need to be inspected.
- In depth study of xylitol producing microbes and their xylitol producing pathway.
- Preliminary design, economic feasibility study and establish economic scale for microbial xylitol production.
- Downstream processes of xylitol production and optimization.
- Scale up of microbial xylitol production.
- Effect of hydrolysis method and conditions

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APPENDICES

Appendix A: Experimental Design Output

Figure. A-1 Concentration Vs absorbance plot

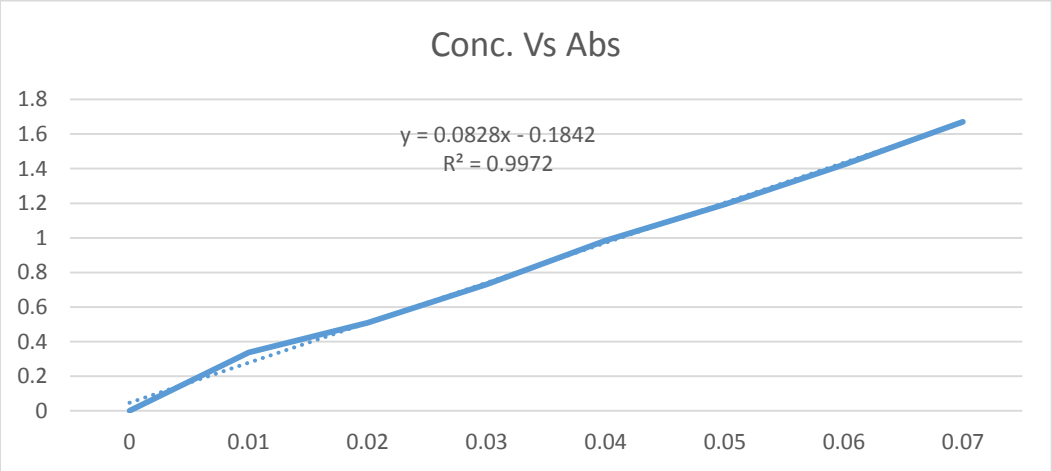
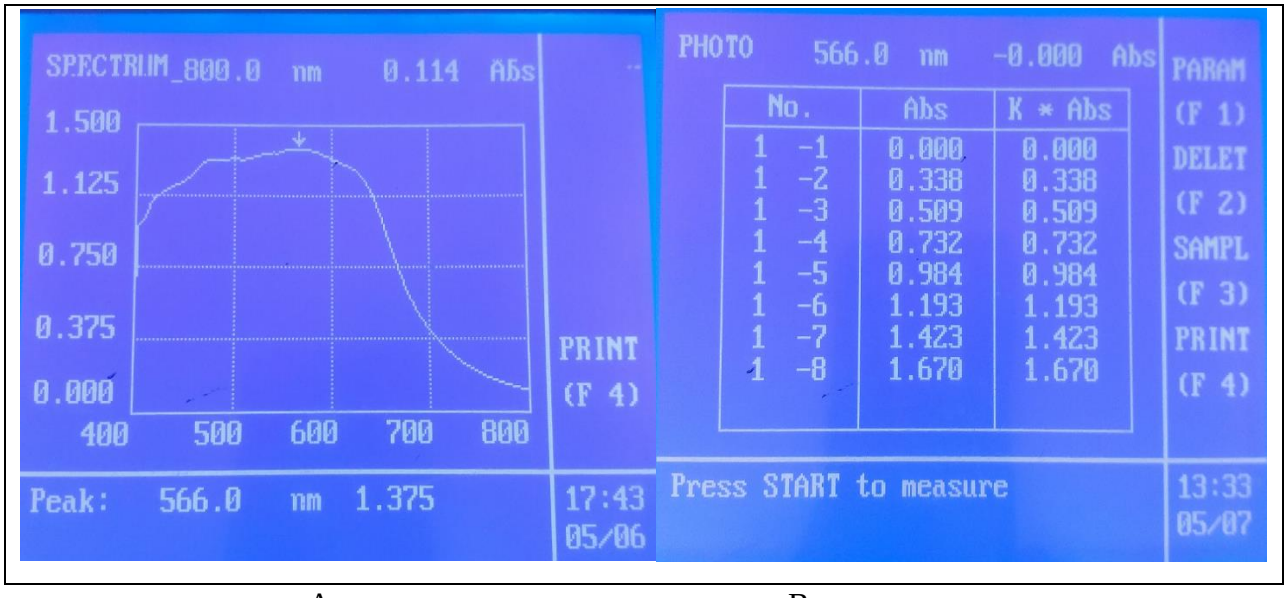


Figure A-2 UV- spectrophotometer analysis

(A) Spectrum analysis for Xylose solution: wavelength range [400 – 800] and (B) Absorbance reading of standard solution at 566 nm



A B

Figure A-3 Standard curve [Con. Vs Area]

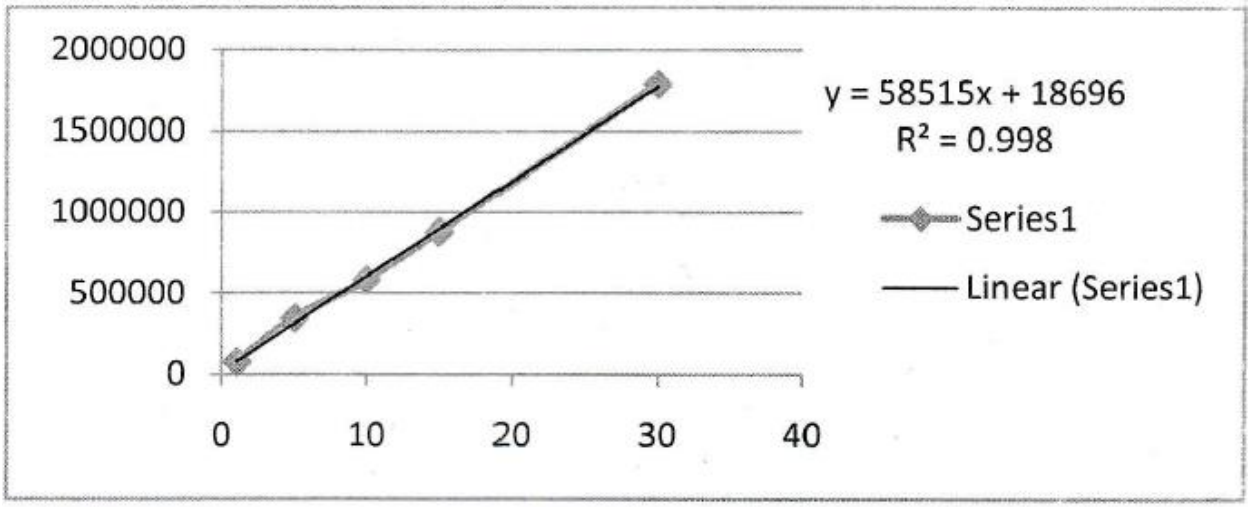


Table A-1 Sample coding

Fermentation Period	Code no.	Yeast dose (%)	pH
24 Hours	01	5	5
	02	7.5	4.5
	03	7.5	5.5
	04	10	5
48 Hours	05	5	5.5
	06	7.5	5
	07	10	4.5
	08	10	5.5
	09	5	4.5
72 Hours	10	5	5
	11	7.5	4.5
	12	19	5
	13	7.5	5.5

Table A-2: Experimental design data

Run No.	Factor A; yeast dose (%)	Factor B; pH	Factor C; fermetation period (hr)	Xylitol Yield (g/L)
1	5.00	4.50	48.00	23.56
2	10.00	4.50	48.00	19.68
3	5.00	5.50	48.00	18.88
4	10.00	5.50	48.00	19.54
5	5.00	5.00	24.00	15.88
6	10.00	5.00	24.00	9.85
7	5.00	5.00	72.00	15.33

8	10.00	5.00	72.00	18.15
9	7.50	4.50	24.00	13.09
10	7.50	5.50	24.00	13.36
11	7.50	4.50	72.00	18.04
12	7.50	5.50	72.00	17.45
13	7.50	5.00	48.00	18.28
14	7.50	5.00	48.00	18.28
15	7.50	5.00	48.00	18.28
16	7.50	5.00	48.00	18.28
17	7.50	5.00	48.00	18.28

Table A-3: ANOVA for Response Surface Quadratic Model

Response: Xylitol yield

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares]

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**
Model	150.37	9	16.71	42.69	< 0.0001
A	*5.17*	*1*	*5.17*	*13.21*	*0.0084*
B	*3.30*	*1*	*3.30*	*8.44*	*0.0228*
C	*35.24*	*1*	*35.24*	*90.05*	*< 0.0001*
A^2	*2.22*	*1*	*2.22*	*5.68*	*0.0487*
B^2	*8.36*	*1*	*8.36*	*21.35*	*0.0024*
C^2	*74.41*	*1*	*74.41*	*190.14*	*< 0.0001*
AB	*5.15*	*1*	*5.15*	*13.17*	*0.0084*
AC	*19.58*	*1*	*19.58*	*50.04*	*0.0002*
BC	*0.18*	*1*	*0.18*	*0.47*	*0.5140*
Residual	2.74	7	0.39		
Lack of Fit	*2.74*	*3*	*0.91*		
Pure Error	*0.000*	*4*	*0.000*		
Cor Total	153.11	16			

Std. Dev.	0.63	R-Squared	0.9821
Mean	17.31	Adj R-Squared	0.9591
C.V.	3.61	Pred R-Squared	0.7137
PRESS	43.83	Adeq Precision	27.739

Factor	**Coefficient Estimate**	**DF**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
Intercept	18.28	1	0.28	17.62	18.94	
A-Yeast dose	-0.80	1	0.22	-1.33	-0.28	1.00
B-pH	-0.64	1	0.22	-1.17	-0.12	1.00
C-Time	2.10	1	0.22	1.58	2.62	1.00

A^2	0.73	1	0.30	5.370E-003	1.45	1.01
B^2	1.41	1	0.30	0.69	2.13	1.01
C^2	-4.20	1	0.30	-4.92	-3.48	1.01
AB	1.13	1	0.31	0.40	1.87	1.00
AC	2.21	1	0.31	1.47	2.95	1.00
BC	-0.22	1	0.31	-0.95	0.52	1.00

Final Equation in Terms of Coded Factors:

Xylitol yield	=
+18.28	
-0.80	* A
-0.64	* B
+2.10	* C
+0.73	* A^2
+1.41	* B^2
-4.20	* C^2
+1.13	* A * B
+2.21	* A * C
-0.22	* B * C

Table A-3: Design Summary

Design Summary

Study Type	Response Surface			**Experiments**	17	
Initial Design	Box Behnken				**Blocks**	No Blocks
Design Model	Quadratic					
Response Model	**Name**	**Units**	**Obs**	**Minimum**	**Maximum**	**Trans**
Y1	Xylitol yield	g/L	17	9.85	23.56	None	No model chosen

Factor	**Name**	**Units**	**Type**	**Low Actual**	**High Actual**	**Low Coded**	**High Coded**
A	Yeast dose	%v/v	Numeric	5.00	10.00	-1.000	1.000
B	pH		Numeric	4.50	5.50	-1.000	1.000
C	Time	hr	Numeric	24.00	72.00	-1.000	1.000

Figure A-4: Normal probability plots

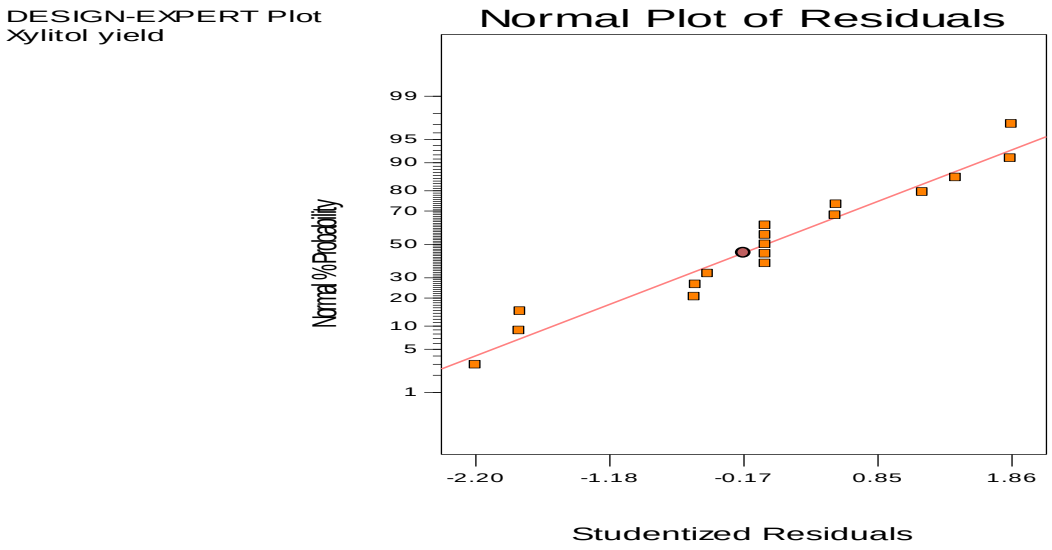


Figure A-5 Predicted vs. Actual plots

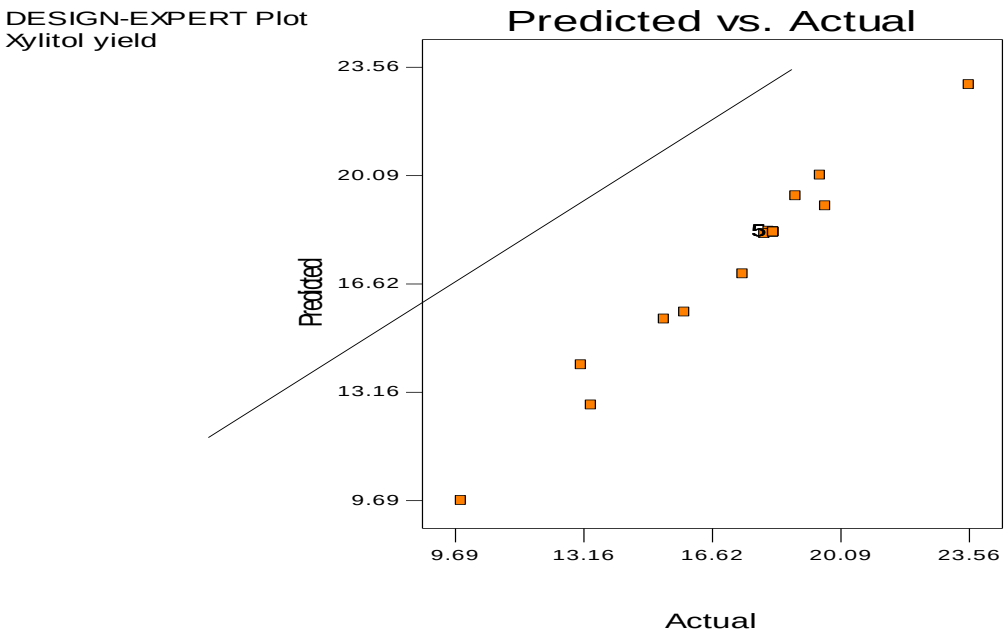
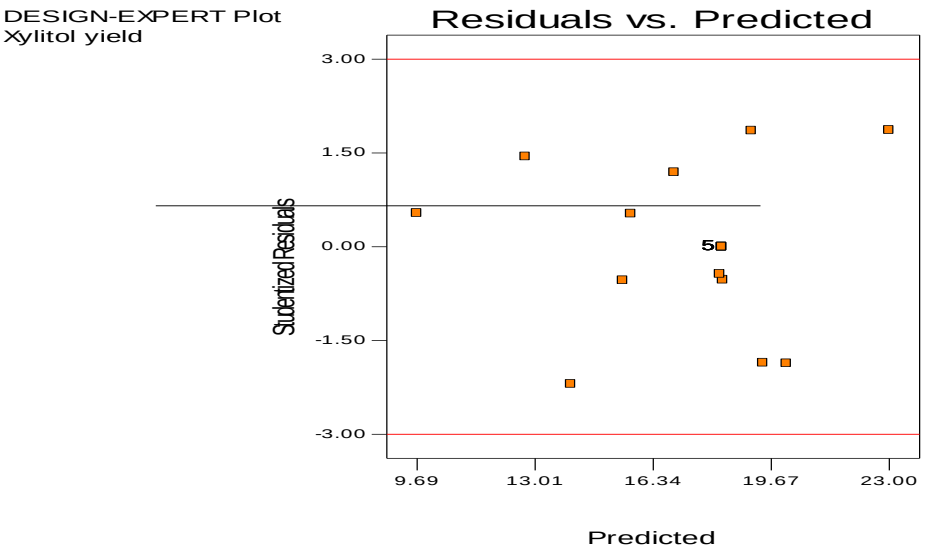


Figure A-6 Residual vs. predicted plot



Appendix B: Analytical result of samples

Figure B-1: Chromatographic plot (RID signal) of Xylitol blank

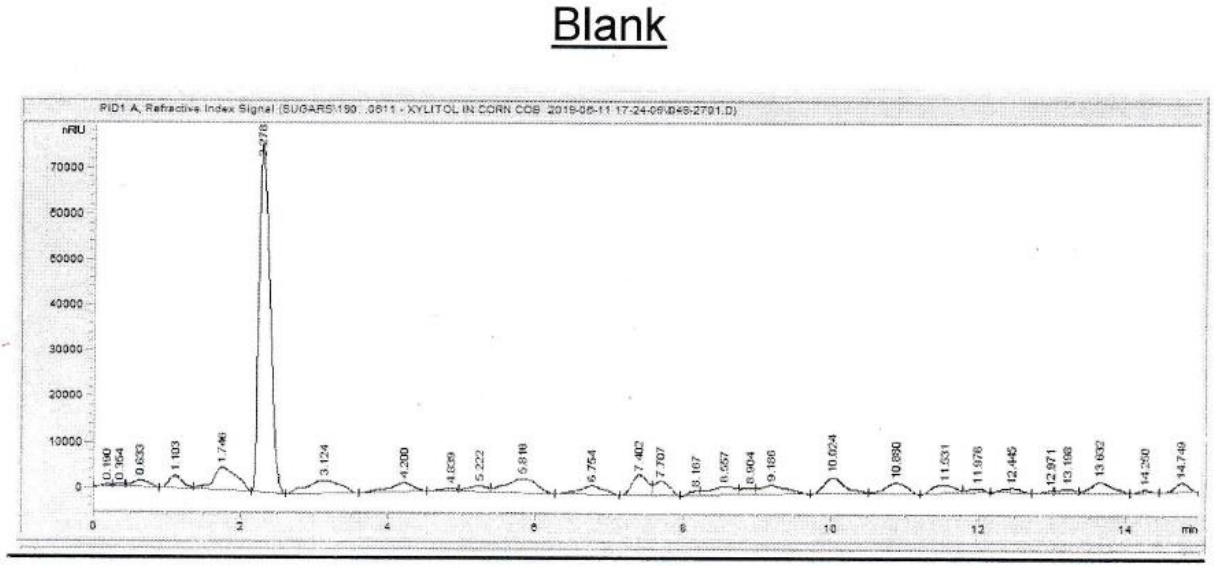
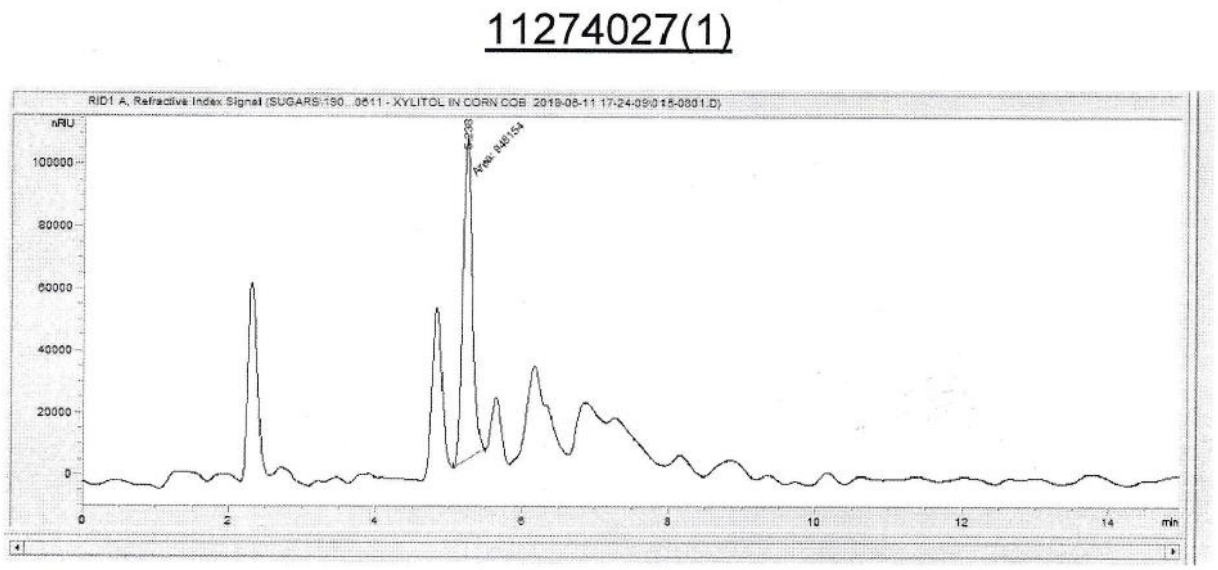


Figure B-1: Chromatographic plot (RID signal) of Xylitol in coded samples



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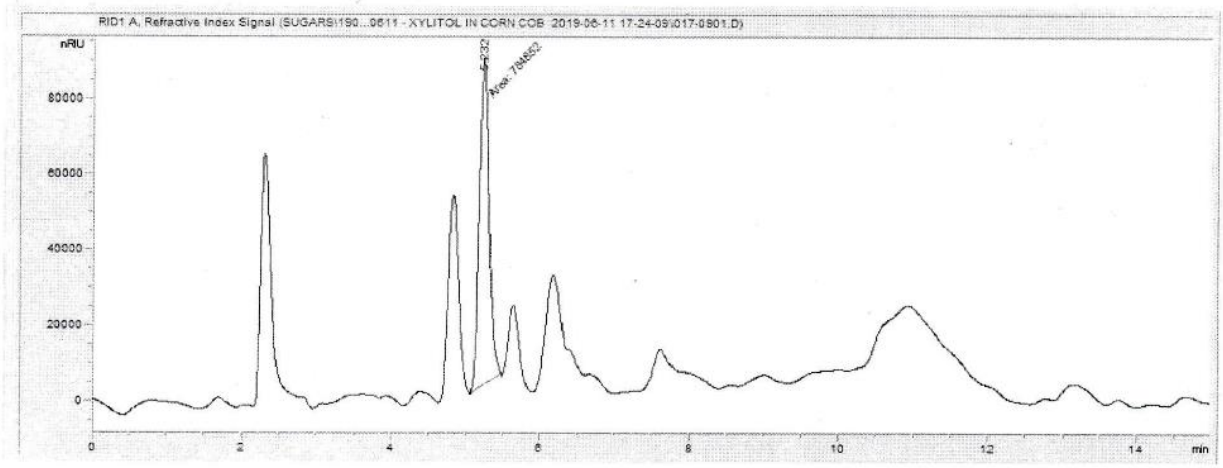
11274028(2)



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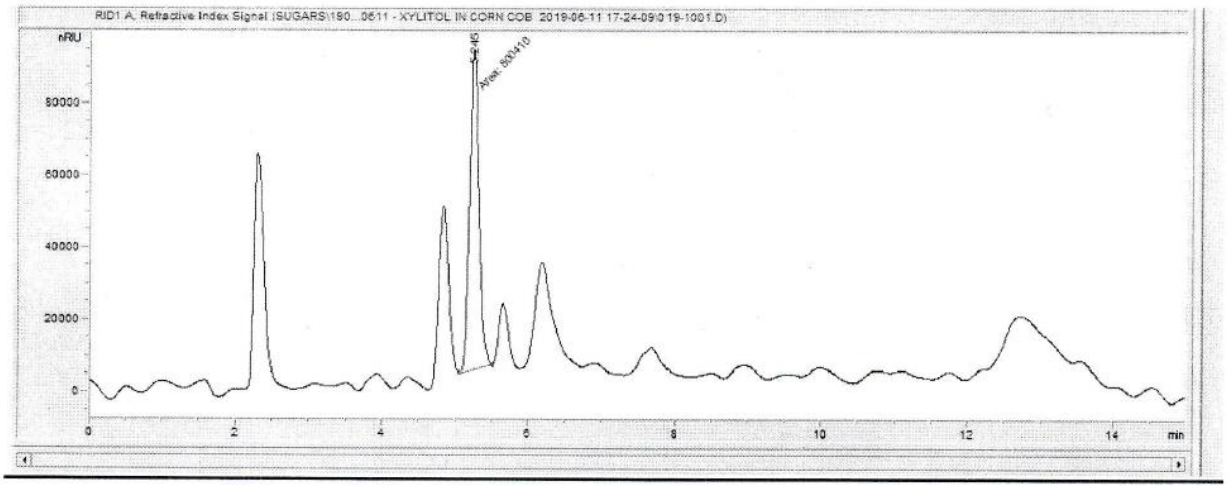

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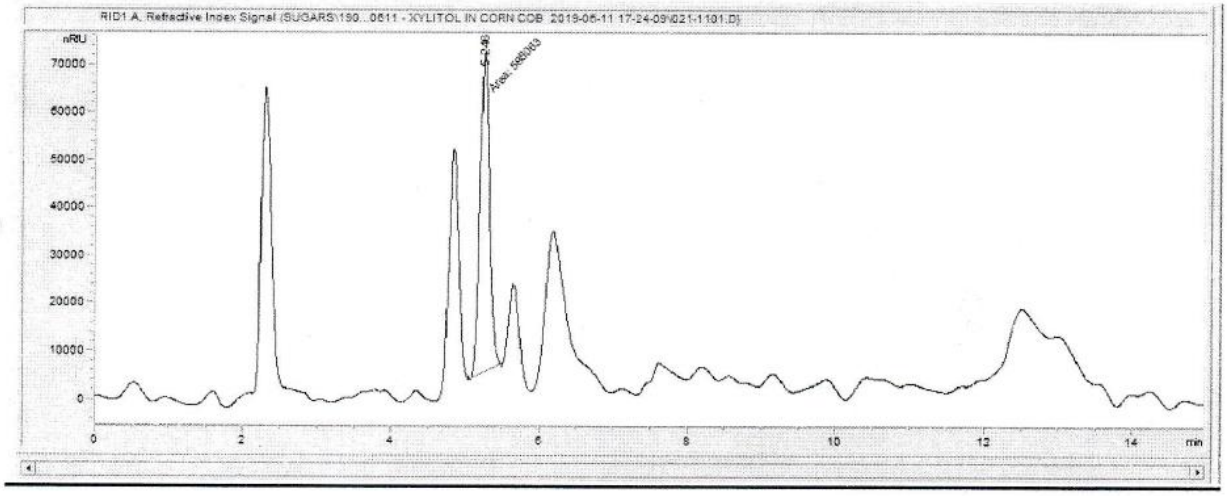

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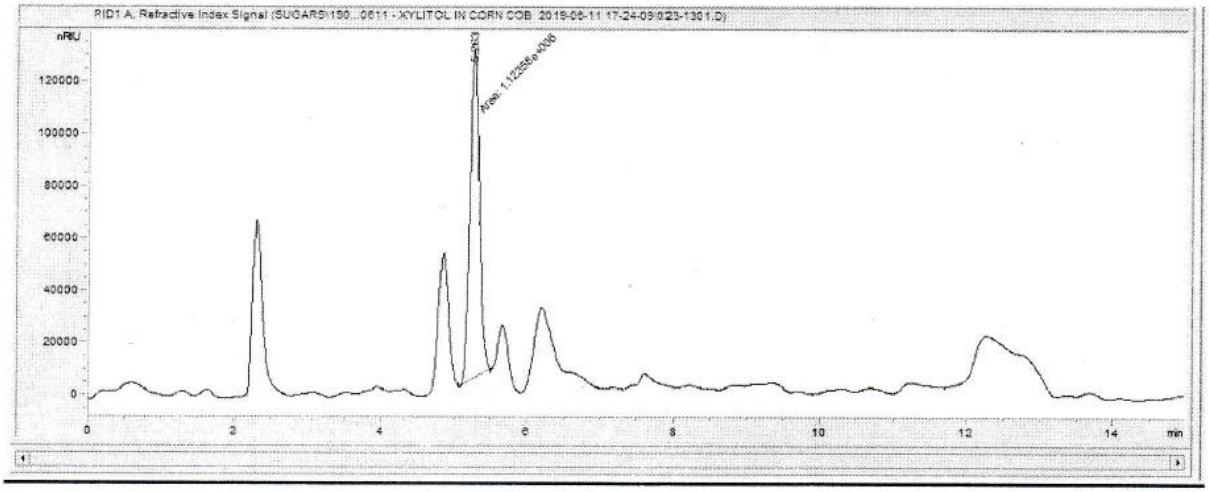

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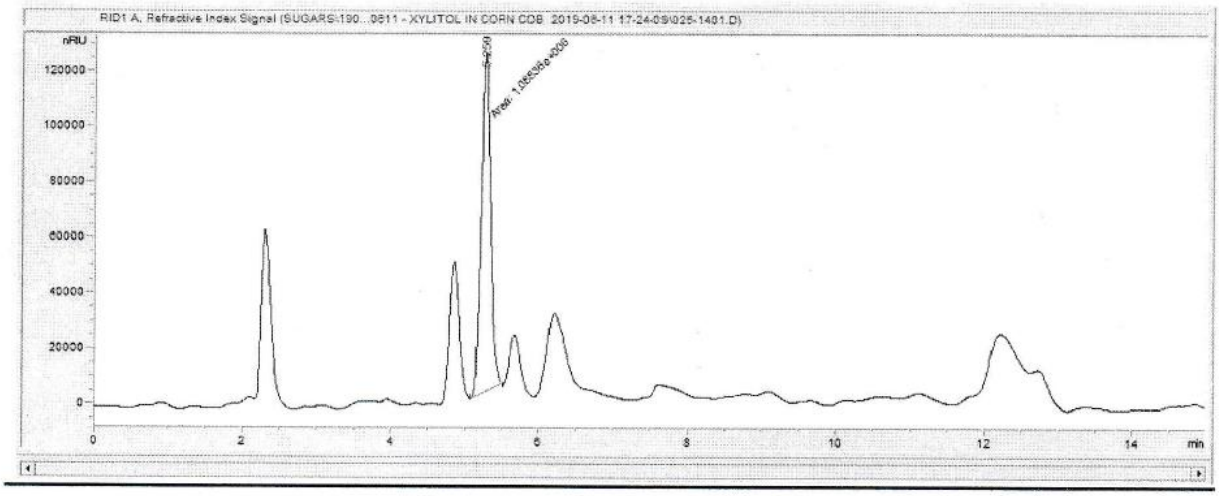

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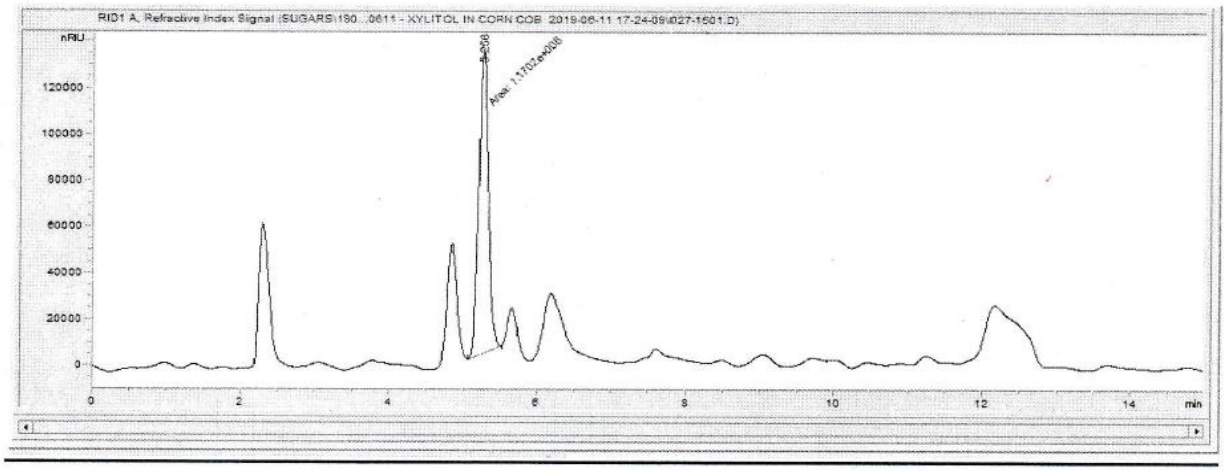

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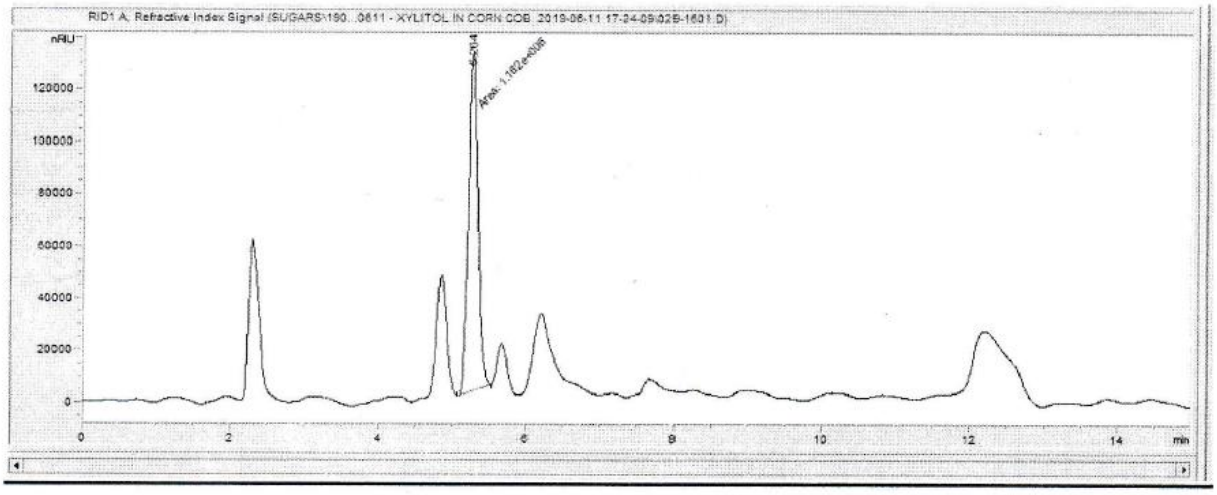

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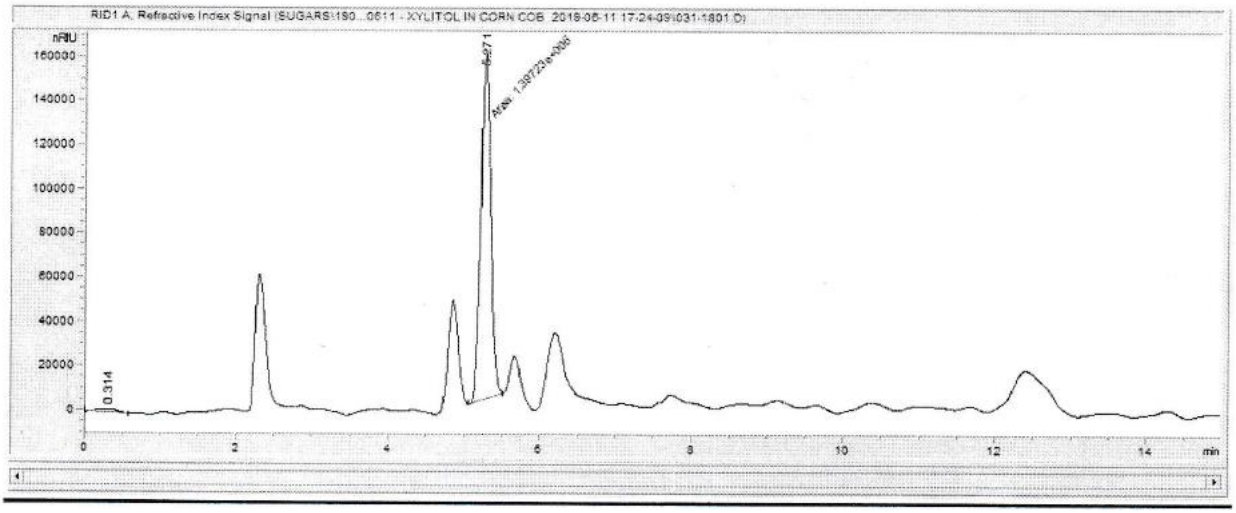

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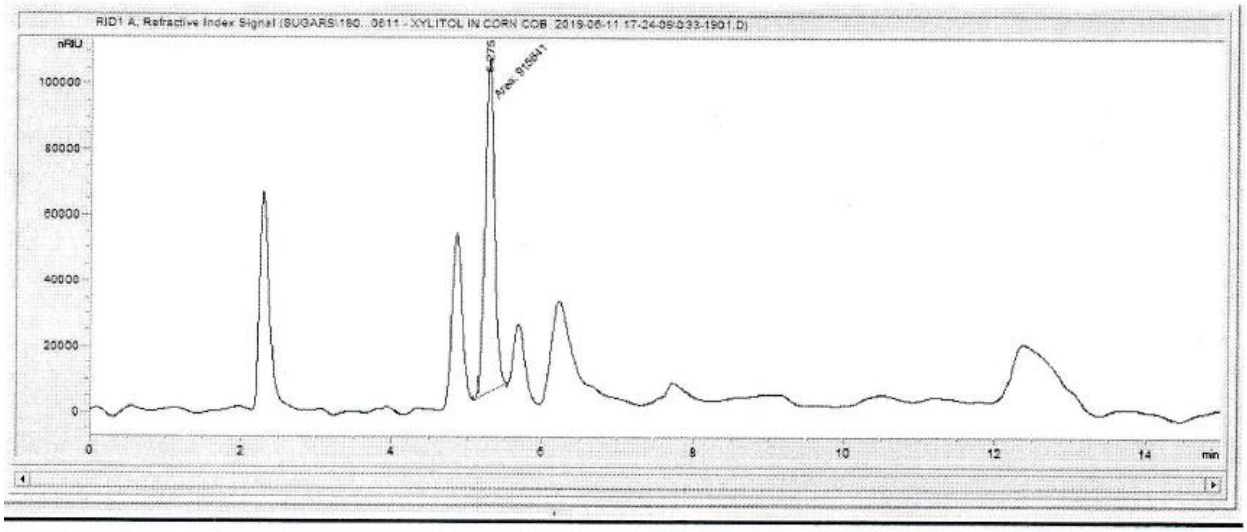
11274036(10)



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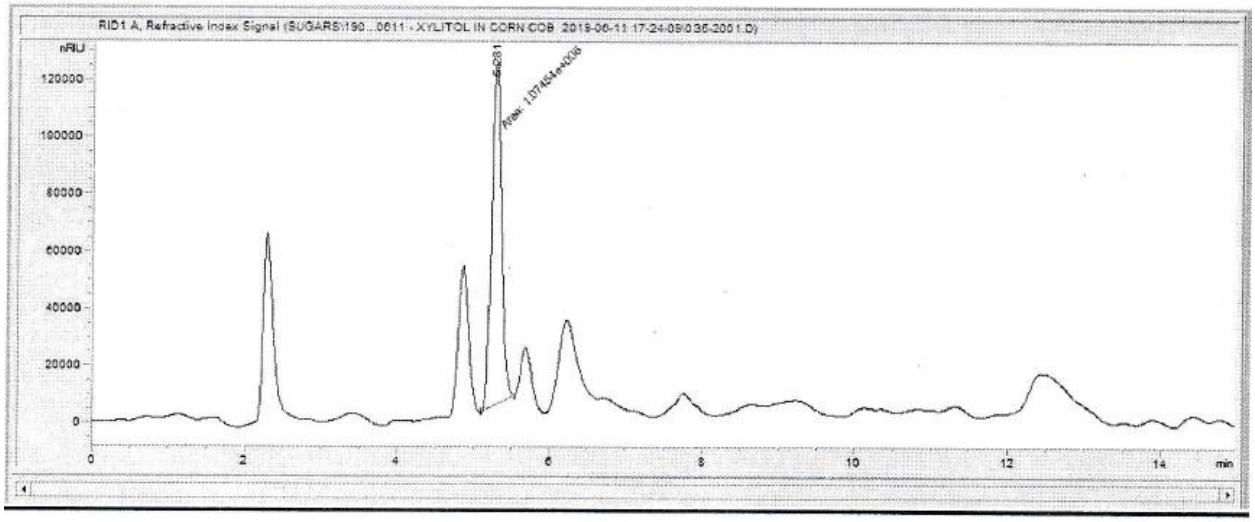

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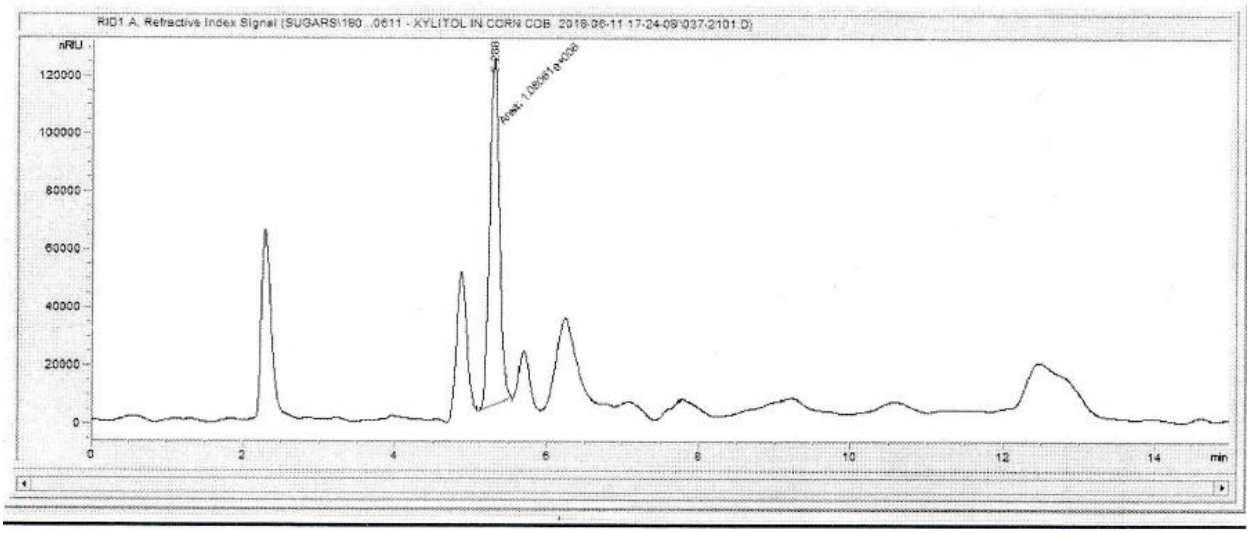

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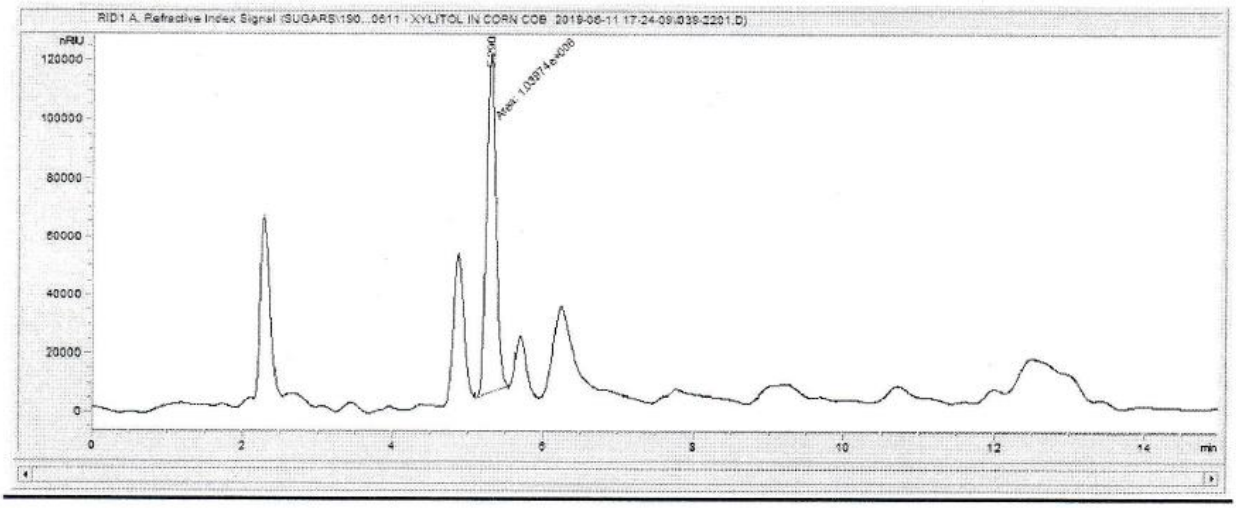

(13)

Data File C:\CHEM32\...RN COB\190611 - XYLITOL IN CORN COB 2019-06-11 17-24-09\031-1801.D
Sample Name: 035

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==========================================================================
Acq. Operator   : Abeba                          Seq. Line :  18
Acq. Instrument : Instrument 1                    Location : Vial 31
Injection Date  : 11-Jun-19 9:58:41 PM                Inj :   1
                                                 Inj Volume : 10.000 µl
Acq. Method     : C:\CHEM32\1\DATA\SUGARS\190611 - XYLITOL IN CORN COB\190611 - XYLITOL IN
                  CORN COB  2019-06-11 17-24-09\SUGAR ANALYSIS METHOD NH2 COLUMN.M
Last changed    : 18-Apr-19 5:21:05 PM by Hawine
Analysis Method : C:\CHEM32\1\METHODS\AFLATOXIN HD.M
Last changed    : 12-Jun-19 11:21:49 AM by Abeba
                  (modified after loading)
Additional Info : Peak(s) manually integrated
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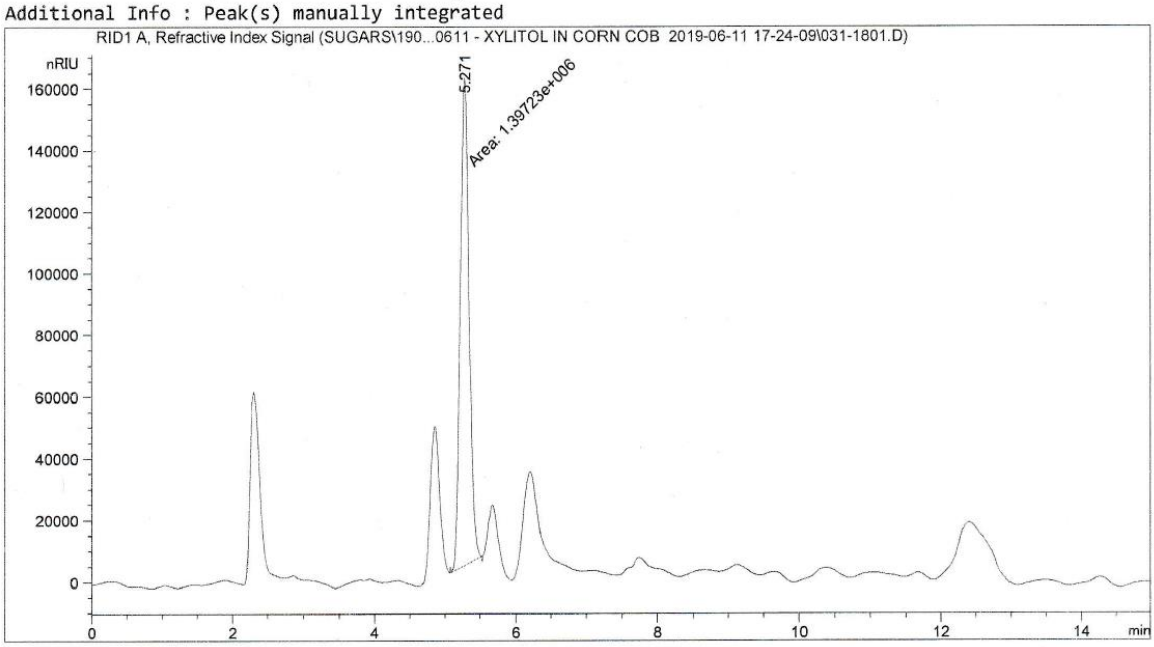
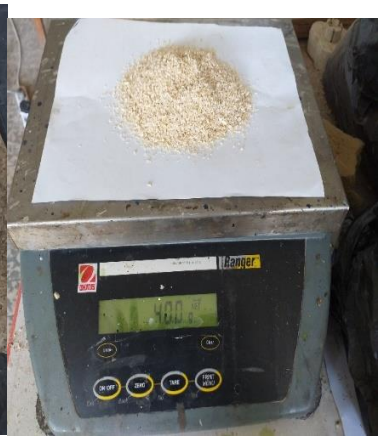



```
==========================================================================
                        Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: RID1 A, Refractive Index Signal

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]  [nRIU*s]    [nRIU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.271 MM    0.1477 1.39723e6  1.57655e5  100.0000

Totals :                  1.39723e6  1.57655e5

==========================================================================
```

Appendix C: experimental Pictures



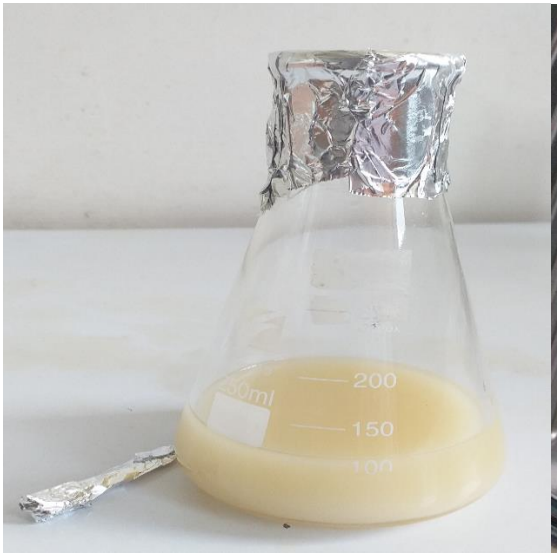
Corn cob **Size reduction**

Corncob Hydrolysis

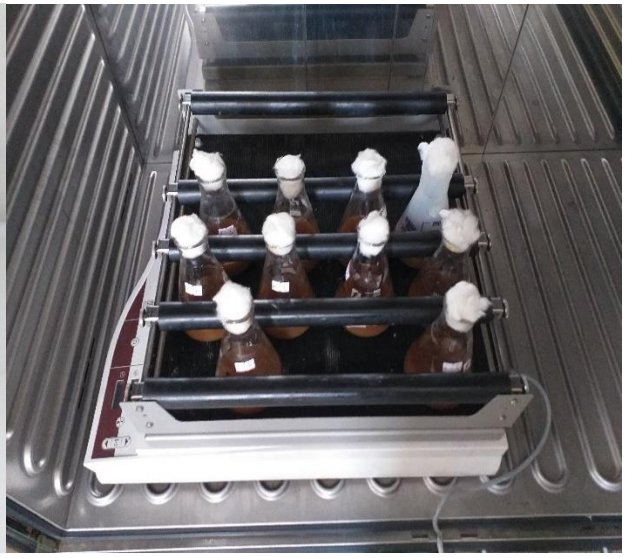
Hydrolysate Detoxification and filtration

Detoxified hydrolysate

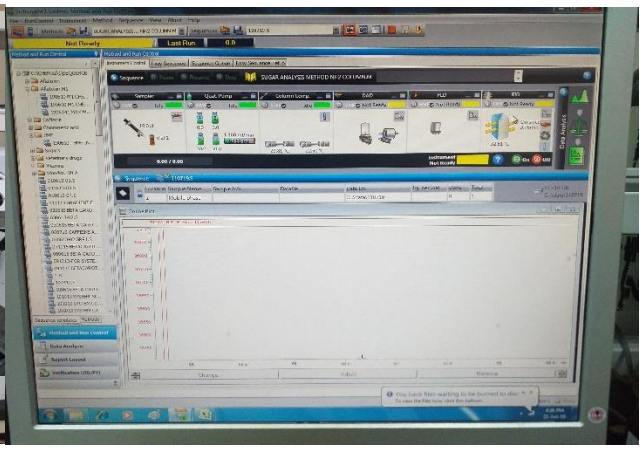
Vacuum Concentration



Inoculum Preparation



Fermentation



HPLC-RID analysis