XYLANASE PRODUCTION BY MIXED CULTURE SUBMERGED FERMENTATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

GÖZDE ÖREN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

FEBRUARY 2014

Approval of the thesis:

XYLANASE PRODUCTION BY MIXED CULTURE SUBMERGED FERMENTATION

submitted by **GÖZDE ÖREN** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Alev Bayındırlı _____
Head of Department, **Food Engineering**

Assoc.Prof.Dr.Deniz Çekmecelioğlu _____
Supervisor, **Food Engineering Dept., METU**

Examining Committee Members:

Prof. Dr.Alev Bayındırlı _____
Food Engineering Dept., METU

Assoc.Prof.Dr.Deniz Çekmecelioğlu _____
Food Engineering Dept., METU

Assist.Prof.Dr.Yeşim Soyer _____
Food Engineering Dept., METU

Assist.Prof.Dr.Halil Mecit Öztop _____
Food Engineering Dept., METU

Assist.Prof.Dr.Deniz Baş _____
Food Engineering Dept., Çankırı Karatekin University

Date: 04.02.2014

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Gözde Ören

Signature

ABSTRACT

XYLANASE PRODUCTION BY MIXED CULTURE SUBMERGED FERMENTATION

Ören, Gözde

M.S., Department of Food Engineering

Supervisor: Assoc.Prof. Dr. Deniz Çekmecelioğlu

February 2014, 89 pages

The growing interest in enzyme production by the usage of microorganisms continues for easier process control and sustainably higher amount of enzyme production. Food and agricultural industry, which produces large amounts of waste materials, is an excellent resource for enzyme manufacture. Among all, xylanase is considered to have 25 % shares in conjunction with pectinolytic enzymes within food enzyme production and extensively used in extraction/clarification of juices and wines, improvement of bread-quality, and improvement of feed/food digestibility etc. Thus, in this study, *Bacillus subtilis* and *Kluyveromyces marxianus* were searched for their potential in xylanase production using mixed culture submerged fermentation method. Effect of inoculum amounts (2-4 ml/100 ml), time (12- 96 h), substrate concentration (10-40 %) and type (arabinose, xylose, sucrose and glucose, synthetic pure xylan and hazelnut shell) as carbon sources, and pH on xylanase production was evaluated at constant temperature of 35^{o}C and agitation of 130 rpm. Xylanase activities of 11.23±0.02 IU/ml

and 12.11±0.05 IU/ml were obtained using 1.0 ml of *K. marxianus* and 1.0 ml of *B. subtilis* cultures, respectively. Enhanced xylanase activity of 17.93±0.04 IU/ml was produced after 24 h of fermentation with mixed cultures of *Bacillus subtilis* (0.5 ml) and *Kluyveromyces marxianus* (0.5 ml). The higher xylanase production of 19.65±0.4 IU/mL was achieved with total inoculum amount of 2.0 ml of *Bacillus subtilis* (1.0 ml) and *Kluyveromyces marxianus* (1.0 ml) after 24 h fermentation. Screening experiments revealed that the highest enzyme activities were obtained using arabinose (26.77± 0.5 IU) and xylose (14.92 ± 0.6 IU/ml) as carbon sources after 24 h fermentation at 35^{o}C, pH 6.5 and 130 rpm. Hydrolyzed hazelnut shell (11.38±0.01 IU/ml) was found the second best raw material after the xylose (14.58±0.06 IU/ml). Optimum conditions suggested by Box-Behnken RSM were found as 4 (ml/100 ml) *B. subtilis*, 4 ml (ml/100ml) *K. marxianus*, and 40 (% w/v) solid load and pH 7 with maximum activity of 49.5 IU/ml. The determination coefficient (R^2) of the model (0.95) and the insignificant lack of fit (p=0.099>0.05) verified that the model fitted well to the experimental data. As a result, xylanase production was increased by 152 % compared to initial unoptimized culture conditions. This study also showed that xylanase production using mixed culture of bacteria and yeast is advantageous over single culturing.

Keywords: mixed culture, *Bacillus subtilis, Kluyveromyces marxianus*, xylanase activity, carbon source

ÖZ

KARIŞIK VE DERİN KÜLTÜR FERMENTASYON YÖNTEMİ İLE KSİLANAZ ENZİMİ ÜRETİMİ

Ören, Gözde

Yüksek Lisans,Gıda Mühendisliği Bölümü

Tez Yöneticisi : Doç. Dr. Deniz Çekmecelioğlu

Şubat 2014, 89 sayfa

Daha kolay proses kontrolü ve sürdürülebilir şekilde daha yüksek miktarda enzim üretimleri için mikroorganizmaların kullanımı ile enzim üretimine artan ilgi devam etmektedir.Büyük miktarlarda atık madde üreten gıda ve tarım sanayi enzim üretimi için mükemmel bir kaynaktır. Ksilanazlar, tüm enzimler içinde pektinolitik enzimlerle birlikte gıda enzim üretiminde % 25 paya sahiptirler ve meyve suları ve şarapların özütlenmesinde/berraklaştırılmasında, ekmek kalitesinin arttırılmasında ve gıdaların/yiyeceklerin sindirilebilirliğinin geliştirilmesinde v.s. yaygın olarak kullanılmaktadırlar. Bu nedenle bu çalışmada, *Bacillus subtilis* and *Kluyveromyces marxianus*'un ksilanaz üretme potansiyelleri karışık kültür derin kültür fermentasyon yöntemi kullanılarak araştırılmıştır. İnokulum miktarları (2-4 ml/100 ml), zaman (12-96 h), karbon kaynağı olarak substrat derişimi (%10-40) ve çeşidi (arabinoz, ksiloz, sükroz ve glikoz, sentetik saf ksilan ve fındık kabuğu) ve pH'ın ksilanaz üretimi üzerine etkileri 35 ^{o}C sabit sıcaklık ve 130 dev/dak karıştırma hızında değerlendirilmiştir. 1.0 ml *K. marxianus* ve 1.0 ml *B. subtilis* kültürleri kullanılarak

sırasıyla 11.23±0.02 IU/ml ve 12.11±0.05 IU/ml ksilanaz aktiviteleri elde edilmiştir. Bacillus subtilis (0.5 ml) ve Kluyveromyces marxianus (0.5 ml) karışık kültürleriyle 24 saatlik fermentasyondan sonra 17.93±0.04 IU/ml aktiviteye sahip arttırılmış ksilanaz aktivitesi üretilmiştir. 19.65±0.4 IU/mL'lik daha yüksek ksilanaz üretimine *Bacillus subtilis* (1.0 ml) and *Kluyveromyces marxianus* (1.0 ml)'tan oluşan toplam 2 ml inokulum miktarı ile 24 saatlik fermantasyondan sonra ulaşılmıştır. Tarama denemelerinde en yüksek enzim aktivitesine, karbon kaynağı olarak arabinoz (26.77± 0.5 IU/ml) ve ksiloz (14.92 ± 0.6 IU/ml) kullanılarak 35^{o}C, 6.5 pH ve 130 dev/dk'da 24 saatlik fermentasyon sonucunda ulaşılmıştır. Hidrolize fındık kabuğu (11.38±0.01 IU) ksilozdan (14.58±0.06 IU/ml) sonra en iyi ikinci hammadde olarak bulunmuştur. Box-Behnken YYY ile optimum koşullar 49.5 IU/ml'lik en yüksek aktiviteyle %4 (ml/100 ml) *B.subtilis*, %1.0 (ml/100 ml) *K.marxianus*, ve %40 (w/v) katı kütle miktarı ve pH 7 olarak bulunmuştur. Modelin deneysel sonuçlara uyumu, hesaplanan varyasyon katsayısı (R^2=0.95) ve önemsiz uyum eksikliği (p=0.099>0.05) sonuçlarıyla doğrulanmıştır. Sonuç olarak, ksilanaz üretimi baştaki optimize edilmemiş kültür şartlarına oranla %152 arttırılmıştır. Ayrıca bu çalışma, bakteri ve maya karışımı kültür kullanarak ksilanaz üretiminin tekli kültür kullanımına göre avantajlı olduğunu göstermiştir.

Anahtar Sözcükler: karışık kültür, *Bacillus subtilis*, *Kluyveromyces marxianus*, ksilanaz aktivitesi, karbon kaynağı

TO MY BELOVED FAMILY

ACKNOWLEDGMENTS

Foremost, I would like to express my greatest thanks to my supervisor Assoc. Prof. Dr. Deniz Çekmecelioğlu for his motivation, enthusiasm, immense knowledge and patience throughout the research. He made this study possible by helping and encouraging me at all the stages of the research.

Besides my advisor, I am deeply grateful to Res. Assist. Sibel Uzuner for her insightful comments and hard questions.

I thank my fellow lab mates who were always willing to help and give their best suggestions. I also thank all the members of staff at Çankırı Karatekin University and Middle East Technical University that provided all kind of necessary support for this research. I would like to thank to METU BAP coordination for their financial support.

My deepest appreciation goes to my family. Words cannot express how grateful I am to my mother Arzu Ören, my father Ramazan Ören and brother Umut Ören for all of the sacrifices that they've made on my behalf. Special thanks to all my friends who have supported me and had their contributions in making this thesis possible.

At the end I owe my deepest gratitude to my beloved husband Metin Cudi Yardımcı who spent sleepless nights with and was supported me in the moments when there was no one to answer my queries.

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ANOVA: Analysis of Variance

ATCC: American Type Culture Collection

CaA: carboxylic acid amide

CMC: Carboxymethyl cellulose

CMCase: carboxymethyl cellulase

CV: Coefficient of Variance

DNS: Dinitrosalicylic Acid

LAB: Laboratory

MAE: Mean Absolute Error

ND: Not determined

NRLL: Agricultural Research Center Culture Collection

RMSE: Root Mean Square Error

RSM: Response Surface Methodology

Rpm: Revolutions per minute

SmF: Submerged Fermentation

SSF: Solid State Fermentation

TRS: Total reducing sugar

U: Enzyme activity unit

CHAPTER 1

INTRODUCTION

The demand for industrial enzymes from microbial sources is increasing due to their applications in various processes and much higher catalytic activities than the enzymes from animal and plants, absence of unwanted by-products, more efficient production, higher stability and economic values. Among all, xylanase is considered to have 25% share in conjunction with pectinolytic enzymes within food enzyme production. Importance of microbial xylanases have also increased due to its biotechnological applications in mostly food, animal feed, paper and pulp industries in bioconversion of lignocellulosic by-products into value-added products.

Although xylanase can be produced by mixed and single cultures of bacteria, mold and yeast, there is no published research in literature that could have been accessed so far about mixed culture production of xylanase with yeast and bacteria. Therefore, this study is the first to describe xylanase production using mixed culture of yeast (*Kluyveromyces marxianus*) and bacteria (*Bacillus subtilis*).

Chapter 2 outlines basic concepts of food enzymes, especially xylanase as the focus of this study. The first two parts of this chapter include general information about food enzymes; properties of food enzymes, production methods, raw material types, and lastly xylanase as a food enzyme.

The third and fourth parts focus on xylanase production with different inoculum types (single and mixed culture). Last two parts explain the xylanase production and raw material relation including examples of mixed culture fermentation in food industry.

Chapter 3 describes material and methods of the study. Medium conditions, raw material properties and methods of the experiments are explained. These methods consist of acid hydrolysis, inoculum preparation and enzyme production, xylanase assay, analytical tests and initial screening of important variables. Optimization with response surface methodology is also explained in this chapter.

Finally, in chapter 4, results are given and discussed according to previous literature review. This chapter includes the xylanase production of single and mixed cultures of *Bacillus subtilis* and *Kluyveromyces marxianus* in synthetic media; focusing on the effects of fermentation time, inoculum level, and raw materials as carbon source on mixed culture xylanase production; and major factors that affect xylanase production to optimize the fermentation conditions for mixed culture xylanase production.

CHAPTER 2

LITERATURE REVIEW

2.1 Food Enzymes

Enzymes are proteins which serve as catalysts without any changes by increasing the rate of chemical reactions. They are crucial for daily and economic life due to their role in important metabolic activities. Enzymes are produced by crops, animals, bacteria, fungi and other organisms (Renge *et al.*, 2012). Although some specific enzymes of plant or animal source take part in industry, microbial sources have gained importance for three decades.

Enzymes of microbial sources are useful due to much higher catalytic activities than enzymes from animal and plants, absence of unwanted by-products, more efficient production, higher stability and economic values. Today, in industry mostly microbial enzymes are used and that cause an increase in the amount of microorganism used. However, the type of the microbial source is important and must be chosen wisely according to their toxicity and pathogenicity (Kirk *et al.*, 2002).

Nowadays, microbial enzymes are used in different types of processes such as dairy processing, brewing, meat production, fruit juice clarification, fructose syrup production, detergent production, textile production, paper processing and medicine as summarized in Table 2.1 (Kirk *et al.*, 2002).

Table 2.1 Industry vs application of various enzymes adapted from Kirk *et al.* (2002).

Industry	**Enzyme**	**Application**
Animal feed	Phytase Phytate	Digestibility–phosphorus
	Xylanase	release
Baking	Amylase	Bread softness and
	Xylanase	volume, Dough stability
	Lipase	and conditioning
Detergent	Protease	Cleaning, color
	Amylase	clarification
	Lipase	stain removal
	Mannanase	
Food	Protease	Milk clotting, infant
	Lipase	formulas
	Lactase	Cheese flavor
	Pectinase	Lactose removal (milk)
	Transglutaminase	Fruit-based products
		Modify visco-elastic
		properties
Personal care	Amyloglucosidase	Antimicrobial,
	Glucose oxidase	Bleaching
	Peroxidase	
Pulp and paper	Lipase	Pitch control, contaminant
	Amylase	control
	Xylanase	Starch-coating, de-inking,
	Cellulase	drainage improvement
		Bleach boosting
		fiber modification
Starch and fuel	Amylase	Starch liquefaction
	Amyloglucosidase	Saccharification
	Glucose isomerase	Glucose to fructose
	Xylanase	conversion
		Viscosity reduction (fuel
		and starch)

The use of enzymes in industrial processes is called "enzyme technology". Enzyme technology covers microbial processes (selection and improvement of producer strains), production of enzymes by fermentation (media, environmental conditions for large scale production) and replacement of three dimensional enzyme structure to enhance catalytic activity (protein engineering), isolation and immobilization of enzymes (Kirk *et al.*, 2002).According to Wiseman (1987), commercially used enzymes consist of proteases (59%), carbohydrases (28%), lipases (3%) and other enzymes (10%). In the carbohydrases group, 13% of the total production is α-amylase.

The food industry uses an extensive diversity of products from animal and plant sources as cornerstone in processing, which are primary for more extensive types of products to consumers. Enzymes from different microorganisms have been used in baking industry, alcoholic beverages, vinegar, dairy products and many other food products in order to qualify and enhance the operational, nutritional and sensory properties of products and their constituents (Kirk *et al.*, 2002).

Enzymes used in food industry have a number of advantages. First of all, they are an alternative to chemicals used in production, therefore they can replace artificial chemicals in an extensive range of processes. This has advantages in environmental aspects via low energy consumption and high biodegradability of products. Secondly, enzymes are more specific than chemicals and produce fewer side reactions and by-products, thus the quality of products is higher. Some enzymes can also work under extreme conditions (e.q. 95-100ºC) without destroying valuable properties of foods and their constituents. Lastly, enzymes are essential for food processes like wine production and cheese production those would be difficult to proceed, otherwise (Whitehurst and Oort, 2009).

Chymosin is the first enzyme that is biotechnologically produced by genetically modified microorganisms and used in cheese making, previously being extracted from the fourth stomach of calves. In food processing, various enzymes are used and the number will increase with the new discoveries of microbial world (Whitehurst and Oort, 2009). A summary of the enzymes used in various food applications is given in Table 2.2.

Table 2.2 Enzymes used in various food applications (Adopted from Whitehurst and Oort, 2009)

Enzyme	**Source**	**Action in food**	**Application**
Amylase	*Aspergillus spp.* *Bacillus spp.* *Microbacterium imperiale*	Wheat starch hydrolysis	Dough softening, bread volume, production of sugars for yeast fermentation
Cellulase	*Aspergillus niger* *Trichoderma spp.*	Hydrolyzes cellulose	Fruit liquefaction
Hemicellulase and xylanase	*Aspergillus spp.* *Bacillus subtilis* *Trichoderma reesei*	Hydrolyzes hemicelluloses (insoluble non-starch polysaccharides in flour)	Bread improvement through improved crumb structure
lactase	*Aspergillus spp.* *Kluyveromyces spp*	Hydrolyzes milk lactose to glucose and galactose	Sweetening for lactose-intolerant
Protease	*Aspergillus spp.* *Rhizomucor miehei*	Hydrolysis of κ-casein	Milk coagulation for cheese making hydrolyzate

Besides the advances which were mentioned above, a few new applications within the food industry should be mentioned (Kirk *et al.*, 2002).By further development of enzyme technology, the diversity and high economic value of the food products and industrial enzymes related to various surveys in the field of food biotechnology are gaining increased attention. As a result, enzymes of various varieties have a wide use in the food industry. Developments in technology are also important that production areas are becoming more widespread. The importance of the microorganisms in the preparation of the enzymes is also increasing day by day.

2.2 Enzyme Production

Enzymes are proteins that act like catalytic agents by dropping the energy required for a reaction to take place and being not spent in the same reaction. Different industries use enzymes for production. Examples of these productions are; wine making, bakery, dairy products, etc. Enzymes may be acquired from animal and plant sources but to produce enzymes for industrialized processes microbial fermentation is the most economic and convenient method.

'Fervere' in Latin means to boil, which defines the look of the action of yeast during production of alcoholic beverages. (Das and Deka, 2012).However, fermentation is taken in a different way by microbiologists. To a microbiologist, fermentation means any procedure for the product formation by the mass culture of microorganisms (Stanbury, 1988). In this section fermentation is used in this microbiological perspective. For example, yeast is a fungus, whose enzymes utility lead to anaerobic breakdown of glucose into ethanol and carbon dioxide.

$$C_6H_{12}O_6\ (aq) \xrightarrow{\text{Yeast}} 2\ C_2H_5OH\ (aq) + 2\ CO_2\ (g)$$

When the reaction above takes place in the absence of oxygen, it is called fermentation (Renge *et al.*, 2012). Fermentation is used in the production of all alcoholic beverages. Fermentation is also used in bakery to increase bread volume. When the dough is ready before putting into the oven, it is left to respite in a warm place that gives the enzymes of yeast a chance to break down the sugar and form carbon dioxide. There are two methods of fermentation for enzyme production; submerged fermentation and solid-state fermentation. In submerged fermentation enzymes are produced in liquid medium which has enough nutrients for microorganisms. On the other hand, in solid state fermentation enzyme production occurs on a solid substrate, whose carbon sources are broken down by microorganisms. Both intracellular and extracellular enzymes can be produced in the two fermentation methods. These enzymes are recovered by downstream processing of appropriate choice depending on location of enzymes.

2.2.1 Methods of Fermentation

2.2.1.1 Submerged Fermentation

Submerged fermentation (SmF) involves growth of microorganisms and production of by products in liquid media. The desired microorganism is inoculated into sterilized, closed fermentation vessels, which have enough nutrients and oxygen. Microorganisms release desired enzymes into the medium by breaking down the nutrients in the solution. Nutrients can be taken from synthetic media or raw materials like wheat straw, rice husk, corn, etc.

Fed-batch and continuous fermentation processes can be used to break down carbon and nitrogen sources in the fermentation media (Renge *et al.*, 2012).

In the fed-batch process, while the biomass is growing, the sterilized nutrients are added to the vessel. In the continuous process, the sterilized nutrients are supplied to the fermenter at controlled flow rate as the fermentation broth exits the system which finally reaches steady-state condition (Renge *et al.*, 2012). Parameters such as carbon dioxide formation, oxygen consumption, temperature, pH, and agitation can be monitored and controlled during fermentation.

Downstream processing is required to recover the desired product; enzyme. The first step is to remove insoluble products like microbial cells from the medium by centrifugation, as the most commercial enzymes are extracellular, meaning that they are secreted into the liquid medium in which they remain after centrifugation.

In industrial scale production, the recovered biomass can be used as fertilizers or animal feed. After centrifugation, the desired enzyme is furthermore concentrated by membrane filtration, evaporation and crystallization according to their intended use. However, gel or ion exchange chromatography can be used to purify enzyme preparations if needed. These enzyme preparations can be in the form of both granules and liquid formulations (Renge *et al.*, 2012).Another way to use enzymes in submerged fermentation is immobilization. Immobilized enzymes are placed on surfaces of inert granules and held in reaction columns. Starch conversion by amylase columns can be an example for this type of process. Immobilization increases the usability of enzymes by allowing them to be repeatedly used (Renge *et al.*, 2012).

2.2.1.2 Solid State Fermentation

An alternative method to submerged fermentation for enzyme production is solid state fermentation (SSF).In this method, microorganisms are cultivated on a solid substrate such as rice, grains, sugarcane bagasse. Both methods are used industrially but SSF has some advantages over SMF because of higher production rate, lower effluent generation and simpler fermentation equipment (Renge *et al.*, 2012).Many substrates can be used for production of enzymes by SSF. The most important factor affecting SSF process is the properties of the solid substrate used. Several factors affect the substrate selection. They are mainly related to cost and availability of the substrate that leads to using agro-industrial wastes. Agro-industrial residues are considered the best substrates to produce enzymes (Mitra *et al.*, 1996). Second task of the substrate is to serve as a port for the biomass in SSF.

Among the numerous factors, particle size and moisture level/water activity are the most critical as they are essential for microbial growth and activity on a specific substrate (Auria *et al.*, 1992; Barrios-Gonzalez *et al.*, 1993; Echevarria *et al.*, 1991; Liu and Tzeng, 1999; Pandey *et al.*, 1994; Pastrana *et al.*, 1995; Roussos *et al.*, 1993; Sarrette *et al.*, 1992; Smail *et al.*, 1995; Zadrazil and Puniya, 1995).Substrates should have relatively smaller particles with a larger surface area. If the particles are too small there will be no efficient respiration that causes poor growth and inevitably poor enzyme production. Also larger particles will provide efficient agitation and respiration, but they have smaller surface area for production. A compromise must be reached, about the size of the particle of the substrate for a specific process (Pandey and Joshi, 1999).

Moisture content is another important aspect for solid state fermentation. It must be optimized for the specific process because higher or lower water activity adversely affects the microbial biomass. Water also has effects for the physicochemical properties of the solid substrate (Renge *et al.*, 2012).

2.2.2 Substrates Used for Production of Food Enzymes

As it can be seen from Table 1.3, to produce specific enzyme by microorganisms, substrates are the most important element in fermentation. Another importance of substrate is that different substrates lead to different products. In other words, microorganisms produce different enzymes according to the substrate properties. (Suganthi *et al.*, 2011). Sometimes the substrate doesn't provide the necessary nutrient for microbial growth. Therefore, to make it ideal for the microorganisms to grow, some supplements should be added externally.

Furthermore, sometimes microorganisms may not use the substrate because nutrients are hold by other structures in the substrate. For this reason, chemical or mechanical pre-treatment methods prior to fermentation process become important. These methods make the substrates more accessible to microbial growth (Pandey, 1994; Chahal and Moo-Yong, 1981).A number of substrates that have been used for cultivation of microorganisms to produce enzymes is listed below (Table 2.3).

Table 2.3 Substrates for cultivation of microorganisms to produce enzymes

Substrate	**Microorganism**	**Enzyme**	**References**
Coconut coir pith	*A. niger*	Cellulase, β-glucosidase	(Muniswaran *et al.*, 1994)
Grapevine trimming dust	*Cerrena unicolor*	Cellulase, xylanase, ligninase	(Zakariasvili and Elisashvili, 1993)
Tea production waste	*Cerrena unicolor, Colorius hirsutus,*	CMCase, xylanase	(Kokhereidze and Elisashvili, 1993)
Cellulose, starch	*T.viride, A. niger*	Cellulase, amylase	(Desgranges and Durand, 1990)
Sweet sorghum silage	*Gliocladium sp., Trichoderma sp.*	Cellulase, xylanase,	(Szakacs and Tengerdy, 1996)
Agro-wastes	*A. niger*	Cellulase, β-glucosidase	(Madamwar *et al.*, 1989)
Sugar beet pulp	*P.capsulatum*	Polysaccharide degrading enzymes	(Considine *et al*., 1988)
Wheat bean + rice straw, spent wheat bran	*Trichoderma sp., Botritis sp., A.ustus,*	Cellulase, β-glucosidase, xylanase	(Shamla and Sreekantiah, 1986)
Palm oil mill waste	*A. niger*	Cellulase, xylanase	(Prasertsan *et al*., 1997)
Bagasse	*T. reesei, A. niger, A. phoenicis*	Xylanase	(Correa and Tengerdy, 1998)
Rice straw, soybean hull, wheat bran	*A. sojae*	Xylanase, α-arabinofuranosidase	(Kimura *et al*., 1995)

2.2.3 Xylanases as the Focus of This Study

Agro-industrial residues mostly consist of lignocellulosic materials (lignin, hemicellulose, cellulose). As it can be seen from the Table 2.3 important enzymes like cellulose, pectinase, xylanase, and ligase are produced from lignocellulosic materials. Hemicellulose is the significant component of lignocellulosic material and its bioconversion is important for economy and industrial use.

Xylan is the main component of plant cell walls and the most abundant renewable hemicellulose.

Approximately 20±40% of total plant biomass constitutes xylan. Therefore, a substantial interest in xylan utilization has grown due to its potential applications in enzyme production, waste treatment, energy generation, production of chemicals and paper manufacture. (Kuhad and Singh, 1993; Sunna and Antranikian, 1997).

Xylan has heterogeneous and complex chemical structure and there are several hydrolytic enzymes to break down the xylan completely with different specificity and action sites. This is due to the fact that xylan from different sources shows major dissimilarity in composition and structure (Figure 2.1).

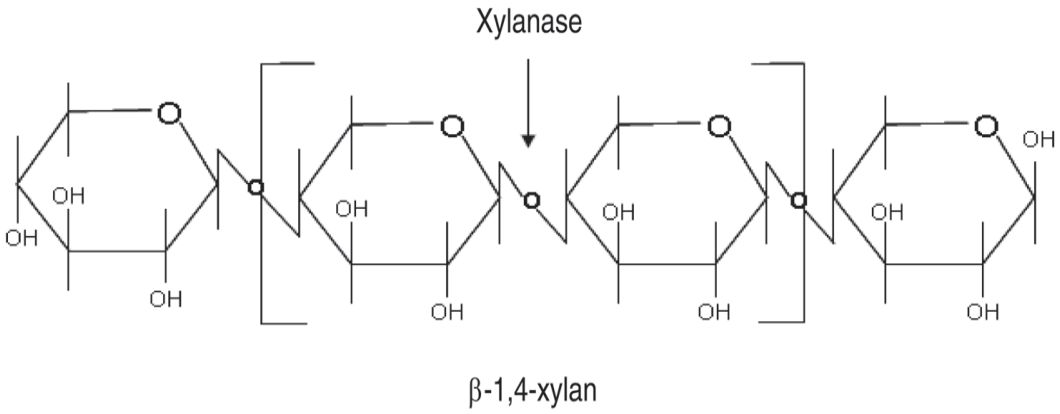


Figure 2.1. Structure of xylan and point of xylanase action (Butt *et al*., 2008).

Xylanases are glycosidases, which catalyze endohydrolysis of 1,4-β-D-xylosidic linkages in xylan, and involved in the production of xylose. They are important for bioconversion of hemicelluloses.

Submerged and solid state fermentation are the two of the numerous biotechnological methods used for xylanase biosynthesis (Cai *et al.*, 1998; Gawande and Kamat, 1999; Kansoh, 2001).

Mostly submerged fermentation is used to produce enzymes. However, there is an increase in using solid state fermentation techniques to produce enzymes, generally xylanases from fungal sources.

The complex structure of xylan needs different enzymes for its complete hydrolysis. The main-chain enzymes involved are Endo-1, 4-β-xylanases (E.C.3.2.1.8), which

depolymerize xylan by random hydrolysis of xylan backbone and 1, 4-β-D-xylosidases (E.C.3.2.1.37), which split off small oligosaccharides.

Depending on both the source and the procedure used in its extraction and purification, the side groups present in xylan are liberated by α-L-arabinofuranosidase, α-Dglucuronidase, galactosidase and acetyl xylan esterase (Figure 2.2).

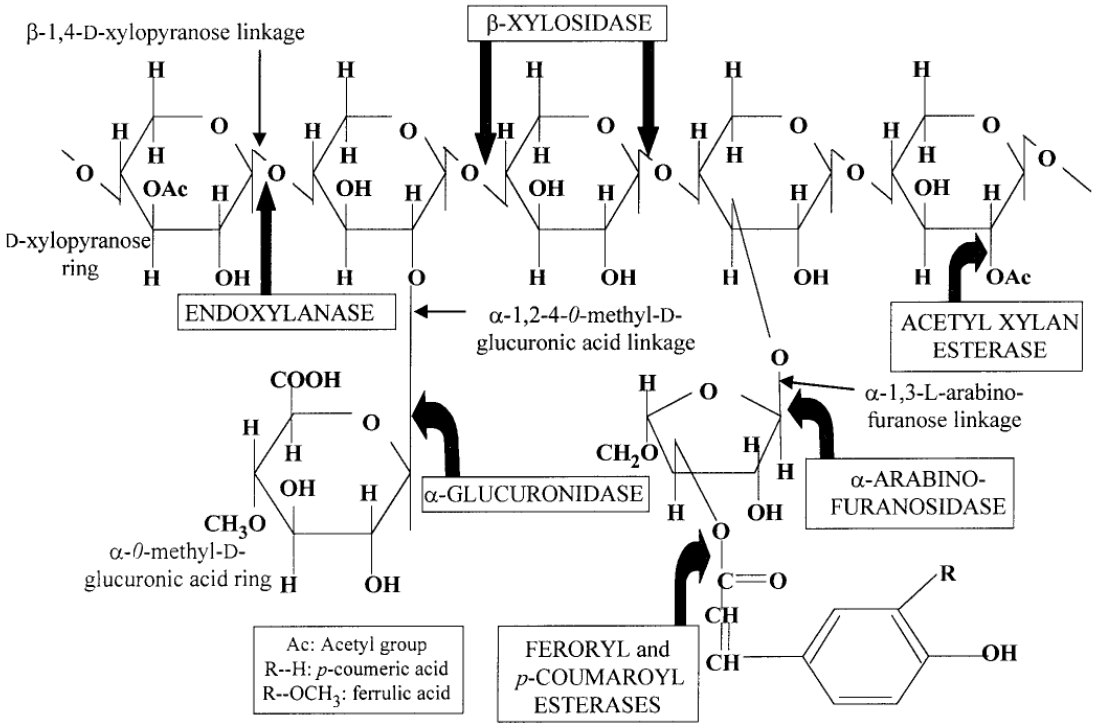


Figure 2.2. A plant xylan structure showing different substituent groups with sites of attack by microbial xylanase (adapted from Beg *et al*., 2001)

Most substrates are large in size that prevents the entrance into the cell, thus xylanases are excreted into the extracellular environment.

In fact, the current belief is that xylanase production is induced by means of the products of their own action (Singh *et al.*, 2000; Biely, 1985; Defaye *et al.*, 1992; Collins *et al.*, 2005). It is believed that small amounts of constitutively produced enzymes liberate xylo-oligomers, which may be transported into the cell where they are further degraded by β-xylosidases, or indeed by intracellular xylanases (Fontes *et al.*, 2000; Teplitsky *et al.*, 2000; Collins, 2005), and thus induce further xylanase synthesis.

The Xylanases have been utilized mostly from bacteria (Saleem *et al.*, 2002; Azeri *et al.* 2010), mold (Deschamps and Huet 1985; Chapla *et al.*, 2010), actinomycetes (Li *et al.*, 2010; Rifaat, 2006), and yeast (Xin and He, 2012; Rajoka, 2007). Some examples of the types of microorganisms used in the production of xylanase are *A. niger* (Deshpande, 2008), *A. phoenicis* (Duenas *et al.*, 1995), *Bacillus pumilus* (Battan *et al.*, 2007), *B. subtilis* (Irfan *et al.*, 2013), *Trichoderma reseii* (Xiong *et al.*, 2004) and *Streptomyces chromofuscus* (Rifaat, 2006), *Kluyveromyces marxianus* (Rajoka, 2007).

Xylanases, first reported in 1955, were termed as pentosanases due to their similarities and assigned the enzyme code EC 3.2.1.8 by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 (Whistler and Masak, 1955).

They are genetically single chain glycoproteins, between 6.5-85 kDa, of molecular weight and temperature of 35-65 °C and active at pH of 4-6.5. The optimum temperature and pH differ for xylanases with different sources. (Butt *et al.*, 2008).

Table 2.4 Characteristics of some xylanases produced by different microorganisms (Butt *et al.*, 2008).

Microorganism	**Molecular mass/ kDa**	**Optimum pH**	**Optimum temp./ºC**	**Reference**
Aspergillus awamori	39	5.5-6.0	40-55	(Kormelink *et al.*, 1993)
Aspergillus nidulans	34	6.0	56	(Fernandes *et al.*, 1994)
Aspergillus nidulans KK-99	nd	8.0	55	(Taneja *et al.*, 2002)
Aspergillus oryzae	35	5.0	60	(Kitamoto *et al.*, 1999)
Aspergillus sojae	32.7	5.0-5.5	50-60	(Kimura *et al.*, 1995)
Aspergillus terreus	nd	7.0	50	(Ghanem *et al.*, 2000)
Aspergillus terreus	nd	4.5	45	(Ghareib and El Dein, 1992)
Myceliophthora sp.	53	6.0	75	(Chadha *et al*., 2004)
Penicillium capsulatum	22	3.48	48	(Ryan *et al*., 2003)
Streptomyces sp.	24.5	6.0-8.0	55-60	(Georis *et al*., 2000)

Nd = not determined

Xylanases have gained attention in biotechnology due to their application in several industries such as paper, feed, food and fermentation (Bajpai, 1999 and Butt *et al.*, 2008), under different commercial names (Table 2.5).

Table 2.5 Examples of commercial preparations containing xylanases. Data obtained from, Beg *et al.* (2001) and Haltrich *et al.* (1996). SmF: Submerged Fermentation, SSF: Solid Stated Fermentation.

Company	**Product**	**Strain And Mode Of Fermentation**	**Applications**
Alltech, Inc, (USA)	"Allzym PT"	*Aspergillus niger* (SmF)	Upgrading animal feed.
Alltech, Inc, (USA)	"Fibrozyme"	*Aspergillus niger* and *Trichoderma viride* (SSF)	Upgrading animal feed.
Amano Pharmaceutical Co, Ltd (Japan)	"Amano 90"	*Aspergillus niger* (SSF)	Pharmaceutical, food and feed industry.
Danisco Ingredients (Denmark)	"Grindazym PF" and "Grindazym GP 5000"	*Aspergillus niger* (SmF)	Supplementation of feed.
Gamma Chemie GmbH (Germany)	"Gammafeed X" "Gammazym X400OL"	*Trichoderma longibrachiatum* (SmF) *Trichoderma reesei* (SSF)	Production of wheat starch, baking and Feed and brewing industry
Röhm GmbH (Germany)	"Rohalasa 7118" "Vernon 191"	*Aspergillus sp.* and *Trichoderma sp.* (SmF). Same as above	Reduction of viscosity in starch processing. Baking industry.
Shin Nihon Chemical (Japan)	"Sumizyme X"	*Trichoderma koningii* (SSF)	Manufacturing of mushroom and vegetable extracts

Potential applications of xylanases in the pulp and paper industry have enhanced gradually. At this time, the most popular application of xylanase is in prebleaching of kraft pulp to minimize use of severe chemicals in the following treatment phases of kraft pulp (Beg *et al.*, 2001).

Xylanases are also used in animal feeds (Bhat, 2000), dough handling (Maat *et al.*, 1992), fruit and vegetable juices (Biely, 1985; Galante *et al.*, 1998), and starch-gluten separation (Frederix *et al.*, 2003).

Their potential efficiency in bread making has increased the applications of xylanase. Previously, different flour quality and deviations in processing factors can affect the dough quality but with xylanase the dough becomes more tolerant to these changes (Dervilly *et al.*, 2002; Harbak and Thygesen, 2002). In the bread making industry starch and non-starch carbohydrate-hydrolyzing enzymes are commonly used as bread improvers (Polizeli *et al.*, 2005 and Javier *et al.*, 2007).

Xylanase enzymes are also used to speed up the baking time by assisting to break down polysaccharides in the dough (Godfrey and West, 1996). Jiang (2005) stated that the crumb firmness was reduced to 57.9 % with the addition of 100 ppm of XynB. Rouau, (1993) explained that water binding capacity of dough is increased with increasing enzyme activity to form a uniform crumb and these changes prevent dough from sticking to the machinery parts.

2.3 Xylanase Production

Xylanases are inducible extracellular enzymes that secreted in media containing pure xylan or residues containing xylan (Balakrishnan *et al.*, 2000). However, constitutive production of xylanase has also been reported (Khanna, 1993; Khasin *et al.,* 1993; Lindner *et al.*, 1994; Gomez de Segura, 1998; Beg *et al*., 2001).

Induction is mostly by xylan in *Trametes trogii* (Levin and Forschiassin, 1998; Beg *et al*., 2001), and *Aspergillus awamori* (Siedenberg *et al.*, 1998; Beg *et al*., 2001). Also a significant level of xylanase is detected with Birchwood (162 U/ml) and beech xylan (367 U/ml) from *T.aurantiacus* (Alam *et al.*, 1994).

According to Rani and Nand (1996), pure xylan and xylose were two of the good inducers; fructose, galactose, glucose, maltose reduced enzyme activity and lastly no activity in the presence of cellobiose, raffinose, mannitol, starch, pectin and CMC was reported.

On the other hand, readily metabolizable sugars like glucose or xylose, can suppress the xylanase synthesis (Bataillon *et al.*, 1998; Beg *et al.* 2001; Fernandez-Espinar *et al.*, 1994; Ishihara *et al.*, 1997).Induction of xylanase by some other compounds including xylose, and lignocellulosic residues, has been reported (Liu *et al.*, 2008). Soy flour and oat spelt xylan in medium induces the xylanase production in *Scytalidium thermophilum* and *Sporotrichum thermophile* respectively (Joshi and Khare, 2011). Xylanase synthesis in yeast *Trichosporon cutaneum* also induced some positional isomers (Hrmova *et al.*, 1984; Beg *et al.*, 2001).

Several microorganisms including mold, yeast and bacteria have been reported to be readily hydrolyzing xylan by synthesising 1, 4-β-D endoxylanases and β-xylosidases as summarized in Table 2.6 (Subramaniyan and Prema, 2002).

Table 2.6 xylanase producing microorganisms (Subramaniyan and Prema, 2002).

Microorganism	**Xylanase IU/ml**	**Reference**
Aspergillus awamori VTT-D-75028	12.00	(Poutanen *et al.*, 1987)
Aspergillus niger sp.	76.60	(Bi *et al.*, 2000)
Fusarium oxysporum VTT-D-80134	3.70	(Poutanen *et al.*, 1987)
Schizophyllum commune	1244	(Steiner *et al.*, 1987)
Talaromyces emersonii CBS 814.70	56	(Tuohy *et al*., 1990)
Thermomyces lanuginosus *	650-780	(Gomes *et al*., 1993)
Trichoderma reesei RUT C-30 ATCC 56765 *	400	(Gamerith *et al*., 1992)
Trichoderma viride	188.10	(Gomes *et al*., 1992)
Bacillus SSP-34	506	(Subramaniyan, 2000)
Bacillus circulans	400	(Rättö *et al*., 1992)
Bacillus stearothermophilus StrainT6 *	2.33	(Lundgren *et al*., 1994)
Bacillus sp.	11.50	(Paul and Varma., 1993)
Bacillus sp. Strain NCL 87-6-10	93	(Balakrishnan *et al*., 2000)
Streptomyces sp.	3.50	(Techapun *et al*., 2002)

*Microorganisms producing 'virtually' cellulose-free xylanases

2.4 Inoculum Type

Microorganisms have been searched for decades as a biological resource. These studies can be divided into two kinds: natural mixed culture studies and single culture studies. A diversity of organisms had been participated in traditional microbial fermentation, before microscope by Leeuwenhock and microbial pure cultivation method by Pasteur (Chen, 2013). Nowadays, in specific processes, pure culture fermentation is used. There are no unstable traits, no complex situations and unwanted mutual interferences between species.

In Microbiology, mixed culture refers to a laboratory culture that contains more than one species of organism that are grown in a medium. Establishment of metabolic synergisms between different microorganisms using mixed cultures is a distinctive and incomparable goal.

In nature, fungi and bacteria normally grow in symbiotic relations on solid substrates such as soils or plant materials. The catalytic processes of microbial metabolism are often completed by two or more microorganisms, closely interacted with each other. These complex substrates need contribution of a wide variety of different enzymes produced by different microorganisms to be degraded (Gupte, 1998; Fedorova *et al.*, 2012). For example, koji-making process is accomplished by the symbiotic group of mold, yeasts and bacteria (Chen, 2013).

2.4.1 Single Culture

In single pure culture xylanase production, several aspects can affect production such as type of the process, temperature, pH and carbon source. Irfan *et al.* (2013) and Saleem *et al.* (2002) reported maximum xylanase activities on sugarcane bagasse using *Bacillus subtilis*-BS05 with addition of sucrose. The outcome of L-arabinose-rich plant hydrolysate for xylanase synthesis by *T. reesi* C-30 was studied (Xiong *et al.*, 2004). Terrasan *et al.* (2010) considered the production of extracellular xylanase, β-xylosidase and α-L-arabinofuranosidase by the mesophilic fungus *Penicillium janczewskii* under SmF with different agro-residual sources.Synthetic calcium-containing zeolite (CaA), aluminosilicate minerals normally used as commercial adsorbents, was used to increase xylanase production up to twofold in *Bacillus sp.* NCL 87–6-10 at a concentration of 0.5% (Balakrishnan *et al.*, 2000).

Mostly pure xylan is the ideal source of xylanase production but in some cases higher results can be obtained from agricultural wastes. For example, Gupta *et al.* (2001) reported an improved xylanase production (five-fold) by *Staphylococcus sp.* SG-13 in a poplar wood medium over xylan alone as carbon source.

Type of the fermentation process is another important element in xylanase production. Mostly fungal xylanase is produced by SSF, whereas bacterial and yeast xylanases are produced by SmF. However, different microorganisms can produce xylanase both in SmF and SSF efficiently by changing medium conditions.

Aspergillus ochraceus produced xylanase using both fermentation methods and purified using ammonium sulphate precipitation and gel filtration (Biswas *et al.*, 1988). A higher xylanase activity in solid substrate fermentation compared to

submerged fermentation was reported from thermophilic *Melanocarpus albomyces* IIS-68 with wheat straw and sugarcane bagasse (Jain, 1995).

The production of thermostable xylanase by thermophilic *Bacillus licheniformis* was described in a SSF process by Archana and Satyanarayana (1997). Enzyme production was found 22-fold higher in SSF system than in SmF system. Cai *et al.* (1998) also described production of a thermostable xylanase in SSF system. More thermostable enzyme was produced in SSF system than in SmF system.

Xylanases normally active at pH 5-7 and 40°C-60°C; however, thermostable and alkali-stable xylanases (pH 7-11, 60°C-90°C) have great potential in industrial applications because of their specificity. On the other hand, cultivation of microorganisms at such extreme conditions might be an advantage in case of rapid growth and contamination (Okazaki *et al.*, 1984).

Using these microorganisms in production of specific enzymes for industrial utilization has promising financial benefits of being capable of breaking down agricultural residues at higher temperatures and pH (Haki and Rakshit, 2003). Okazaki *et al.* (1984) used alkalophilic and thermophilic *Bacillus sp.* to produce xylanase under extreme conditions and reported no growth and xylanase production below pH 7 and above 55°C. Another thermo-stable xylanase was reported by Azeri *et al.* (2010) with maximum activity at 60°C and pH 9 from *Bacillus species.* Enzyme was stable at 60°C for more than 60 min.

Fungi can be a source for alkali-stable xylanase, as Senthilkumar *et al.* (2005) used *A. fischeri* to produce alkali-stable xylanase at pH 9.0 with wheat bran as a carbon source in solid state fermentation. A solid state fermentation process for the production of

xylanase by thermostable *Melanocarpus albomyces* was also defined by Jain (1995). Alam *et al*. (1994) isolated a thermostable cellulase-free xylanase from *T. lanuginose* via SSF process.

2.4.2 Mixed Culture

There are a number of reports describing mixed-culturing of two microorganisms for improved enzyme production. An enhancement in xylanase production by fungal mixed culture *Trichoderma reesei* LM-UC4 E 1, *Aspergillus niger* ATCC 10864, and *A. phoenicis QM 329* using solid substrate fermentation was reported (Gutierrez-Correa and Tengerdy, 1998). Panda *et al.* (1987) used *Aspergillus wentii* to produce a polysaccharide, mainly involving glucose monomeric units, which was essential for the expression of maximum xylanase activity and stated that an important increase in extracellular xylanase activity was detected in the mixed culture fermentation of *Trichoderma reesei* D1-6 and *Aspergillus wentii* Pt 2804.

Gupte *et al.* (1998) and Madamwar *et al.* (1989) cultured two strains of *Aspergillus ellipticus* and *A. fumigatus* together and informed better hydrolytic and β-glucosidase activities compared to separately cultured strains using SSF method. Duenas *et al.* (1995) also used SSF method to produce xylanase with *Trichoderma reesei* and *Aspergillus phoenicis* from sugarcane bagasse. It is important to produce enzyme separately and together with organisms to compare the effectiveness of mixed culture. For example, Kaushal *et al.* (2012) found relatively similar results for single and mixed culture enzyme production with *Aspergillus niger and Fusarium oxysporum* using forest waste as the substrate.

Also, the medium components play an important role in mixed culturing. Gutierrez-Correa and Tengerdy (1998) stated that single culture of *Trichoderma reesei* and *Aspergillus phoenicus*, when enhanced with inorganic nitrogen source, produced similar xylanase levels to mixed cultures. Enzymes can be produced and used at the same time if the environment is suitable such as using water hyacinth, a type of water flower that reproduces rapidly and disturbs natural balance of the rivers, to produce xylanase by *Trichoderma reesei* and *Aspergillus niger* (Desphande, 2008).

Genetic engineering has an important role in both single and mixed culture enzyme production. Recombinant DNA techniques and gene manipulation give the ability of producing higher amount of specific enzymes. Kanotra and Mathur (1995) succeeded enhanced enzyme titres when a mutant of *Trichoderma reesei* was mixed-cultured with a strain of *Pleurotus sajor-caju* using wheat straw as a substrate.

Although most of the researches include fungal mixed culture due to the higher product formation and more predictable pathways, there are also some researches that includes bacteria. Moses Jeyakumar and Rajesh (2012) produced xylanase by mixed culture of *Bacillus polymyxa* and *Cellulomonas uda* on sugarcane leaf extract.

2.5 Raw Material

Carbon source plays a key role in economics of xylanase production; therefore, cost effective alternatives have to be considered for large scale processes. In order to decrease the cost of the xylan, lignocellulosic substrates like wheat bran, sugarcane bagasse, rice straw, corn cobs etc., can be used for production of xylanase.

In cultures on solid substrate, wheat bran and rice are regarded as inducers (Beg *et al.,* 2001).A high level of thermostable xylanase is produced by *Thermoascus aurantiacus ATCC 204492* with sugar cane bagasse as substrate (Milagres *et al.*, 2004). Streptomyces sp. QG-11-3 (Beg *et al.,* 2001) produces maximum xylanase yield (84.26 U/ml) at substrates of wheat bran and eucalyptus craft pulp in solid state fermentation.

In this study, use of hazelnut shell as a renewable and low cost lignocellulosic substrate for xylanase production was used for the first time. The world's annual production of hazelnuts (*Corylus avellana L.*) averages nearly one million tons. Hazelnut fruits have a hard, smooth shell with about 27.5% hemicellulose by weight. A sample composition of hazelnut shells is presented in Table 2.7

Table 2.7. Composition of hazelnut shells (Adapted from Uzuner and Cekmecelioglu, 2013).

Chemical components	**Hazelnut Shell (%)**
Dry matter	90.30±0.24
Ash content	1.13±0.04
Moisture	9.7±0.24
Fat	6.06±0.48
Total lignin	34.64±0.43
Extractive	6.09±0.16
Cellulose	24.20±0.99
Hemicellulose	28.20±0.14
Crude fiber	68.22±1.86

Currently hazelnut shells are used for interior home heating in Black sea region (Pütün and Pütün, 1998). Today, approximately 66 % of hazelnut production performed in Turkey in only Black sea region and it means nearly 453,150 tons of hazelnut shells (Ozturk and Bastancelik, 2006).

2.6 Mixed Culture Applications in Food Industry

Mixed cultures are capable of utilizing multistep transformations that can be impossible for a single microorganism. The most important food application of mixed cultures is fermentation of them. Fermented foods are generated from consumable products that are thermally treated or untreated food raw materials of plant or animal origin. They have distinctive sensory and nutritional properties that are affected by microorganisms and enzymes.

Although mixed cultures can be used naturally, pure cultures have been used in industrial applications up to now. However, biotechnological and microbiological improvements increase the usage of mixed cultures in food industry. Furthermore, food scientists have discovered some advantages of mixed cultures as summarized below:

• Higher production yield: Yogurt is produced by fermentation of milk with *Streptococcus thermophilus* and *Lactobacillus bulgaricus.* Albers (2001) reported that when they were grown individually, 24 mmol and 20 mmol, acid were produced respectively. On the other hand when grown together, with the same amount of inoculum, a yield of 74 mmol was obtained. The number of *S. thermophilus* cells increased from 5 x 10^8 per milliliter to 8.8 x 10^8 per milliliter with *L. bulgaricus* (Albers, 2001).

• Increased shelf-life of the product: Antagonistic effect prevents the growth of unwanted microorganisms. For example, yeast and bacteria produce alcohol and lactic acid respectively, which then change the environmental conditions like pH and oxygen, provide anaerobic conditions and inhibits the growth of unwanted bacteria and fungi. In contrary, this mechanism allows the growth of desirable bacteria and fungi (DFG Senate Commission on Food Safety).

Table 2.8 Examples of fermented foods in the European market (DFG Senate Commission on Food Safety)

Raw material	**Product**	**Microorganism**
Olives, cabbage, cucumbers, tomatoes	Fermented olives, sauerkraut, pickles	Lactactic acid bacteria (LAB)
Dough and pastes from cereals	Sourdough, yeast dough, Kisra	LAB, yeasts
Malt, Koji, made from cereals	Beer, sake, spirits	LAB, yeasts, moulds
Beer, wines and spirits	Vinegar	Acetic acid bacteria
Grapes and other fruits	Wine	Yeasts, LAB
Soya, Carob	Soy sauce, tempeh, natto, Dawadaya	LAB, *Bacillus spp.*, moulds, yeasts
Milk	Sour milk products:	Lab, yeasts
	Sour milk, sour cream, yogurt, kefir, kumis	Acetic acid bacteria
	Sour cream butter	LAB
	Cheese	LAB, yeasts, moulds, propionic acid bacteria
Meat	Fermented sausages	LAB, yeasts, moulds, staphylococci Micrococci, *Streptomyces*
	Ham	LAB, yeasts, moulds, staphylococci
Fish	Fish sauce, fermented fish	Staphylococci, Vibrio costicolai LAB

Another fermented product with increasing commercial attention is kefir. It is a sour fermented milk product with low or no alcohol content. It is obtained by incubation of milk with kefir grains. *Lactococcus lactis sub sp. lactis, Lactobacillus kefir, Lactobacillus plantarum, Acetobacter* and *Saccharomyces* isolated from kefir grains were associated in a starter culture for kefir (Garrote *et al.*, 2001).

Nowadays, consumers are concerned about their health and prefer healthy foods to eat. Probiotics are live microorganisms which when consumed in adequate amounts confer health effects on the host" (FAO/WHO, 2001). Many studies reported that the best way to convey probiotics are milk and milk products. According to their supposed health benefits probiotic bacteria have been more and more included in yoghurts and fermented milks for the past two decades (DFG Senate Commission on Food Safety).

Arihara *et al.* (1998) reported that LAB strains are suitable for meat fermentation to enhance product safety.

As fermentation process involves mixed cultures such as yeast, lactic acid bacteria (LAB) and mold (Blandino *et al.*, 2003), traditional fermented foods are microbial source and some of them show probiotic characteristics (De Valdez *et al.*, 1990; Psani and Kotzekidou, 2006; Todorov *et al.*, 2008).

The probiotic bacteria used in commercial products today are mainly members of the genera *Lactobacillus* and *Bifidobacterium* (Reuter, 1997; Bonaparte and Reuter, 1997).

2.7 Aim of This Study

Xylans are the main component of hemicellulose structure; a complex of polymeric carbohydrates including xylan, cellulose, lignin, glucomannan, galactoglucomannan and arabinogalactan, which also form the major polymeric constituents of plant cell walls degradable by xylanolytic enzymes.

Xylanase belongs to glycosidase group that catalyzes the endo-hydrolysis of 1,4-β-D-xylosidic bonds. There are different xylanases, which take part in xylose production of primary carbon source for cell metabolism. They are produced by widespread group of organisms like bacteria, algae, molds and yeasts.

Microbial xylanase have many commercial uses in paper manufacturing, feed production, baking, and juice and wine industries. Microorganisms which produce extracellular enzymes provide industrial xylanases.

This enzyme can be produced both in solid state and submerged media. Furthermore, substrate concentration, substrate source, pH, temperature and ions in the media can affect the production.

The first xylanase produced in commercial terms in 1991 by Novo Nordisk from *Trichoderma Reese* (mold) (Dhiman *et al.*, 2008). In our country, xylanase is not produced commercially, not produced from wastes and also there is no published research in literature that could have been accessed so far about mixed culture production of xylanase with yeast and bacteria.

Therefore, this study is the first to describe xylanase production using mixed culture of yeast (*Kluyveromyces marxianus*) and bacteria (*Bacillus subtilis*). Objectives of the study to fulfill this aim were;

1. To evaluate the potential xylanase production of single and mixed cultures of *Bacillus subtilis* and *Kluyveromyces marxianus* in synthetic media.

2. To study the effects of fermentation time, inoculum level, and raw materials as carbon source on mixed culture xylanase production.

3. To determine the major factors that affect xylanase production and to optimize the fermentation conditions for mixed culture xylanase production.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Media

Chemicals used in this study were analytical grade, and purchased from Sigma-Aldrich (N.Y., USA) or Merck Chemical Companies (Deisenhofen, Germany) unless otherwise stated. The list of the chemicals and the suppliers is given in Appendix A. The preparation of used buffers and solutions are given in Appendix B.

3.1.1.1 Activation Medium

Bacillus subtilis was obtained from Refik Saydam Hıfzıssıhha Merkezi, Ankara and *Kluyveromyces marxianus* was provided by United States Department of Agriculture, Agricultural Research Service, ARS Culture Collection (also known as the NRRL Collection), NRRL number: Y-8287, ATCC number: ATCC 22296. Both microorganisms were activated in nutrient broth incubating overnight at 35 ºC under shaking at 130 rpm.

3.1.1.2 Growth Medium

To prepare the seed cultures of fermentation experiments, overnight grown stock cultures of both microorganisms were incubated in 250 ml flasks containing 50 ml of growth medium at 35 ºC for 24 h with agitation rate of 130 rpm. The composition of the growth medium is given in Table 3.1 below.

Table 3.1 Growth medium composition*

Components	**Amount (g/50 ml)**
Yeast extract	0. 02
$MgSO_4$	0.002
K_2HPO_4	0.046
KH_2PO_4	0. 01
D-xylose	0.5

*(50 ml in 250 ml flask)

3.1.1.3 Fermentation Medium

Xylanase enzyme was produced by *Bacillus subtilis* and *Kluyveromyces marxianus* in submerged fermentation. Thus the following fermentation media was used as follows (g/50 ml);

Yeast extract 0.01, $MgSO_4$ 0.001, K_2HPO_4 0.023, KH_2PO_4 0.005 and predefined amount of carbon source.

3.1.2 Hazelnut Shells

Hazelnut shells were obtained from Ordu in Black Sea Region of Turkey. They are known to contain 45.59 % C, 4.59 % H, 38.14 % O, 1.26 % ash and 10.07 % moisture (Midilli *et al.*, 2000). Hazelnut shells have 43.1% lignin, 27.5% hemicellulose, 24.7% cellulose, 3.4% alcohol-benzene and 1.4% (Uzuner & Cekmecelioglu, 2013). In this study, hazelnut shells were first ground into fine particles (1 mm) and then hydrolyzed by autoclaving in dilute acid solution (see section 3.2.1).

3.2 Methods

3.2.1 Acid Hydrolysis

Ground hazelnut shells at prespecified solid loads were hydrolyzed using 100 ml 0.6 M sulphuric acid solution for 30 minutes at 130 °C in an autoclave (Uzuner & Cekmecelioglu, 2013). After hydrolysis, the solid fraction of the slurry was separated by vacuum filtration and then the pH of the supernatant was adjusted to desired pH levels.

3.2.2 Inoculum Preparation and Enzyme Production

Aliquots (50 ml) of growth medium was inoculated with 2 ml of stock cultures of *Bacillus subtilis* and *Kluyveromyces marxianus*, and incubated overnight at 35°C, pH 6.5 in an incubator shaker adjusted to 130 rpm for 24 h and used as seed culture in 250 ml flasks.

Fermentation medium was then inoculated with predefined amount of seed culture ($6*10^7$ CFU/ml) and kept at 35ºC, pH 6.5 and 130 rpm for 24 h.

Different fermentation variables were screened for their effects on xylanase production using *B.subtilis* and *K.marxianus* as inoculum. Incubation time (12 h and 24 h), inoculum level of 1-4 % (ml/100 ml), and various sole carbon sources (xylose, glucose, arabinose, sucrose, xylan, ground hazelnut shell and hydrolyzed hazelnut shell) were tested. After fermentation, liquid medium was centrifuged at 2000 xg at 15 °C for 15 min to recover the crude enzyme solution.

3.2.3 Xylanase Assay

Xylanase activity was determined using 1% birchwood xylan solution in 100 ml acetate buffer with a pH of 5.0 as the substrate in xylanase assay. A half milliliter of diluted enzyme sample was incubated with 0.5 ml of the substrate solution at 50°C and 130 rpm for 30 min. The liberated reducing sugars were measured at 550 nm with a spectrophotometer according to DNS method (Miller, 1959).One unit of the xylanase is defined as the amount of enzyme that releases 1 μmole of xylose equivalents per minute under assay conditions.

3.2.4 Analytical Tests

Dinitrosalicylic acid method (DNS) was used for determination of reducing sugars released during enzymatic reaction (Miller, 1959). Samples from xylanase assay were diluted to 1/100 and 3 ml of resulting sample was used in DNS method. The DNS reagent prepared by dissolving 1.0 g of dinitrosalicylic acid, 0.2 of g phenol, 0.05 g of

sodium sulfite (Na_2SO_3), and 1.0 g of sodium hydroxide in 100 ml of distilled water. Three milliliters of DNS solution was added to 3 ml of the sample and then boiled at 90°C in water bath for 15 min to terminate the enzymatic reaction.

One milliliter of potassium-sodium tartrate solution prepared by dissolving 36 g of potassium-sodium tartrate in 100 ml of distilled water, was added instantly to DNS samples and placed in the cold water bath for cooling to room temperature. Finally, reducing sugars were measured at 550 nm with a spectrophotometer against the standard curve presented in Appendix C and reported as xylose equivalent.

3.2.5 Initial Screening of Important Variables

The potential xylanase production of single and mixed cultures of *Bacillus subtilis* and *Kluyveromyces marxianus* was assessed at varying inoculum levels using constant temperature of 35°C, pH of 6.5 and agitation rate of 130 rpm.

3.2.6 Effects of Fermentation Time and Substrate Type

Effects of fermentation time, different synthetic sugars, polymeric substrate (xylan) and organic wastes (hazelnut shells and its hydrolyzate) as carbon source were studied by one-factor at a time approach for initial screening purposes. Fermentation periods of 12, 24, 36, 48, 72, 96 h and carbon sources of xylose, glucose, arabinose, sucrose, xylan, ground and hydrolyzed hazelnut shell (Table 3.2) were tested.

Table 3.2 Fermentation medium composition (g/50 ml)*

Flask #	Yeast Extract	$MgSO_4$	K_2HPO_4	KH_2PO_4	Carbon source
F1	0. 01	0.001	0.023	0.005	D-xylose (2. 0)
F2	0. 01	0.001	0.023	0.005	Sucrose (2. 0)
F3	0. 01	0.001	0.023	0.005	Glucose (2. 0)
F4	0. 01	0.001	0.023	0.005	Arabinose (2. 0)
F5	0. 01	0.001	0.023	0.005	Xylan (2. 0)
F6	0. 01	0.001	0.023	0.005	ground hazelnut shells (5. 0)
F7	0. 01	0.001	0.023	0.005	hydrolyzed hazelnut shells (5.0)

* (50 ml – in 250 ml flask)

Finally, the results from screening activities were analyzed and experimental plan was prepared by Response Surface Method (RSM) for optimization of xylanase production.

3.2.7 Optimization of Xylanase Production by Response Surface Model (RSM)

The specific and joint effects of inoculum amounts of both microorganisms, pH and hazelnut shell concentration were evaluated by Box-Behnken response surface methodology (Box and Behnken, 1960). The independent variables, which were inoculum amounts of both microorganisms, pH and hazelnut shell concentration are shown in coded and uncoded forms in the Table 3.3. Minimum, middle, and maximum levels of these variables are represented by coded levels using integers (-1, 0, +1). Means, standard deviations, and lack of fit were measured by the combination of points of (±1, 0, 0), (0, ±1, 0), (0, 0, ±1) and center point (0, 0, 0) (Khuri and Cornell, 1996).

Table 3.3 Coded and uncoded forms of independent variables in used response surface method

Variables		**Coded levels**		
		-1	0	+1
Uncoded levels				
Inoculum amounts of *B. subtilis*	(ml/100 ml)	1.0	2.5	4.0
Inoculum amounts of *K. marxianus*	(ml/100 ml)	1.0	2.5	4.0
pH		4.0	5.5	7.0
Hazelnut shell concentration	(%w/v)	10.0	25.0	40.0

A second order polynomial equation was used to develop a predictive model for xylanase production was as follows:

$$Y=\beta_0+\beta_B X_B+\beta_K X_K+\beta_S X_S+\beta_{pH} X_{pH}+\beta_{BB} X_B^2+\beta_{KK} X_K^2+\beta_{SS} X_S^2+\beta_{pHpH} X_{pH}^2+\beta_{BK} X_B X_K+\beta_{BS} X_B X_S+\beta_{BpH} X_B X_{pH}+\beta_{KS} X_K X_S+\beta_{KpH} X_K X_{pH}+\beta_{SpH} X_S X_{pH} \quad \text{(Equation 3.1)}$$

where Y is the response (enzyme activity), X_B, X_K, X_S, X_{pH} represent independent variables of inoculum amounts of *B.subtilis*, inoculum amount of *K.marxianus*, hazelnut shell concentration, and pH, respectively and β's are regression coefficients.

To define the significant terms and the coefficients of predictive model, analysis of variance (ANOVA) and regression analysis were performed by the statistical software MINITAB® 16.1 (Minitab Inc. State College, PA, USA). The optimum conditions for maximizing the enzyme production were determined by response optimizer in MINITAB® 16.1.

Analysis of variance (ANOVA) was used at α=0.05 in order to identify any differences in sample measurements and the fitting of experimental data to the regression model was evaluated by root mean square error (RMSE) and mean absolute error (MAE) values as illustrated in the following equations;

$$RMSE = \sqrt{\frac{1}{N} x \sum_{i=1}^{N} \left[(P)_i - (O)_i \right]^2} \qquad \text{(Equation 3.2)}$$

$$MAE = \frac{1}{N} \sum_{i=1}^{N} \left| P_i - O_i \right| \qquad \text{(Equation 3.3)}$$

where P_i and O_i are predicted and experimental xylanase activity values respectively, and N represents the number of data points.

The reproducibility of the verification experiments was calculated using coefficient of variation (CV) values as follows;

$$CV = \frac{\sigma}{\overline{X}} x100 \qquad \text{(Equation 3.4)}$$

where σ is sample standard deviation, and $\overline{X}$ is sample mean. Low standard deviation values give low CV values meaning that high reproducibility is revealed.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Initial Screening for Selection of Important Factors

To select the important factors to be used in RSM, initial screening experiments were carried out and also the potential xylanase production by single and mixed cultures of *Bacillus subtilis* and *Kluyveromyces marxianus* were evaluated. Therefore, inoculum amount ranging from 1.0 ml to 2.0 ml were studied at 35°C, pH 6.5 and with agitation of 130 rpm.

When fermentation medium (F1) was inoculated separately with 1.0 ml of *K. marxianus* and *B. subtilis*, xylanase activities of 11.23±0.02 IU/ml and 12.11±0.05 IU/ml was obtained respectively after 24 h of fermentation (Table 4.1).

Higher xylanase activity of 17.93±0.04 IU/ml was produced after 24 h of fermentation when mixed cultures of *Bacillus subtilis* (0.5 ml) and *Kluyveromyces marxianus* (0.5 ml) were used. Similar trend was observed as inoculum level increased; 2.0 ml of *K. marxianus* and 2.0 ml of *B. subtilis* resulted in xylanase activities of 13.69±0.5 IU/ml and 15.63±0.1 IU/ml, respectively.

The maximum xylanase production of 19.65±0.4 IU/mL was observed with total inoculum amount of 2.0 ml of *Bacillus subtilis* (1.0 ml) and *Kluyveromyces marxianus* (1.0 ml) after 24 h fermentation.

Evaluation of the individual enzyme production by both microorganisms is an important step while working with mixed cultures to report any benefits if exist. Garcia-Kirshner *et al.* (2002) reported that single cultures of *Penicillium sp CH-TE-001* and *Aspergillus terreus CH-TE-013* produced lower xylanase activities (1.45 IU/ml, 3.96 IU/ml) than mixed culture of both microorganisms (5.02 IU/ml).

Table 4.1 Initial screening results

Total Inoculum amount (ml)	**Microorganism**		**Xylanase production (IU/ml)**
	B. subtilis	***K.marxianus***	
1.0	-	1.0	11.23±0.02
1.0	1.0	-	12.11±0.05
1.0	0.5	0.5	17.93±0.04
2.0	-	2.0	13.69±0.5
2.0	2.0	-	15.63±0.1
2.0	1.0	1.0	19.65±0.4

*reported in replicates

4.2 Effect of Fermentation Time

Keeping substrate concentration, amount of inoculum, temperature, agitation and pH of the fermentation medium constant, enzyme activities were measured at the end of 12, 24, 36, 48, 72 and 96 h to determine the effect of time on xylanase production (Figure 4.1).

These results indicated that the highest enzyme production (12. 41 ± 0. 1 IU/ml) was achieved after 24 h at 35°C, pH 6.5 and with agitation of 130 rpm. Afterwards, a decrease was observed in xylanase production. The growth and xylanase production were parallel up to 24 h, thereafter enzyme production ceased. However, the growth increased up to 72 h, and then decreased slowly with similar trend to xylanase production (Fig. 4.1).

Different culture conditions such as pH, type of substrate, temperature or type of the microorganism can affect the xylanase production. For example, Nadia *et al.* (2010) observed the highest xylanase activity after 5 days of incubation (15.00 IU/ml) with *S.lividans*.

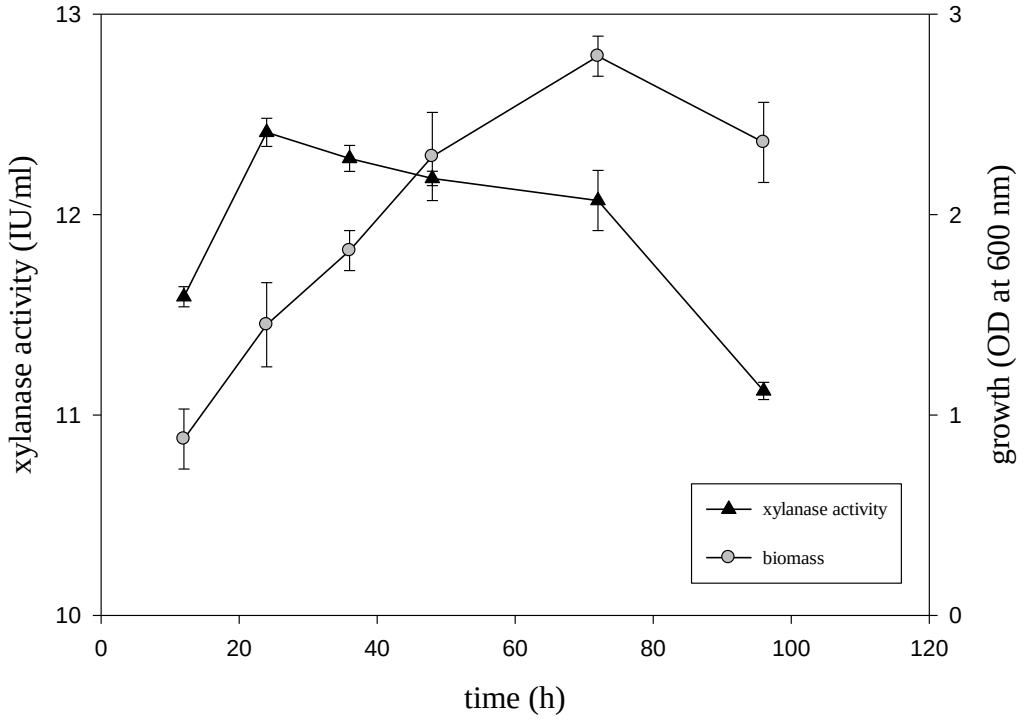


Figure 4.1 Time-course of xylanase production by mixed culture of *B.Subtilis* and *K.Marxianus* vs growth on F1 medium at pH 6.5, 35 °C and with shaking at 130 rpm.

4.3 Effect of Different Sugars

To study the effect of raw material on enzyme production using mixed culture, different types of sugar were used in F1, F2, F3 and F4 media, incubated at 35 °C, pH 6.5 and 130 rpm for 24 h and the total reducing sugar and enzyme activities were reported (Figure 4.2).

The results revealed that the highest enzyme activities were obtained by arabinose (26.77± 0.5 IU/ml) and xylose (14.92 ± 0.6 IU/ml) respectively. Rifaat (2006) used *Streptomyces chromofuscus* to produce xylanase using different sugars, and reported similar results with xylose (12.31 IU/ml) and glucose (10.26 IU/ml).

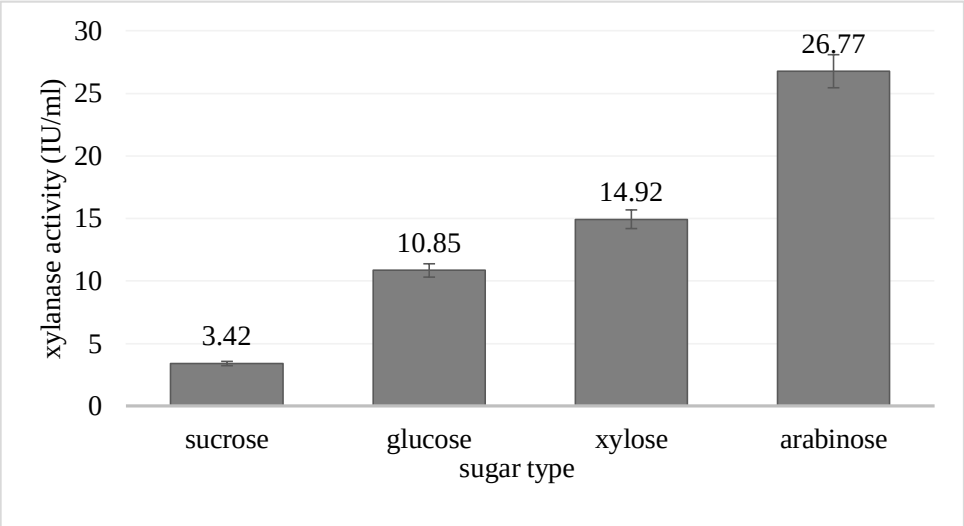
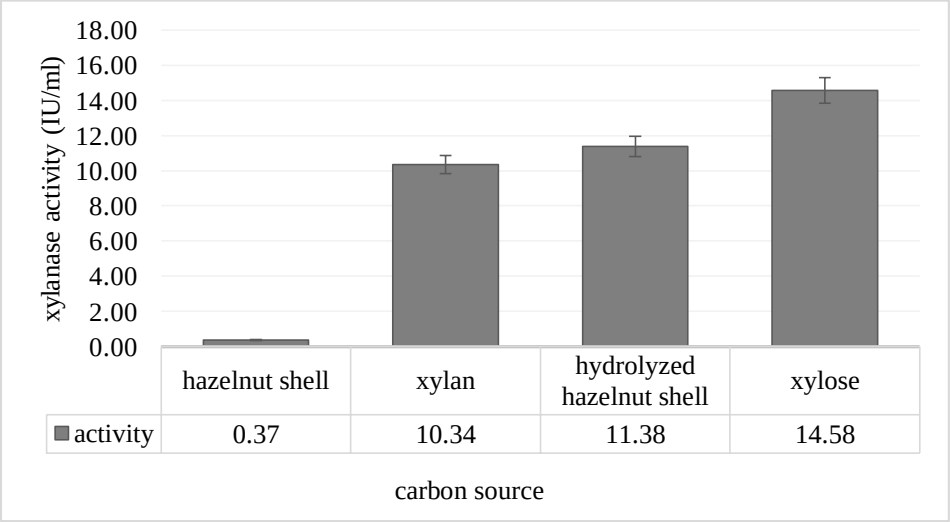


Figure 4.2 Xylanase enzyme production after 24 h (IU/ml) at 35ºC, pH 6.5 and 130 rpm.

4.4 Effect of Raw Material Type

The xylanase production by mixed culture of *B.Subtilis* and *K.Marxianus* grown on different carbon sources is shown in Figure 4.3. The highest xylanase production was obtained with xylose (14.58±0.06 IU/ml) followed by hydrolyzed hazelnut shell (11.38±0.01 IU/ml). It was clear from Figure 4.3 that hydrolysis of hazelnut shell was effective compared to ground form based on xylanase production increased remarkably from 0.37 IU/ml to 11.38 IU/ml. The positive effect of hydrolysis was also shown by Rifaat (2006) with *Streptomyces albus* who produced higher xylanase (32.53 IU/ml) using treated pulp than untreated pulp (13.25 IU/ml).High xylanase activity of 11.38±0.01 IU/ml also showed that agricultural waste can be used as raw material for cost-effective xylanase production.



*Reported in triplicate

Figure 4.3 Effect of raw materials on xylanase production at 35ºC, pH 6.5 and 130 rpm after 24 h fermentation.

4.5 Optimization by Response Surface Method

The experimental design by Box-Behnken response surface method (RSM) and xylanase activity values are shown in Table 4.2. A second order polynomial equation was found appropriate as to characterize the data and the equation became as follows;

$$Y = -41.3795 - 6.18242X_B - 3.70466X_K - 1.10841X_S + 21.8258X_{pH} + 0.0730159X_B^2 + 0.444081X_K^2 + 0.0210430X_S^2 - 2.31257X_{pH}^2 + 0.909707X_BX_K + 0.00239397X_BX_S + 0\ .861828X_BX_{pH} - 0.0574552X_KX_S - 9.59000E-16X_KX_{pH} + 0.225751X_SX_{pH}$$

(Equation 4.1)

Table 4.2 Box-Behnken experimental design and experimental vs predicted xylanase activity values for various combination of fermentation conditions

Run No	B [1,4] (ml/100 ml)	K[2,4] (ml/100 ml)	Solid (%w/v)	pH	Activity (IU/ml)	
					Experimental[3]	predicted
1	2.50	4.00	25.0	7.0	17.01 ± 0.86	18.93
2	4.00	2.50	10.0	5.5	7.96 ± 0.43	8.84
3	1.00	1.00	25.0	5.5	21.75 ± 0.86	20.12
4	2.50	2.50	25.0	5.5	17.44 ± 0.86	17.83
5	4.00	2.50	25.0	7.0	24.12 ± 0.65	22.89
6	2.50	2.50	10.0	7.0	5.52 ± 0.28	2.84
7	2.50	2.50	10.0	4.0	3.65 ± 0.43	0.44
8	2.50	1.00	10.0	5.5	4.51 ± 0.43	7.52
9	2.50	1.00	25.0	4.0	9.25 ± 0.86	8.31
10	2.50	2.50	40.0	4.0	20.46 ± 0.86	21.72
11	4.00	2.50	40.0	5.5	40.49 ± 0.65	40.38
12	2.50	2.50	25.0	5.5	18.52 ± 0.65	17.83
13	1.00	4.00	25.0	5.5	17.01 ± 0.43	14.09
14	2.50	1.00	25.0	7.0	22.18 ± 0.43	20.87
15	1.00	2.50	25.0	7.0	13.56 ± 0.43	15.24
16	4.00	1.00	25.0	5.5	18.30 ± 0.43	19.80
17	2.50	2.50	40.0	7.0	42.65 ± 0.65	44.43
18	2.50	4.00	40.0	5.5	39.42 ± 0.43	37.01
19	2.50	2.50	25.0	5.5	17.44 ± 0.86	17.83
20	2.50	1.00	40.0	5.5	42.00 ± 0.86	41.54
21	1.00	2.50	25.0	4.0	4.73 ± 0.17	6.56
22	4.00	2.50	25.0	4.0	7.53 ± 0.43	6.45
23	2.50	4.00	10.0	5.5	7.10 ± 0.43	8.17
24	2.50	4.00	25.0	4.0	4.08 ± 0.43	6.37
25	1.00	2.50	40.0	5.5	36.40 ± 0.43	36.50
26	1.00	2.50	10.0	5.5	4.08 ± 0.43	5.18
27	4.00	4.00	25.0	5.5	21.75 ± 0.86	21.95

[1] *B.Subtilis*, [2]*K.Marxianus*, [3]results are in triplicate, [4] growth medium contains $6*10^7$ cfu/ml

Table 4.3 ANOVA results and estimated regression coefficients for xylanase production. (The analysis was done using uncoded units)

Term	Coefficient	P
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.000
Lack-of-fit		0.099
Constant	-41.3795	0.000
B (^{XB})	-6.18242	0.000*
K (X_2)	-3.70466	0.031*
Solid (X_3)	-1.10841	0.000*
pH (X_4)	21.8258	0.000*
B*B	0.0730159	0.801
K*K	0.444081	0.132
solid*solid	0.0210430	0.000*
pH*pH	-2.31257	0.000*
B*K	0.909707	0.009*
B*solid	0.00239397	0.943
B*pH	0.861828	0.014*
K*solid	-0.0574552	0.092
K*pH	-9.59000E-16	1.000
solid*pH	0.225751	0.000*

*result is significant when P<0.05

Another run with excluded insignificant terms according to Table 4.3 expressed by Equation 4.2.

$$Y=-45.1128-5.75749X_B-2.92064X_K-1.18142X_S+23.2478X_{pH}+0.0197502X_S^2-2.44184X_{pH}^2+0.909707X_BX_K+0.861828X_B\,X_{pH}+0.225751X_S\,X_{pH} \quad \text{(Equation 4.2)}$$

Where Y is the response (enzyme activity), X_B, X_K represent inoculum amount of *B.Subtilis* and *K.Marxianus*, respectively and X_S is solid load and X_{pH} indicates the pH levels.

According to Table E.1, the coefficients of X_B, X_K, X_{pH} and the constant increased slightly; the coefficients of X_S, X_S^2, X_{pH}^2 changed at decimal value while the coefficients of X_BX_K, $X_B\,X_{pH}$, $X_S\,X_{pH}$ remained same.

ANOVA results (Table 4.3, Table E.1) indicated that the quadratic model was found statistically significant ($P<0.05$) at the 95% confidence level. Linear (p=0.000), quadratic (p=0,000), and interaction effects (p=0.000) were highly significant. The determination coefficient (R^2) of the model was calculated to be 0.95, indicating that the model could explain 95% of the variability in the response (*Li et al.*, 2007).

The insignificant lack of fit ($p=0.099>0.05$) also verified that the model fitted well to the experimental data. All the factors, inoculum amounts of both microorganisms, pH and hazelnut shell concentration showed significant effects ($P<0.05$). Interactions between solid-solid, pH-pH, B-K, B-pH, and solid-pH showed significant effects ($P<0.05$); while interactions between B-B, K-K, B-solid, K-solid, and K-pH were

insignificant (P>0.05). Similar results can be seen in factor plots shown in Appendix D. The constructed model was also evaluated with error analysis. Root mean square error (RMSE) and mean absolute error (MAE) values were calculated as 1.77 and 0.004, respectively. Low values of RMSE and MAE also showed that the model was fitted well (Uncu & Cekmecelioglu, 2011).

The response surface plots for the effects of inoculum amounts, hazelnut shell concentration and pH, and their interactions are presented in Figure 4.4 – 4.9. In Figure 4.4 and 4.5, it can be seen that inoculum amounts of *B.subtilis* and *K.marxianus* had lower effects on xylanase production and xylanase activity increased sharply with increasing solid concentration. Also in Figure 4.6, the increase of pH from 4 to 7 nonlinearly increased the xylanase production from 8 IU/ml to 20 IU/ml, respectively.

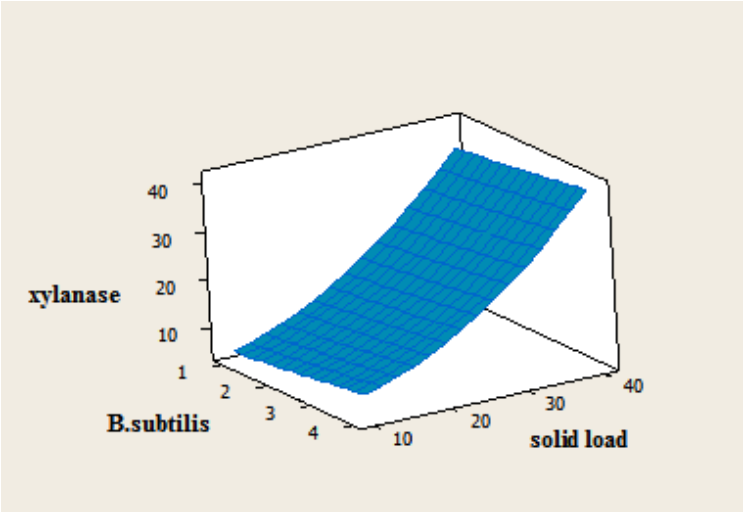


Figure 4.4 Surface plot displaying the effect of *B.subtilis* (ml/100 ml) and solid concentration (%w/v) on xylanase activity (IU/ml) (Constant values: pH 5.5, *K.marxianus* 2.5 (ml/100 ml))

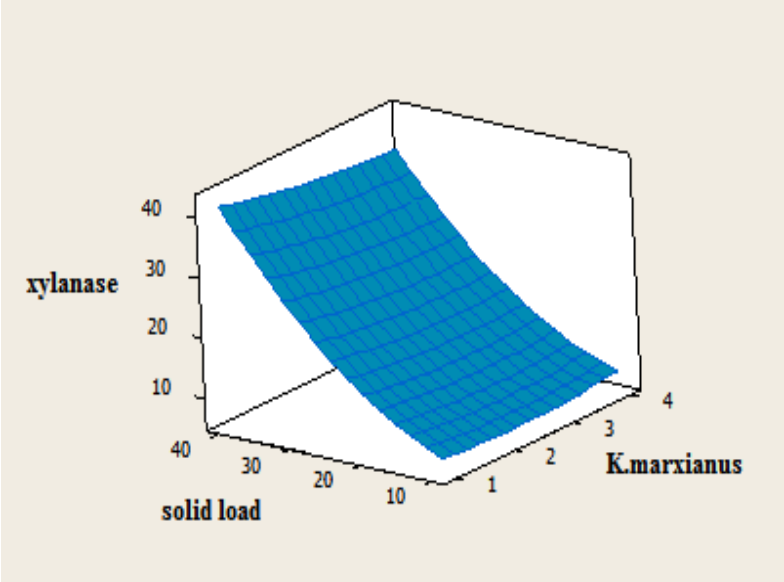


Figure 4.5 Surface plot displaying the effect of *K.marxianus* (ml/100 ml) and solid concentration (%w/v) on xylanase activity (IU/ml) (Constant values: pH 5. 5, *B.subtilis* 2.5 (ml/100 ml))

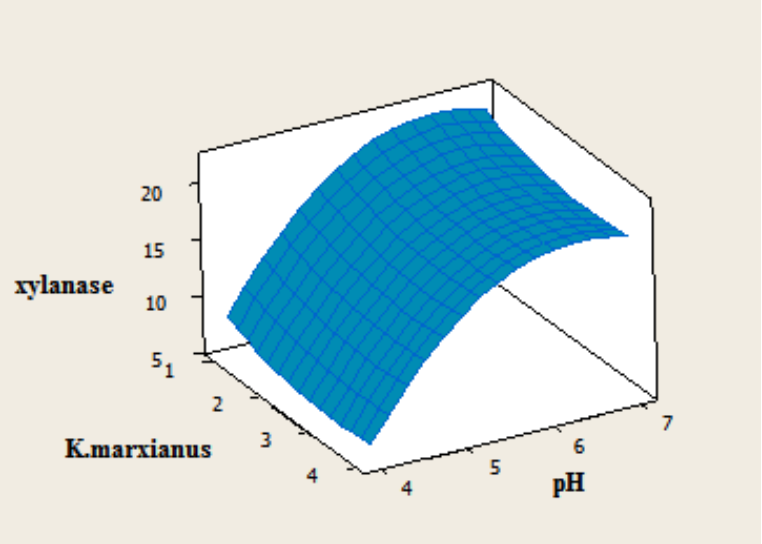


Figure 4.6 Surface plot displaying the effect of *K.marxianus* (ml/100 ml) and pH on xylanase activity (IU/ml) (Constant values: solid concentration 25 (%w/v), *B.subtilis* 2.5 (ml/100 ml))

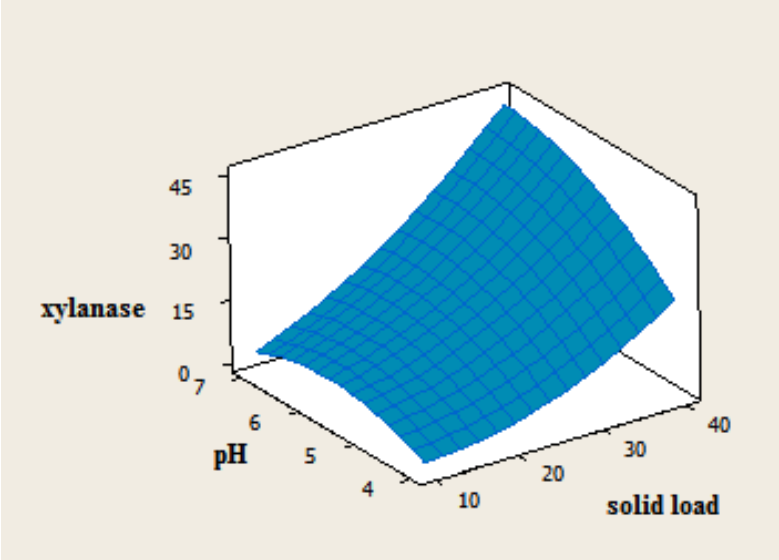


Figure 4.7 Surface plot displaying the effect of solid concentration (%w/v) and pH on xylanase activity (IU/ml) (Constant values: *K.marxianus* 2.50, *B.subtilis* 2.50)

In Figure 4.7, the xylanase activity showed high dependence on varying pH and solid concentration. That is, xylanase activity sharply increased with increasing solid concentration at pH 7, whereas xylanase activity increased smoothly with an increase in the solid concentration from 18% to 40% at pH 4. Similar xylanase activity (41.6 IU/ml) at pH 7 with 10 g/100 ml of solid load was reported by Nadia *et al. (2010)* with *Streptomyces lividans.* Surface plot displaying the effect of *K.marxianus* and *B.subtilis* on xylanase activity is shown in Figure 4.8. Xylanase activities with increasing inoculum amount of *K.marxianus* from 2% to 4% were 14.69 (IU/ml) and 21.74 (IU/ml) respectively at constant volume of *B. subtilis.* In Figure 4.9, there is slight change in the enzyme activity at pH 5 while inoculation amount of *B.subtilis* was increased. On the other hand, xylanase activity increased linearly with increasing inoculum amount of *B.subtilis* between pH 5-7.

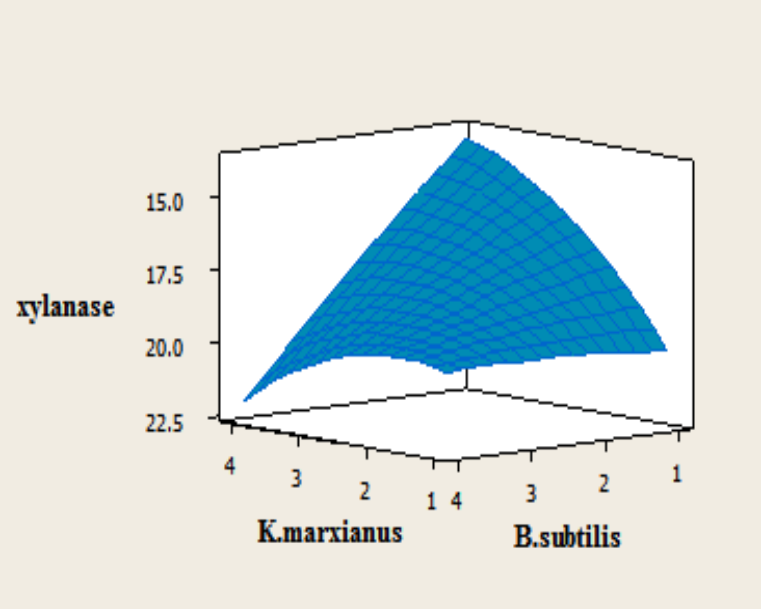


Figure 4.8 Surface plot displaying the effect of *K.marxianus* (ml/100 ml) and *B.subtilis* (ml/100 ml) on xylanase activity (IU/ml) (Constant values: solid concentration 25 (%w/v), pH 5.5)

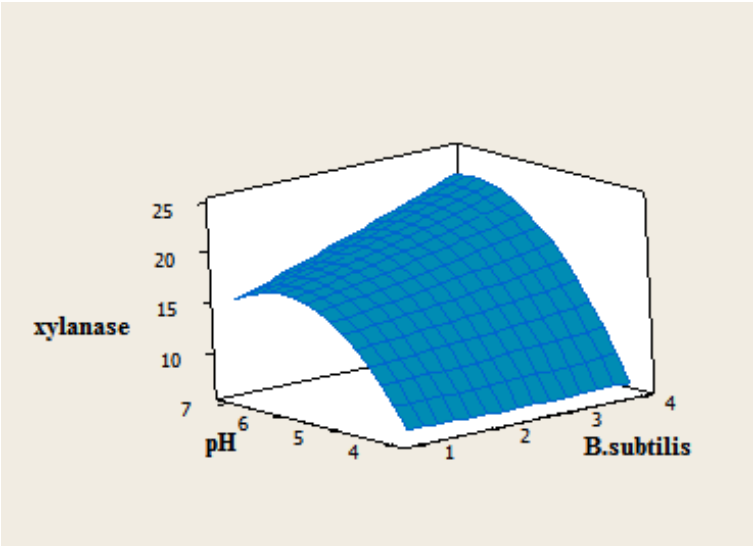


Figure 4.9 Surface plot displaying the effect of *B.subtilis* (ml/100 ml) and pH on xylanase activity (IU/ml) (Constant values: solid concentration 25 (%w/v), *K.marxianus* 2.5 (ml/100 ml))

The effect of process variables, when two varied by keeping the third constant at mid value, can also be seen clearly from the contour plots in Figure 4.10- 4.15. The highest enzyme activity of 40 IU/ml was observed in the combination of solid concentration (40.0) and pH (7.0). The effect of *B.subtilis*, pH, and solid concentration on enzyme activity is also visible in the contour plots. At pH 7, xylanase activity abruptly increased from 10 IU/ml to 40 IU/ml with increasing solid concentration from 12 (%w/v) to 39 (%w/v), but at pH 4, an increase in the solid concentration from %29 to %39 increased the xylanase activity smoothly from 10 IU/ml to 20 IU/ml.

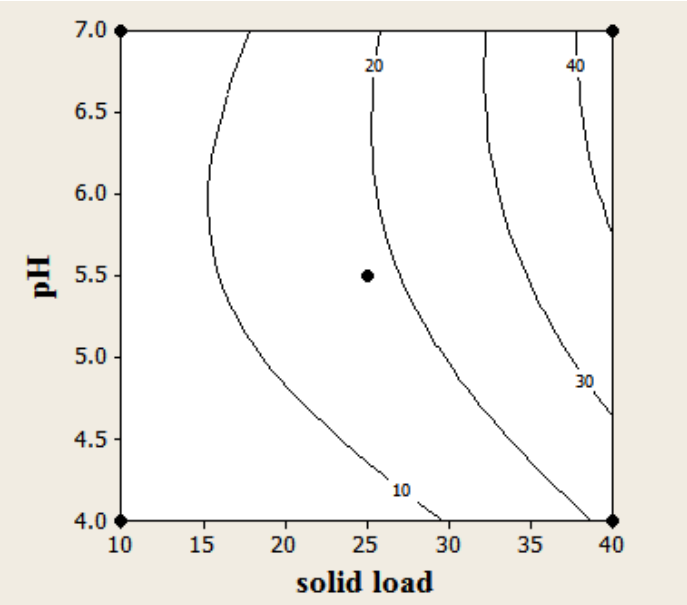


Figure 4.10 Contour plot displaying the effect of solid concentration (%w/v) and pH on xylanase activity (IU/ml) (Constant values: *K.marxianus* 2.5 (ml/100 ml), *B.subtilis* 2.5 (ml/100 ml))

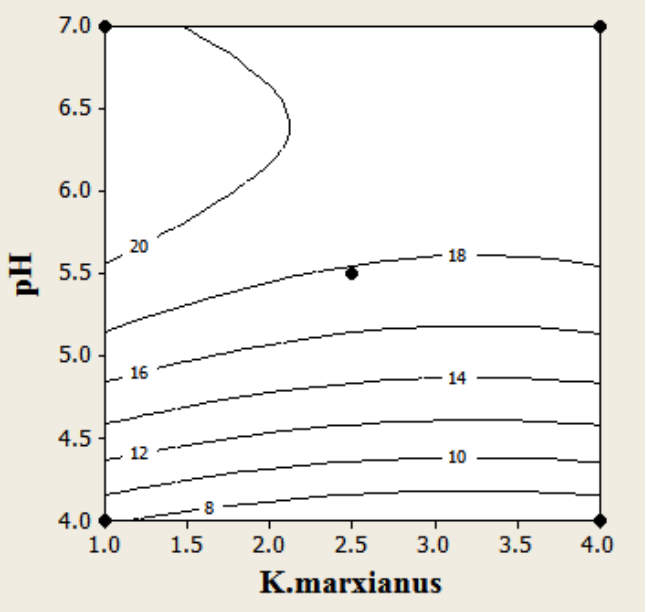


Figure 4.11 Contour plot displaying the effect of *K.marxianus* (ml/100 ml) and pH on xylanase activity (IU/ml) (Constant values: solid concentration 25 (%w/v), *B.subtilis* 2.5 (ml/100 ml))

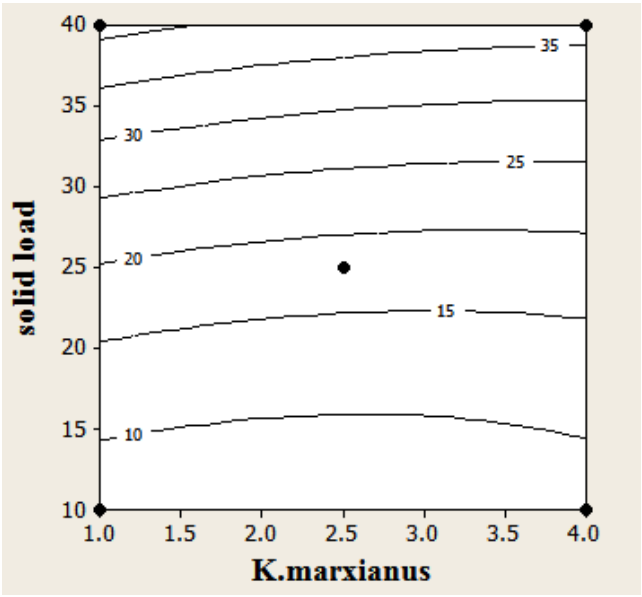


Figure 4.12 Contour plot displaying the effect of *K.marxianus* (ml/100 ml) and solid concentration (%w/v) on xylanase activity (IU/ml) (Constant values: pH 5. 5, *B.subtilis* 2.5 (ml/100 ml))

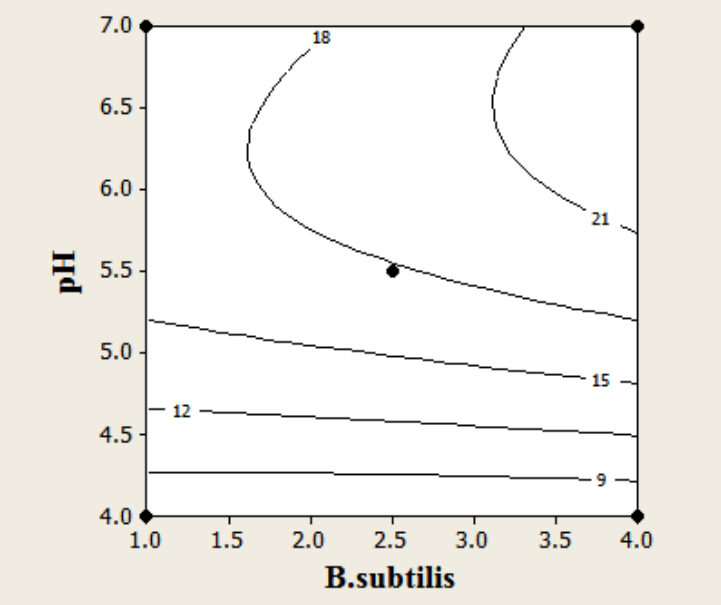


Figure 4.13 Contour plot displaying the effect of *B.subtilis* (ml/100 ml) and pH on xylanase activity (IU/ml) (Constant values: solid concentration 25 (%w/v), *K.marxianus* 2.5 (ml/100 ml))

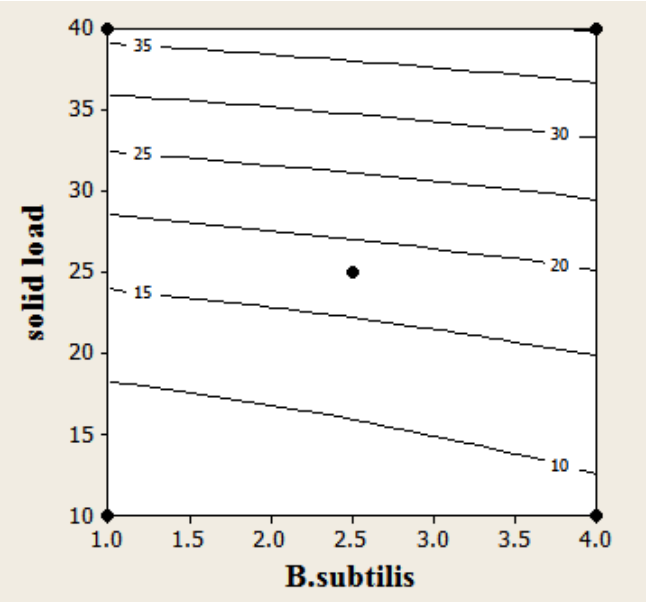


Figure 4.14 Contour plot displaying the effect of *B.subtilis* (ml/100 ml) and solid concentration (%w/v) on xylanase activity (IU/ml) (Constant values: pH 5.5, *K.marxianus* 2.5 (ml/100 ml))

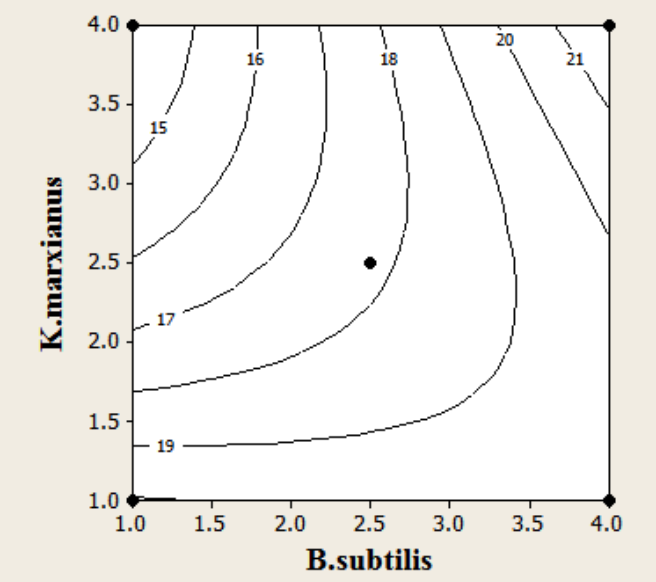


Figure 4.15 Contour plot displaying the effect of *K.marxianus* (ml/100 ml) and *B.subtilis* (ml/100 ml) on xylanase activity (IU/ml) (Constant values: solid concentration 25 (%w/v), pH 5.5)

Optimal conditions for xylanase production were determined by response optimizer tool in MINITAB® 16.1 (Minitab Inc.State Collage,PA,USA). As it can be seen in the Table 4.4, the optimum conditions were found as 4 % (ml/100 ml) *B.subtilis*, 4% (ml/100 ml) *K.marxianus*, and 40 %(w/v) solid load and pH 7, giving a maximal xylanase activity of 49.5 IU/ml as predicted by second order predictive model.

Table 4.4 Response optimization

	Goal	Lower	Target	Upper
Xylanase	Maximum	3	43	43

Global Solution

B = 4.0

K = 4.0

Solid = 40

pH = 7

Predicted Responses = 49.4991

Desirability = 1.000000

According to the R^2 (0.95), lack of fit (P=0.099>0.05), RMSE (1.77) and MAE (0.004), the model was adequate to predict the xylanase production at a definite condition and xylanase production was increased by % 152 compared to initial unoptimized culture conditions. Therefore, it can be concluded that the validated model can be used to effectively foresee xylanase production at various combinations of inoculum amount, solid load, and pH.

Xylanase can be produced by submerged or solid state fermentation by fungi and bacteria. Different carbon sources can be utilized by microorganisms but it is more cost-effective to use agricultural residues instead of pure carbon sources. There are a number of reports (Table 4.5) describing mixed-culturing of two microorganisms including molds and bacteria for improved enzyme production; however, no published research has been reported so far about mixed culture production of xylanase with yeast and bacteria which can grow faster than molds.

Table 4.5 summarizes xylanase production amounts and conditions with single and mixed culture of microorganisms reported in literature.According to Table 4.5, Jeyakumar and Rajesh (2012) reported xylanase activity of 44.773 IU/ml with mixed culture fermentation of *Bacillus polymyxa* and *Cellulomonas Uda* at 50^{o}C, pH 8 after 48 h fermentation using sugarcane leaf extract as carbon source. Desphande (2008) produced xylanase (33.6 IU/ml) by mixed culture of *Trichoderma reesei* and *Aspergillus niger* using cellulose rich medium at 29^{o}C, pH 4.8 after 6 days of fermentation. In the light of this results, xylanase production with mixed culture of *B. subtilis* and *K. marxianus* is more preferable due to higher activity and productivity values than current studies (Panda *et al.,* 1987; Garcia-Kirshner *et al.,* 2002; Moses Jeyakumar and Rajesh, 2012 and Rifaat, 2006)

Table 4.5 Xylanase production with single and mixed culture of microorganisms

Organism	**Substrate**	**Cultivation Conditions**	**Xylanase Activity (IU/ml)**	**Reference**
T. reesei D-1-6 / Awenti Pt 2804	cellulose	Mixed culture Ph 4.8, 29ºC 144 h	33,6 IU/ml	(Panda *et al.*, 1987)
Penicillium sp. and A.Terreus	*sugarcone bagasse*	Mixed, 29 ºC 180 rpm, 2 days	5.02 IU/ml	(Garcia-Kirshner *et al.*, 2002)
T.reesei A.niger	water hyacinth	Mixed, SSF 30 ºC, 85% RH, 10 days	57,2 IU/g	(Desphande , 2008)
Streptomyces albus	rice straw pulp	28 ºC 200 rpm, 5 days	32,53 IU/ml	(Rifaat, 2006)
S. chromofuscus	rice straw pulp	28 ºC 200 rpm, 5 days	43,01 IU/ml	(Rifaat, 2006)
Bacillus subtilis	wheat bran	37 ºC, 140 rpm, 24 hour	36,8 IU/ml	(Irfan *et al*., 2013)
Bacillus thermoalkaloplilus	rice husk bagasse	24h, 60 ºC, 200 rpm	82.30 IU/ml 56.9 IU/ml	(Paul and Varma, 1993)
Bacillus subtilis	*wheat straw*	14h, 180 rpm, 50 ºC	3.2 IU/ml DNS	((Saleem et al., 2002)
Bacillus polymyxa Cellulomonas Uda	Sugarcone leaf extract +xylan (0,2%)	ph:8, 50 ºC, 2 days mixed culture	44.773 IU/ml DNS	(Moses Jeyakumar and Rajesh, 2012)
Bacillus subtilis Kluyveromyces marxianus	**Hazelnut shell**	**Ph:7, 35 ºC,24 h,mixed culture**	**46.5 IU/ml** **DNS**	**This study**

CHAPTER 5

CONCLUSIONS

In this study, the potential of xylanase production by single and mixed cultures of *Bacillus subtilis* and *Kluyveromyces marxianus* were evaluated at variable inoculum levels, fermentation time and substrates as carbon sources by traditional one-factor at a time approach and Box-Behnken response surface method. As the total inoculum amount was increased to 2.0 ml using 1.0 ml of *Bacillus subtilis* and 1.0 ml *Kluyveromyces marxianus*, xylanase production was relatively improved. No remarkable increase in xylanase was observed beyond 24 h fermentation at 35°C, pH 6.5 and with agitation of 130 rpm by mixed culture fermentation. Xylanase production was favored in the order of arabinose, xylose, and hazelnut shells used as carbon sources by mixed culture system in unoptimized medium conditions.

Response surface analysis revealed that optimum xylanase production can be achieved at 4 % (ml/100 ml) *B.subtilis*, 1.0 % (ml/100 ml) *K.marxianus*,and 40 %(w/v) solid load and pH 7 after 24 h fermentation. The optimum result was found and it was 152 % higher in comparison to the activity obtained with the initial experiments.

In conclusion, this study show that for xylanase production by mixed culture submerged fermentation lignocellulosic food processing wastes can be preferred as carbon sources alternatively to synthetic materials (xylan, xylose, arabinose, etc.)

Further study is needed to carry out kinetic analysis of mixed culture fermentation to identify the underlying mechanism of xylanase production in relation to carbon sources used. Scale up studies to higher culture volumes under controlled fermentation conditions will also be needed to fulfill industrial needs.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Table A.1 chemicals and suppliers

Chemical	Supplier
Ammonium acetate	Applichem, USA
3'-5'-Dinitrosalicylic acid (DNS powder)	Sigma, USA
Ethanol	Merck, Germany
Magnesium sulfate-heptahydrate	Merck, Germany
Potassium sodium tartrate	Sigma, USA
Sodium chloride	Merck, Germany
Sodium hydroxide,pellets	Merck, Germany
Dipotassium phosphate	Merck, Germany
Potassium dihydrogen phosphate	Merck, Germany
Phenol	Fluka, USA
Acetic acid	Merck, Germany
Sodium sulfite	Merck, Germany
Birchwood xylan	Merck, Germany

APPENDIX B

COMPOSITION OF BUFFERS AND SOLUTIONS

1. Composition of DNS reagent:

1.00 g Dinitrosalicylic acid

0.2 g phenol

0.05 g sodium sulfite (Na_2SO_3)

1.00 g sodium hydroxide

36 g potassium-sodium tartrate ($C_4H_4KNaO_6.4H_2O$)

100 ml H_2O

2. Composition of acetate buffer for 2 L

54.43 g Sodium Acetate

12 ml Glacial Acetic Acid

1988 ml H_2O

NaOH for adjusting pH.

APPENDIX C

TOTAL REDUCING SUGAR ESTIMATION BY DNS METHOD

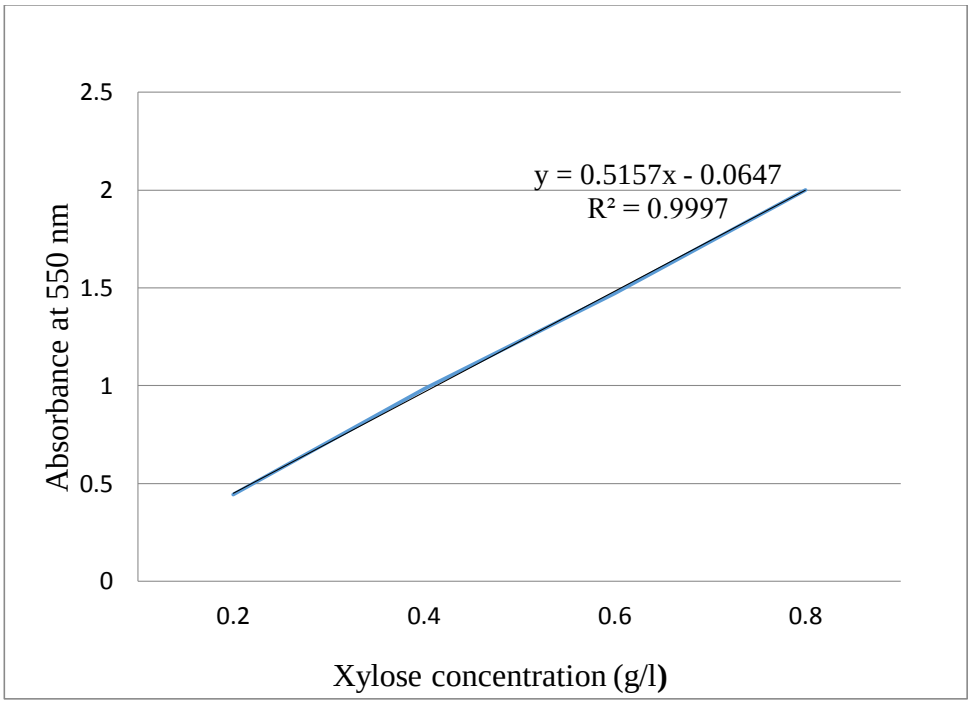

Figure C.1 The standard curve for DNS Method used to estimate total reducing sugar.

According to standard curve, total reducing sugar concentration was calculated as follows:

$$\text{Total reducing sugar (g/l)} = \frac{\text{Absorbance} + 0.0647}{0.5157} \times \text{dilution rate}$$

APPENDIX D

MAIN EFFECTS PLOTS FOR XYLANASE

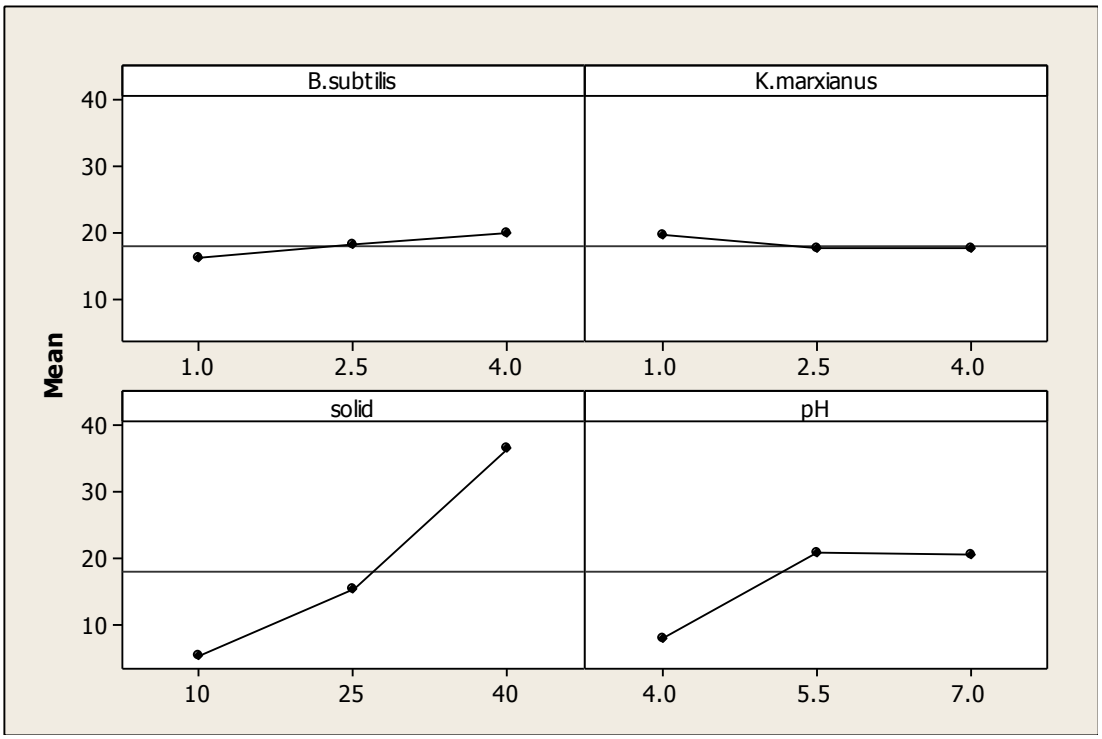

Figure D.1 Main effect plots for xylanase

APPENDIX E

REVISED ANOVA RESULTS AND ESTIMATED REGRESSION COEFFICIENTS

Table E.1 Revised ANOVA results and estimated regression coefficients for xylanase production without insignificant terms

Term	**Coefficient**	**P**
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.000
Lack-of-fit		0.099
Constant	-45.1128	0.000
B (X_B)	-5.75749	0.000
K (X_K)	-2.920	0.031
Solid (X_S)	-1.18142	0.000
pH (X_{pH})	23.2478	0.000
solid*solid	0.0197502	0.000
pH*pH	-2.44184	0.000
B*K	0.909707	0.009
B*pH	0.861828	0.014
solid*pH	0.225751	0.000

*result is significant when P<0.05