

الجمهورية العربية السورية
جامعة دمشق
كلية الهندسة الزراعية
التقانات الحيوية

تأثير معدل سرعة الدوران على إنتاج أنزيم الكسيلاناز من الفطر *Aspergillus niger* المعزول من التربة السورية في المخمر

رسالة أعدت لنيل درجة الماجستير في التقانات الحيوية

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Declaration

I hereby declared that the research work, described in this thesis titled: **“Effect of Agitation rates on Xylanase Production by *Aspergillus niger* Strain Isolated from Syrian Soil in Bioreactor”** has not been accepted for any degree yet, nor has it been submitted concurrently to any other degree.

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تصريح

أصرح بأن هذا البحث الموصوف في هذه الرسالة المعنون (تأثير معدل سرعة الدوران على إنتاج أنزيم الكسيلاناز من الفطر *Aspergillus niger* المعزول من التربة السورية في المخمر) لم يسبق أن قُبل للحصول على أي شهادة. ولا هو مُقدم حالياً للحصول على أية شهادة أخرى.

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Certificate

It is hereby, certificated that the work described in this thesis is the result of **Alaa Mofeed Mekaeel** own investigation under the supervision of **Dr. Amal Koreih** from Damascus University - Faculty of Agriculture, and **Dr. Yasser Bakri** from Atomic Energy Commission of Syria (AECS), and any references to other research work has been dully acknowledged in the text.

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شهادة

نشهد بأن العمل الموصوف في هذه الرسالة هو نتيجة بحث علمي قام به المرشح علاء مفيد ميكائيل بإشراف الدكتورة أمل كورية من جامعة دمشق – كلية الزراعة، والدكتور ياسر بكري من هيئة الطاقة الذرية في سورية، وأي مراجع أخرى بُحثت في هذه الرسالة موثقة في النص.

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تأثير معدل سرعة الدوران على إنتاج أنزيم الكسيلاناز من الفطر *Aspergillus niger* المعزول من التربة السورية في المخمر

الخلاصة:

الكسيلان هو مكون رئيسي من الهيمي سيللوز، وهو عبارة عن متعدد سكريات يتركب في أغلب الأحيان من سلسلة أساسية من الكسيلوز ترتبط فيما بينها بروابط β - 1,4. من أهم الأنزيمات التي تساهم في تحليل الكسيلان هي إنزيم β - 1,4 كسيلاناز التي تحلل الروابط β - 1,4 بين وحدات الكسيلوز.

إن استخدام الكسيلان كركيزة للإنتاج الحيوي للكسيلاناز يزيد من تكلفة الإنتاج الأنزيمي، وكنتيجة لذلك، ومن أجل التطبيقات التجارية، هناك محاولات لتطوير طرائق حيوية لإنتاج أنزيم الكسيلاناز بكميات كبيرة ابتداءً من ركيزات رخيصة الثمن، لذا تم التوجه إلى استخدام المخلفات النباتية رخيصة الثمن الموجودة بكثرة لكونها الخيار الأمثل، ومن بين هذه المخلفات قشور الذرة المستخدمة في بحثنا.

تعد أنزيمات الكسيلاناز من الأنزيمات الهامة في الصناعات التطبيقية، حيث بدأ استخدام هذه الأنزيمات في تطبيقات التقانة الحيوية عام 1980، وذلك في تحضير أغذية الحيوانات ولاحقاً امتد استخدامها ليشمل أغذية الإنسان، وصناعة الخبز، والعصائر والتطبيقات الكيميائية، والصيدلانية، كما دخل في صناعة الأنسجة والورق وكذلك في إنتاج الوقود الحيوي وغير ذلك من الاستخدامات التي تظهر أهمية هذه الأنزيمات.

أجريت دراسات عديدة على إنتاج هذا الأنزيم بواسطة المفاعل الحيوي حيث أثبتت فعالية الأحياء الدقيقة (بكتيريا- خمائر- فطور خيطية) في إنتاج أنزيم الكسيلاناز، إلا أن الفطور الخيطية هي أكثر ما درست في هذا المجال.

يهدف هذا البحث إلى دراسة تأثير معدلي سرعة الدوران، والتهوية في إنتاج أنزيم الكسيلاناز بواسطة الفطر *Aspergillus niger* المعزول من التربة السورية في المفاعل الحيوي، وذلك بهدف الانتقال مستقبلاً للإنتاج الكبير لهذا الأنزيم في المخمرات الكبيرة على المستوى التجاري.

وقد تم في هذه الدراسة إجراء التجارب التالية:

1- تجارب الزراعة المغمورة:

تم إنتاج الأنزيم في المفاعل الحيوي سعة 3 لترات بواسطة الفطر *Aspergillus niger* عن طريق استخدام زراعات الدفعة Batch- culture لمدة 5 أيام لكل زراعة، وبحجم عمل 1.5 لتر في المفاعل الحيوي، وقد أجريت هذه التجارب في مخمر يحتوي على توربين واحد وآخر يحتوي على توربينين. استخدمت فيهما معدلات سرعة الدوران التالية (100- 200 -300 -400 -500 دورة/دقيقة) والتي تقابل السرعات المحيطية التالية (0.26-0.52-0.79-1.05-1.31 متر/ثانية) على التوالي للمخمر الأول و(0.26- 0.52 متر/ثانية) للمخمر الثاني. أما معدلات التهوية فكانت (0.5vvm - 1vvm - 1.5vvm) للمخمرين. درجة الحرارة داخل المخمرين كانت مضبوطة على 30 °م.

وكان الوسط المستخدم في المخمرين يحتوي على 30 غ/ل قشور ذرة مطحونة مغمورة في محلول ملحي عدلت درجة حموضته pH إلى 6.5. هذا المحلول الملحي مكون من مستخلص خميرة (0.4% وزن/حجم)، ببتون (0.2% وزن/حجم)، فوسفات البوتاسيوم الأحادية (0.15% وزن/حجم)، كبريتات المغنيزيوم المائية (0.05% وزن/حجم)، كلورامفينيكول (0.03% وزن/حجم) بعد ذلك يعقم المخمر مع الوسط بالأتوكلاف على درجة حرارة 121 °م لمدة 15 دقيقة.

أما بالنسبة لتلقيح المخمر بالفطر المستخدم فكانت تتم على الشكل التالي:

- تحضير معلق بوعي بلغ تعداد الأبواغ فيه 8.75×10^7 بوغ/مل باستخدام ماء ببتوني معقم، حيث يتم توزيع هذا المعلق في أنابيب سعتها 15 مل وحفظها في الثلاجة لحين الاستخدام.
- تحضير دوارق لإعادة الزراعة سعتها 250 مل والمحتوية على 75 مل من وسط مكون من غلوكوز (2% وزن/حجم)، ببتون (1% وزن/حجم)، مستخلص الخميرة (1% وزن/حجم)

ثم تعقم هذه الدوارق بالأتوكلاف على درجة حرارة 121 °م لمدة 15 دقيقة. بعد ذلك تلقح هذه الدوارق بشكل عقيم بالمعلق البوغي المحضر سابقاً (بحيث يبلغ تعداد الأبواغ 10^6 بوغ/مل) ، ثم توضع الدوارق في حاضنة هزازة على درجة حرارة 30 °م وسعة دوران 150 دورة/دقيقة لمدة 48 ساعة.

• يتم نقل محتويات الدورق بشكل عقيم الى المخمر المحتوي على 1.5 ليتر من وسط التخمير.

استمرت كل تجربة 96 ساعة.

تم سحب العينات خلال عمل المخمر. هذه العينات كانت ترشح بوساطة ورق الترشيح، حيث تؤخذ الرشاحة ويضاف إليها أزيد الصوديوم (1 ميكروليتر لكل 1 مل رشاحة) وذلك لمنع نمو الفطر، ثم تحفظ الرشاحات في البراد (4 °م) لتجرى عليها اختبار قياس النشاط الأنزيمي.

تم قياس النشاط الأنزيمي وفقاً لطريقة (Bailey et al., 1992) وذلك باستخدام 1% كسيلان خشب البتولا كركيزة. وتمت معايرة السكريات المرجعة بطريقة حمض ثنائي نترو ساليسيليك باستخدام الكسيلوز كعيار، وقدرت الفعالية على أساس وحدة الفعالية الأنزيمية، وهي كمية الأنزيم التي تحرر 1 ميكرومول من السكر في كل دقيقة من التفاعل، وفي شروط المعايرة.

2- تجارب الزراعة السائلة:

استخدم في هذه التجارب مستخلص قشور الذرة مضافاً إليه بعض الأملاح كوسط تخمير. لتحضير هذا المستخلص تم نقع قشور الذرة المطحونة بالماء المقطر بنسبة 10% في أوعية خاص لهذا الغرض، بعد ذلك وضعت هذه الأوعية في حاضنة هزازة بسرعة دوران 150 دورة/دقيقة ودرجة حرارة 30 °م لمدة 24 ساعة. ثم أجريت عملية ترشيح لهذا المنقوع للحصول على المستخلص المطلوب، وذلك لاستخدامه كوسط تخمير في التجريبتين التاليتين:

- في التجربة الأولى اسخدم مخمر يحوي توربينين بنظام الدفعة (Batch-culture) بنفس شروط تجارب قشور الذرة المطحونة من حيث أخذ العينات، والمعايرة، ودرجة الحرارة، و pH وسط التخمير المكون من مستخلص خميرة (0.4% وزن/حجم)، بيتون (0.2% وزن/حجم)، فوسفات البوتاسيوم الأحادية (0.15% وزن/حجم)، كبريتات المغنيزيوم المائية (0.05% وزن/حجم)،

كلورامفينيكول (0.03% وزن/حجم) مضافاً إليها المستخلص ليصبح حجم العمل في المخمر 1.5 لـ لتر. أما بالنسبة لسرعة الدوران فكانت 100 دورة/دقيقة ومعدل التهوية 1vvm، وهي الظروف التي أعطت أفضل فعالية أنزيمية في الزراعة المغمورة. استمرت التجربة 144 ساعة.

- في التجربة الثانية استخدم مخمر يحوي توربينين بنظام الزراعة المستمرة (Continuous-culture) بشروط تجربة المستخلص السابقة نفسها ووسط التخمر، حيث أنه في هذا النظام تم تزويد المخمر بصورة مستمرة بوسط تغذية مماثل لوسط التخمر من حيث التركيب بمعدل إضافة قدره 75 مل/ساعة أي بمعدل تخفيف وقدره 0.05 سا⁻¹، من جهة أخرى تم التفريغ المستمر لوسط التخمر بمعدل تفريغ مماثل لمعدل الإضافة بحيث يتم تبديل وسط التخمر 5 مرات. استمرت التجربة 144 ساعة. أما بالنسبة للعينات المأخوذة فأجريت عليها معايرة للأنزيم.

تشير نتائج دراسة الزراعة المغمورة إلى أن إمكانية تحسين إنتاج أنزيم الكسيلاناز من الفطر *Aspergillus niger* يمكن أن تتم بأمثلة معدلي التهوية وسرعة الدوران ضمن المفاعل الحيوي. كما أكدت هذه النتائج أن زيادة سرعة الدوران أدت إلى تقليل إنتاج الأنزيم وأن زيادة معدل التهوية من 0.5 vvm إلى 1 vvm زاد إنتاج الأنزيم، أما زيادة معدل التهوية أكثر من ذلك أدى إلى تقليل إنتاج الأنزيم. كما أشارت النتائج نظام التوربينتين أعطى أعلى إنتاج للأنزيم (182 IU/ml) بزيادة وقدرها 13% عن نظام التوربينة الواحدة (158.9 IU/ml) وذلك عند سرعة دوران منخفضة (100 دورة/دقيقة)، وهذا بدوره يمكن أن يقلل استهلاك الطاقة، ويقلل الضرر الذي يصيب الفطر نتيجة زيادة سرعة الدوران، كون هذا الفطر حساس للسرعات العالية.

أما نتائج دراسة الزراعة السائلة فقد أشارت إلى أن نظام الدفعة (Batch-culture) أعطت فعالية أنزيمية (8.53 وحدة أنزيمية/مل) أعلى من نظام الزراعة المستمرة (Continuous-culture) (5.1 وحدة أنزيمية/مل) في مرحلة الثبات، لكن الإنتاجية الأنزيمية كانت أفضل في نظام الزراعة المستمرة (0.255 IU/ml/h) بـ 3.5 مرة من نظام الدفعة (0.077 IU/ml/h).

أخيراً تُشير هذه الدراسة إلى إمكانية إنتاج أنزيم الكسيلاناز على المستوى الصناعي من هذا الفطر ضمن ظروف التهوية وسرعة الدوران المثلى التي حصلنا عليها، كما ويمكن زيادة المردودية في وحدة الزمن باستخدام نظام الزراعة المستمرة.

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Effect of Agitation rates on Xylanase Production by *Aspergillus niger* Strain Isolated from Syrian Soil in Bioreactor

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Abstract

The effects of agitation and aeration rates on xylanase production in 3 l stirred tank was investigated. Locally isolated "*Aspergillus niger* " SS7 was used in this study. The agitation rates tested were 100, 200, 300, 400 and 500 rpm at each airflow rates of 0.5, 1 and 1.5 vvm. Two fermentation techniques were studied: submerged and liquid fermentation.

In submerged culture, the culture medium used for xylanase production composed of 30 g/L of corn cob hulls, in mineral salt medium. The maximum xylanase production by *Aspergillus niger* SS7 in mono agitator system was found at agitation speed of 200 rpm and aeration rate of 1 vvm. While in bi-agitator system, at low agitation speed (100 rpm), the xylanase production enhanced by 13% compared with mono agitator system for an aeration rate of 1 vvm.

In liquid culture, batch and continuous culture were carried out on a corn cob hulls hydrolysates medium and extra cellular xylanase activity were determined during the both culture processes and the results showed that batch liquid culture gave xylanase activity higher than continuous culture after 144h. But, the xylanase productivity of *Aspergillus niger* SS7 was approximately 3.5 times higher in chemostat culture than in batch culture on the same medium.

1. Introduction

1.1. Lignocelluloses composition and structure:

The large quantities of lignocellulosic wastes and residues generated annually represent a large untapped source of food, fuel and chemical feedstocks. Lignocellulosic residues are formed from three main components, cellulose, hemicellulose and lignin. The exploitation of such materials is clearly of scientific as well as of economic interest. The chemical properties of the components of lignocelluloses make them a substrate of enormous biotechnological value (**Malherbe and Cloete, 2003**).

The structure of wood is usually used as an example for lignocellulose. The basic model of the wood cell wall structure is well described and understood (**Hopkins, 1999**).

The wood cells are composed of different layers, which differ from one another with respect to their structure and chemical composition. In general, the layers of the cell wall structure are illustrated using a wood model shown in (Figure 1).

There are three major types of cell wall layers:

1. Middle lamella: This is the first layer formed during cell division. It makes up the outer wall of the cell and it is shared by adjacent cells. It is composed of pectic compounds and protein.
2. Primary wall: This is formed after the middle lamella before and during active growth. The primary wall consists of a rigid skeleton of cellulose microfibrils embedded in a gel-like matrix composed of pectic compounds, hemicellulose, and glycoprotein (**Dravill *et al.*, 1980**). The actual content of the wall components varies with species and age. All plant cells have a middle lamella and primary wall.
3. Secondary Wall: it is formed after cell enlargement is completed. The secondary wall is extremely rigid and provides compression strength.

They contain up to 45 percent cellulose, correspondingly less hemicellulose, and relatively little pectic substance.

Most secondary walls also contain lignin, which may account for as much as 35 % of the dry weight of woody tissues (Hopkins, 1999).

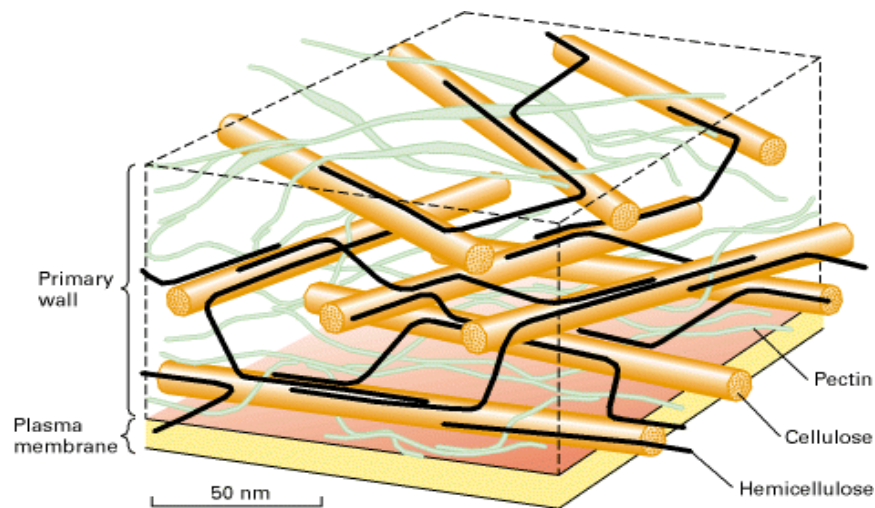


Figure 1. A simplified model to illustrate cell wall.
(Hopkins, 1999).

The lignocelluloses composition depends on the plant species, ages and growth conditions. Lignocelluloses consist of lignin, hemicelluloses and cellulose. Table (1) shows the typical compositions of the three components in various lignocellulosic materials. Based on weight percentage, cellulose and hemicelluloses are higher in hardwoods compared to softwoods and wheat straw. While, softwoods have higher lignin content.

Table 1. Lignocelluloses contents of common agricultural residues and wastes.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Corn cobs	45	35	15
Paper	85-99	0	0-15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Cotton seeds hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Fresh bagasse	33.4	30	18.9
Grasses (average values for grasses)	25-40	25-50	10-30

(Howard *et al.*, 2003)

1.1.1. Cellulose

Cellulose is composed of polymer of glucose units (Figure 2). Typically, consisting of 1,000 to 10,000 beta-D-glucose residues (degree of polymerization) - major component of primary and secondary wall layers. Cellulose polymers are held together by hydrogen bonds. The orientation of fibers could be parallel or antiparallel.

Due to the linkages beta-D-glucose, cellobiose is established as the repeat unit for cellulose chains (Figure 3). The content of cellulose in wood varies between species in the range of 40-50 %.

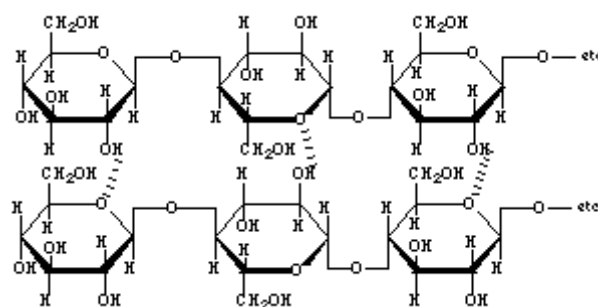


Figure 2. Cellulose structure

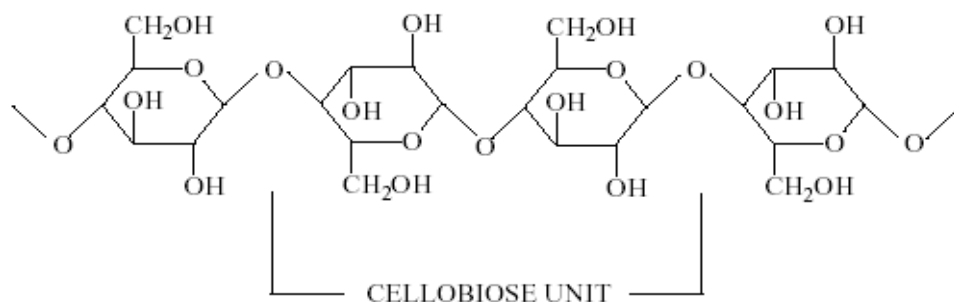


Figure 3. Cellobiose unit

1.1.2. Hemicellulose

Hemicellulose is the second most renewable biomass polymer next to cellulose. It represents about 20-35% of biomass. It could be converted to a number of value-added fermentation products such as fuel ethanol, xylitol, and lactic acid (**Saha, 2003**).

Hemicelluloses are major constituents of plant cell wall polysaccharides. Hemicelluloses are usually classified according to the main sugar residues in the backbone, e.g. xylans, glucomannans, galactans and glucans. (**Buchert *et al.*, 1994**). The structure of various types of hemicelluloses depends on the type of plant and may even vary between different parts of the same plant.

1.1.2.1. Xylan

Xylan is the major constituent of hemicellulose and it is considered with cellulose and chitin as being among the most abundant polysaccharides in nature with a high potential for degradation into useful end products (**Rawashdeh *et al.*, 2005**).

Xylans are heterogeneous polysaccharides found in cell wall as a part of the lignocellulosic complex. The composition of xylans found in cell walls of plants depends on the type of plant, plant tissue and age. The structure of xylans consists of B-1, 4-linked D-xylose pyranose chains containing several side chains at the O-2 and O-3 position (Figure 4) (Biely, 1985).

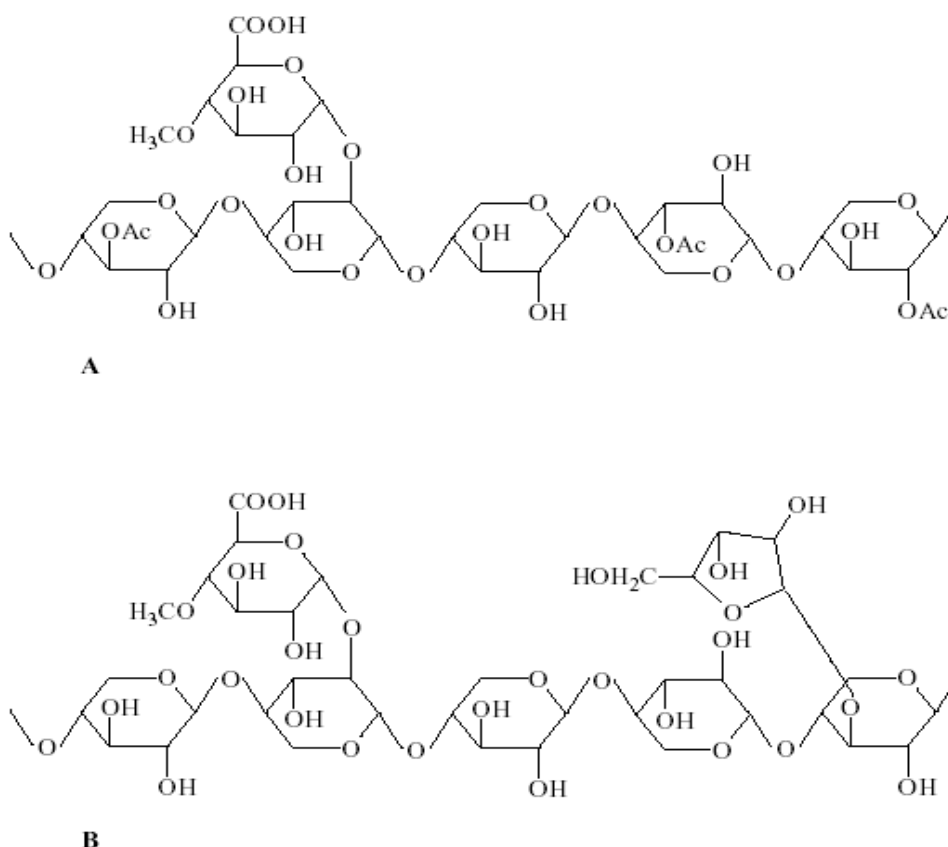


Figure 4. Schematic illustration of xylan.

A Partial xylan structure from hardwood chain

B Partial xylan structure from softwood chain

The xylan proportion in wood amounts up to 35% in some residues of annual plants. However, the xylan content may surpass the cellulose content to reach more than 45% (Puls *et al.*, 2006).

The xylans in many angiosperms may account for 20-30% of the dry weight of the woody tissue. In these cases, they are found mainly in the secondary wall, but in monocotyledonous plants they are also found in the primary cell wall.

1.1.2.2. Glucomannans

Glucomannans, the second group of hemicellulosic polysaccharides, consist mostly of mannose and glucoses units joined in chains by 1, 4 linkages. However, small amounts of other sugars, particularly galactose, have been found, joined in principle chain by G-1, 6 linkages (Figure 5).

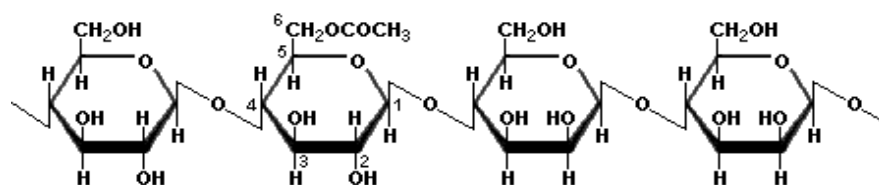


Figure 5: A portion (GGMM) of the glucomannan repeating unit.

The second glucose has an acetate group.

1.1.3. Lignin

Lignin has been described as a random three-dimensional network polymer comprised of variously linked phenylpropane units (**Sjöström, 1993**). The phenylpropane units in lignin are connected by C-C and ether (C-O-C) linkages.

1.2. Xylanolytic enzymes

Xylanases is the name given to a class of enzymes which degrade the linear polysaccharide Beta-1, 4-xylan into xylose, those break down hemicellulose, which is the major component of the plant cell wall (**Belfaqui *et al.*, 2002**).

The complete breakdown of xylan requires the action of a large variety of hydrolytic enzymes (**Biely, 1985; Coughlan and Hazlewood, 1993**). Those enzymes can be classified into two main groups: those act on the xylose backbone, and those cleave the side chains. Degradation of the xylose backbone depends on xylanases(endo- β -1,4-xylanases, EC 3.2.1.8) that

cleave bonds within the polymer and β -xylosidases that release xylose units from xylobiose and xylooligomers.

Removal of xylan side chains is catalysed by α -L-arabinofuranosidases, α -D-glucuronidases, acetyl xylan esterases, ferulic acid esterases (Figure 6).

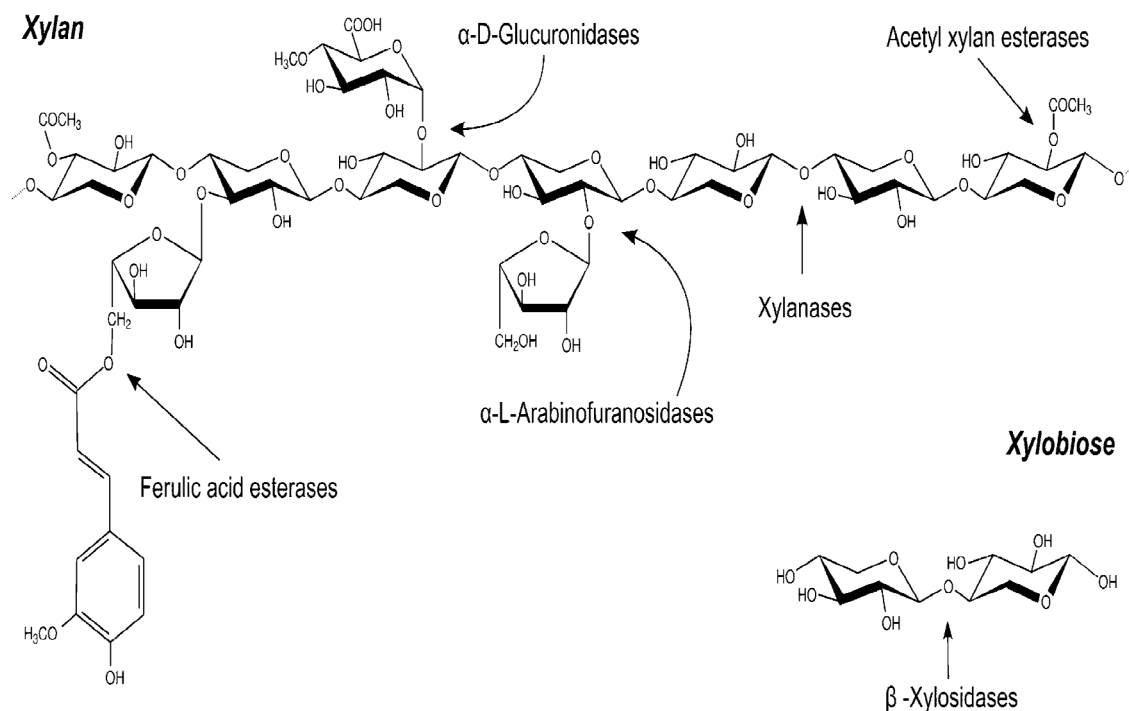


Figure 6: Xylanolytic enzymes

1.2.1. Xylanases applications

Microbial hemicellulases, especially xylanases, have important applications in industry due to their enormous potential to modify and transform the lignocelluloses and cell wall materials abundant in vegetal biomass which is used in a wide variety of industrial processes. The biotechnological application of xylanases began in the 1980s in the preparation of animal feed, and later it expanded to the food, textile and paper industries. Since then the biotechnological use of these enzymes has increased dramatically covering a wide range of industrial sectors. At present, xylanases together with cellulases and pectinases account for 20% of the global industrial enzyme market (Polizeli *et al.*, 2005).

1.2.1.1. Xylanases in animal feed:

Xylanases are used as additives in animal feeds for monogastric animals, together with cellulases, pectinases and many other depolymerizing enzymes. Enzyme degradation of arabinoxylans, commonly found as ingredients of feeds, reduces the viscosity of the raw materials; thus, facilitating better mobility and absorption of other components of the feed and improving nutritional value (**Polizeli *et al.*, 2005**).

* Improving animal feed. Adding xylanase stimulates growth rates by improving digestibility which also improves the quality of the animal litter. Xylanase thins out the gut content and allows increased nutrient absorption and increased diffusion of pancreatic enzymes in the digesta. It also changes hemicellulose to sugars. So that, nutrients formerly trapped within the cell walls are released (**Medel *et al.*, 2002**).

1.2.1.2. Manufacture of bread, food and drinks:

Baking industry: xylanases are used for improving desirable texture, loaf volume and shelf-life of bread (**Romanowska *et al.*, 2003**).

Xylanases may be employed in bread-making together with α -amylase, malting amylase, glucose oxidase and proteases. With the use of xylanases, there has been an increase in bread volumes, greater absorption of water and improved resistance to fermentation (**Maat *et al.*, 1992; Harbak and Thygesen, 2002; Camacho and Aguilar, 2003**).

Nowadays, xylanases, in conjunction with cellulases, amylases and pectinases, lead to an improved yield of juice by means of liquefaction of fruit and vegetables, stabilization of the fruit pulp, increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc., reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate (**Polizeli *et al.*, 2005**).

Xylanases can be also used in brewing to reduce beer's haze and viscosity, and to increase wort filterability (**Polizeli *et al.*, 2005**).

1.2.1.3. Pharmaceutical and chemical applications:

Xylanase and xylan are little used in the pharmaceutical industry. Xylanases are sometimes added in combination with a complex of enzymes (hemicellulases, proteases and others) as a dietary supplement or to treat poor digestion, but few medicinal products can be found with this formulation (**Polizeli *et al.*, 2005**).

Bioconversion of xylan to the low calorie sweetener xylitol is a promising field where xylanases can also play a key role (**Polizeli *et al.*, 2005**).

Xylanases are thus of increasing importance for the bioconversion of lignocellulosic biomass to xylose and other fermentable sugars for the production of biological fuels (ethanol) (**Lee, 1997**).

1.2.1.4. Textiles:

The xylanolytic complex can be used in the textile industry to process plant fibres, such as hessian or linen. For this purpose, the xylanase should be free of cellulolytic enzymes. One process consists of incubating dried ramee (China grass) stems with xylanase to liberate the long cellulose fibres intact. After using this method, there is no need to use the strong bleaching step, since the lignin does not undergo oxidation, which would lead to darkening of the fibres (**Prade, 1995; Brühlmann *et al.*, 2000; Csiszár *et al.*, 2001**).

1.2.1.5. Cellulose pulp and paper:

The major current industrial application of xylanases is in the cellulose pulp and paper industry where xylanase pretreatment facilitates chemical bleaching of cellulose pulps, resulting in important economic and environmental advantages over the non-enzymatic process (**Viikari *et al.*, 1994; Bajpai, 2004**).

Xylanases do not remove lignin-based chromophores directly but instead degrade the xylan network that traps the residual lignin. Degradation of xylan, in xylan-lignin complexes or reprecipitated on the surface of fibres after kraft cooking, allows a more efficient extraction of lignin by the bleaching chemicals.

* Bioleaching paper pulp: The use of xylanase leads to a reduction in organo-chlorine pollutants such as dioxin from the paper making process. In addition, chlorine-free bleaching (such as dioxide or ozone bleaching) can achieve brighter results with the addition of xylanase. Because xylanase does not harm cellulose, the strength of the paper product is not adversely affected (Vicuña *et al.*, 1997).

1.2.1.6. Bio-fuel:

Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels in 2050 (Campbell and Laherrere, 1998). The technology development focus on the production of ethanol has shifted towards the utilization of residual lignocellulosic materials and saccharification process involves using xylanase and cellulase to breakdown (hydrolyse) the polysaccharide into their component sugars for fermentation by microbes to ethanol.

1.3. Fermenter

Bioreactor is a system consisted of few pieces of equipment which provide controlled environmental conditions for the growth of microbes (and/or the production of specific metabolites) in liquid culture whilst preventing entry and growth of contaminating microbes from the outside environment.

A bioreactor enables optimal fermentation conditions to be maintained, allowing addition of nutrients, removal of products and insertion of measuring and/or control probes as well as other necessary equipment (e.g.

for heating, cooling, aeration, agitation, sterilization, etc.) under sterile conditions.

1.3.1. Submerged bioreactors- stirred-tank

Stirred tank bioreactors are commonly used in fermentation processes (**Liu *et al.*, 1994**). Stirred tank bioreactors are widely used in the chemical process industry to carry out many different operations.

A fully functional bioreactor requires perfect integration of several components to ensure that cultures will finish at the desired productivity (**Fisher, 2006**).

These components include:

- a jacketed stainless steel vessel - temperature control - agitation - sparge gas control - headsweep gas control - pH and dissolved oxygen control - a sampling mechanism - weight indication and control - cleaning hardware - sterilization hardware - piping and tubing to deliver all services, add media, pH control solutions, and gases - feed and addition pumps, a supervisory control and a data acquisition system.

For laboratory experiments with smaller volumes, the mixing vessel is typically made of glass. However stainless steel tank construction is the standard for industrial applications involving larger volumes. The height-to-diameter ratio of the vessel can vary depending on heat removal requirements.

Within each vessel, the impeller is connected to an external motor which drives the stirrer system. Agitation and aeration in a stirred tank bioreactor always cause foaming. Excess foaming will force the broth out of the fermenter and contaminate the system.

The operating principles of the stirred-tank bioreactor are relatively simple. As shown in (Figure 7). The sterile medium and inoculum are introduced into a sterilized tank and the air supply typically enters from the bottom.

For optimal mixing, the tank features are not only an agitator system but also baffles which help to prevent a whirlpool effect that could impede proper mixing.

Antifoam which destroys the foam by reducing sulfate tension is always applied in fermentations. However, antifoam also affects the rate of oxygen transfer and cell growth. Therefore, the addition of antifouam is detrimental to fermentation operations (**Liu *et al.*, 1994**).

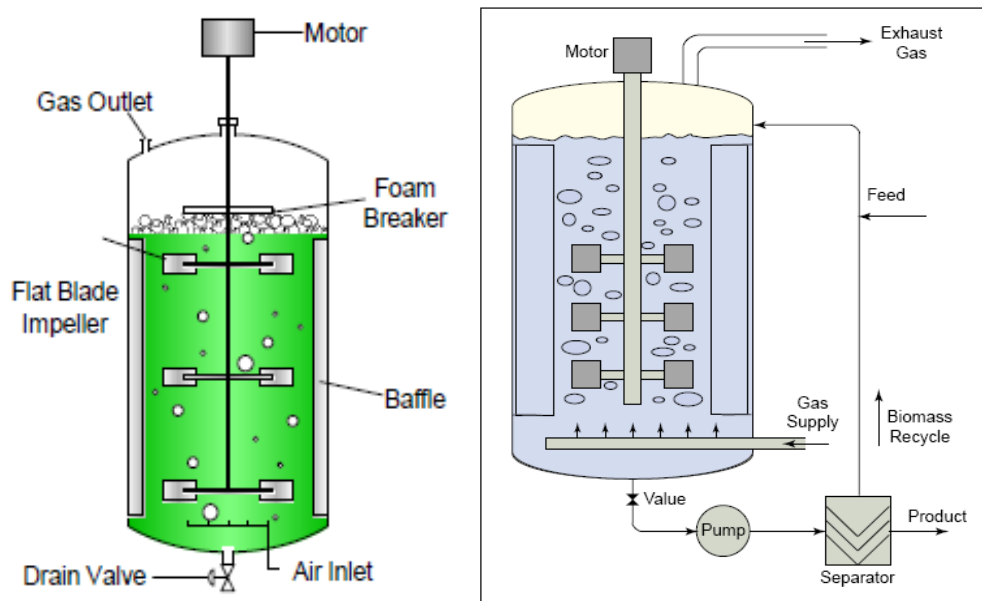


Figure 7: Stirred-tank bioreactor uses baffles and an agitator for optimal mixing, and recycles biomass

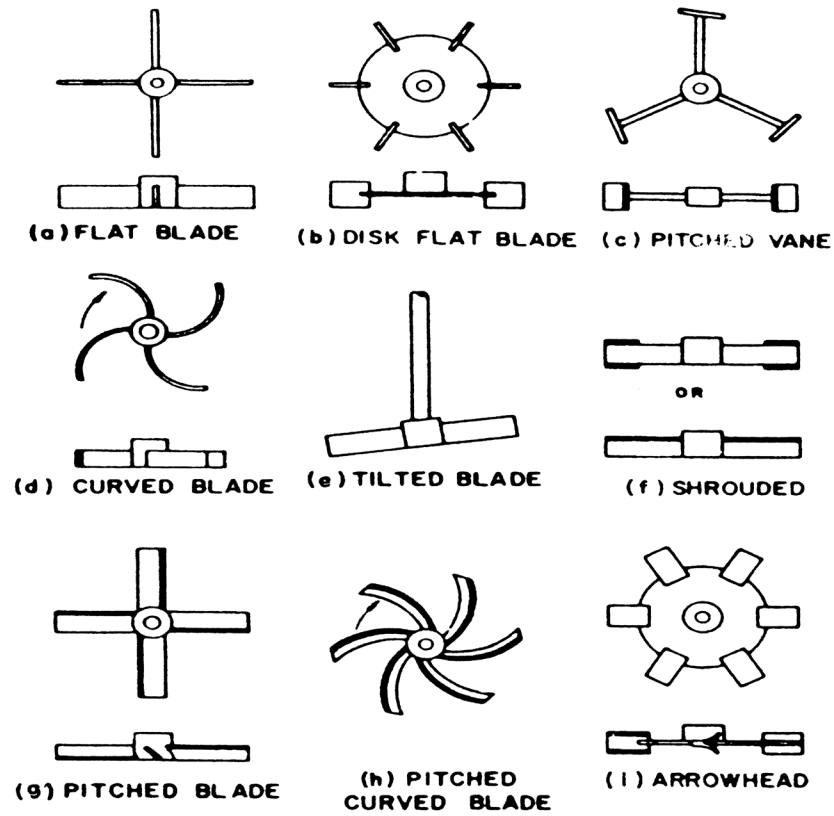
1.3.2. Types of impeller:

Impeller design and choice are critical for maintaining proper hydrodynamic conditions and gas–liquid mass transfer rates in the reactor. Figure 8 gives a schematic representation of the commonly used impellers. Impellers are classified according to the mixing regimes, laminar (i.e. creeping flow), or turbulent mixing (**Gogate *et al.*, 2000**). For laminar mixing, the impeller diameters approach the diameter of the tank, since the transport of momentum by laminar flow is poor. On the other hand, as turbulent flow transports momentum well, the impellers in turbulent media

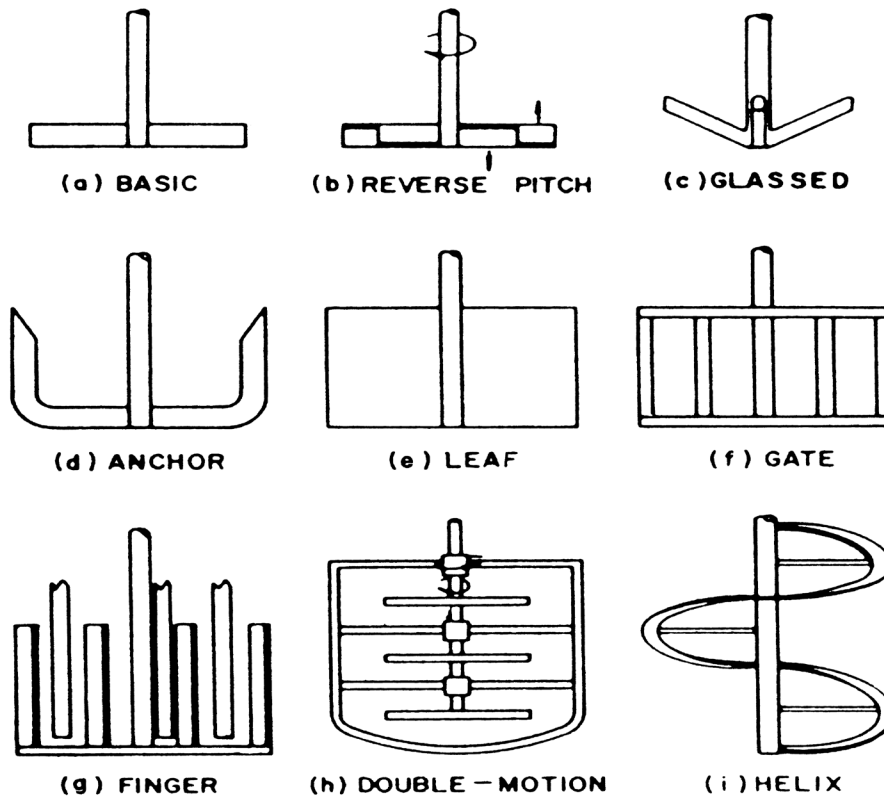
are typically one-fourth to one-half the tank diameter. Typical laminar impellers are helical ribbons, screws, helical ribbon screws and anchor impellers. Discs, paste rollers, high shear and gate impellers are also used in laminar mixing. Turbulent flow impellers which are most commonly used in fermentation applications are further classified into axial and radial flow impellers, depending on the direction of the discharge flow (**Gogate *et al.*, 2000**). Typical radial flow impellers are disc style, flat blade and curved blade impellers. The pitched-blade turbines and propellers are commonly axial or mixed flow impellers. Pitched-blade turbines can generate mixed flow and the extent of axial flow depends on the angle of attachment of the blade to the hub.

Weetman and Oldshue (1988) have also classified turbulent mixing impellers as flow impellers, which have large pumping capacities, pressure impellers, which work against the hydrostatic and dynamic pressure gradients and shear impellers which produce shear. There are also hydrofoil or fluid-foil impellers, which have blades shaped as a hydrofoil giving a low angle of fluid attack (**Weetman and Oldshue, 1988**). This minimizes drag on the blade resulting in a minimum power consumption, but generates larger bulk recirculation rather than the local turbulence. Speciality impellers include high shear impellers with pointed blades and disc-style turbines with concave blades for gas dispersion (**Shieu and Wong, 1984**).

Rushton and Rushton-type impellers are commonly used in fermentations of cell lines that are not considered shear-sensitive, including yeasts, bacteria, and some fungi (**Mirro and Voll, 2009**).



Turbine impeller designs.



Paddle impeller designs.

Figure 8: Different types of impellers commonly used in industrial applications.
(Uhl and Gray, 1966)

1.4. Production of xylanase in fermenter

1.4.1. Fermentation

Fermentation processes attract an increasing interest, being at the heart of both classical industrial activities and some new applications such those related with environment, pharmacy, etc.

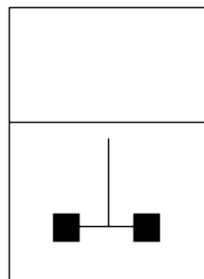
Technically, fermentation processes in bioreactor may be classified as Submerged fermentation, liquid and solid substrate fermentation.

Based on the mode of operation, fermentation processes may be classified into 3 types: Batch processes, continuous processes and fed-batch processes.

1.4.1.1. Batch fermentation:

Batch fermentation is a suspension culture in which cells grow in a finite volume of liquid nutrient medium and follow a sigmoid pattern of growth. All cells are harvested at the same time. Thus, In batch cultures, a bioreactor is filled with fresh medium and then inoculated.

At the end of the fermentation, the contents are removed for downstream processing, the reactor is then cleaned, sterilized and refilled for the next fermentation.



Batch

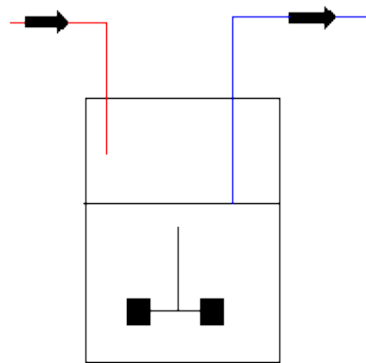
The Batch fermentation has the advantage of simplicity. Since the medium is the same in shake flasks and fermentors, only the physical parameters have to be optimized during scale-up (**Hutter *et al.*, 1978**).

1.4.1.2. Continuous fermentation:

Continuous fermentation is a process in which cells or microorganisms are maintained in culture in the exponential growth phase by the continuous addition of fresh medium that is exactly balanced by the removal of cell suspension from the bioreactor.

Thus, with continuous cultures, fresh media is *continuously added* into the bioreactor and at the same time bioreactor fluid is *continuously removed*.

A continuous cultivation system could have the advantage of reducing the time during which the fermentors are empty and of increasing the overall productivity (**Hutter *et al.*, 1978**).



Continuous

In continuous cultivation, contamination risk is higher due to an increase in the number of vessels, pipes and pumps, a more demanding control and the necessity to change the isolation procedure are additional critical aspects of a continuous fermentation process (**Hutter *et al.*, 1978**). Furthermore, investment costs for continuous systems are higher, and contaminations will lead to longer interruptions of production than with batch processes (**Hutter *et al.*, 1978**).

One of the most important features of chemostats is that micro-organisms can be grown in a physiological steady state. In steady state, all culture parameters remain constant (culture volume, dissolved oxygen concentration, nutrient and product concentrations, pH, cell density, etc.). Micro-organisms grown in chemostats naturally strive to steady state: if a low amount of cells is present in the bioreactor, the cells can grow at growth rates higher than the

dilution rate, as growth isn't limited by the addition of the limiting nutrient. The limiting nutrient is a nutrient essential for growth presented in the media at a limiting concentration (all other nutrients are usually supplied in surplus). However, if the cell concentration becomes too high, the amount of cells removed from the reactor cannot be replenished by growth as the addition of the limiting nutrient is insufficient.

This results in an equilibrium situation (steady state), where the rate of cell growth is equal to the rate of cell removal.

Because obtaining a steady state requires at least 5 volume changes, chemostats require large nutrient and waste reservoirs.

Dilution Rate

At steady state the specific growth rate (μ) of the micro-organism is equal to the dilution rate (D) h^{-1} . The dilution rate is defined as the rate of flow of medium over the volume of culture in the bioreactor as the following:

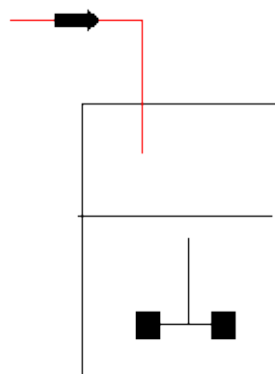
$$D = \frac{\text{Medium flow rate}}{\text{Culture volume}} = \frac{F}{V}$$

1.4.1.3. Fed- batch fermentation:

Fed- batch fermentation is a culture of cells or microorganisms where nutrients are added periodically to the bioreactor (i.e. With fed batch cultures, fresh media is added to the bioreactor without continuous removal). When the reactor volume is full, the fermenter is emptied, either partially or completely, and the process restarted.

When a part of the culture broth is removed at intervals and slowly replaced with fresh medium, the method is called a repeated fed-batch (**pirt, 1974**).

This type of approach has been used successfully for many types of fermentation processes, such as lactic acid production (**Liu *et al.*, 2005**), biomass production (**Demirci *et al.*, 1999**), antibiotic (**Colombie *et al.*, 2005**), and recombinant proteins (**Choi and Park, 2006**).



Fed-batch

Thereby, the feed supplies the cells with the energy required for production, and it is used, at the same time, to prevent the concentration of the medium from increasing due to evaporation (**Hutter *et al.*, 1978**).

This method has the advantage that the additions vary according to the metabolic activity of the culture (**Hutter *et al.*, 1978**).

1.4.2.Effects of agitation and aeration rates on xylanase production in bioreactor

Xylanases may be industrially produced in submerged liquid culture or on a solid substrate. but it should be noted that about 80–90% of all xylanases are produced in submerged culture. In cultures on solid substrate, wheat bran and rice are regarded as inducers. Alternative substrates for enzyme production have also been reported, such as sugarcane bagasse, rice husks and wood pulp (**Kadowaki *et al.*, 1995; Damaso *et al.*, 2000; Medeiros *et al.*, 2000; Pandey *et al.*, 2000; Singh *et al.*, 2000; Anthony *et al.*, 2003**).

In liquid culture, xylanase is produced in response to xylans from various sources (**Gomes *et al.*, 1994a; Liu *et al.*, 1999; Rani and Nand, 2000**).

Haltrich *et al.* (1996) gave an overview of fungal xylanases and showed that the enzyme can be produced by a number of microorganism including bacteria, yeasts and filamentous fungi such as *Trichoderma*, *Bacillus*, *Cryptococcus*, *Aspergillus*, *Penicillium*, *Aureobasidium*, *Fusarium*,

haetomium, *Phanerocha*, *Rhizomucor*, *Humicola*, *Talaromyces* and many more.

These Filamentous fungi are preferred for commercially important enzyme products because the level of the enzymes produced by these culture is higher than those obtained from yeast and bacteria (**Bakri *et al.*, 2003**).

The filamentous fungus *Aspergillus niger* is a widely applied industrial strain for large-scale biotechnological production, e.g. citric acid production (**Papagianni *et al.*, 1998**). In addition, this strain is known for high secretion capability, resulting in high yields of homologous and heterologous proteins like glucoamylase or antibiotics (**Amanullah *et al.*, 2001**; **Punt *et al.*, 2002**; **Wongwicharn *et al.*, 1999**).

Aeration and agitation rates are important factors during xylanase production in bioreactor. The agitation and aeration rates have been thought to influence enzyme productivity in a bioreactor (**Gomes *et al.*, 1993**; **Hoq *et al.*, 1994**; **Purkarthofer *et al.*, 1993a**; **Gomes *et al.*, 1994b**; **Hoq *et al.*, 1995**). So many studies were carried out to optimize the aeration and agitation rates.

Agitation has two main effects on the cultivation of filamentous fungi in stirred tank reactors. First, it is important to ensure the supply of nutrients, especially oxygen. Furthermore, good mixing, mass and heat transfer require a threshold level of agitation.

On the other hand, high agitation rates lead to high energy dissipation rates connected with high shear stress, which may result in fragmentation and damage of cells and mycelial networks (**Amanullah *et al.*, 2000**; **Li *et al.*, 2000**; **Mitard and Riba, 1988**; **Nielsen *et al.*, 1995**).

It is well known that the formation of many products depends on the morphological structure of the macroscopic growth of filamentous fungi. For example, *penicillin* production of *Penicillium chrysogenum* requires small and dense pellets (**Johansen *et al.*, 1998**; **Nielsen *et al.*, 1995**), in contrast to the formation of citric acid with *A. niger*, where freely dispersed mycelia cause

maximum production rates (**Papagianni *et al.*, 1998**).

While high energy dissipation rates are required for optimum mass transfer, they can increase the apparent viscosity of the fermentation broth. This is caused by rising amounts of free filaments and leads to problems like insufficient mass transfer and oxygen limitation (**Bhargava *et al.*, 2003; Li *et al.*, 2000**). These aspects show the necessity to understand the mechanisms of pellet formation and behaviour at different agitation intensities. The mechanism of pellet formation is not well understood yet, but two general types have been described (**Cui *et al.*, 1998b; Nielsen *et al.*, 1995**):

1. Coagulating type: In the early stage of cultivation, spores and hyphae aggregate form aggregates which grow into pellets later on.
2. Non-coagulating type: A single spore grows to become one pellet *Aspergillus niger* is, so far, considered to belong to the coagulating type (**Dynesen and Nielsen, 2003; Metz and Kossen, 1977**). The coagulation or aggregation takes place in the early stage of cultivation by forming aggregates. These aggregates consist of spores and hyphae. As a consequence of further growth, these loose clumps form pellets (**Grimm *et al.*, 2003**).

Agitation has another effect on the growth of pellets due to fragmentation and damage of pellets and cells.

For the fragmentation of pellets, three main mechanisms are described in literature (**Cui *et al.*, 1997; Metz and Kossen, 1977**):

1. Interaction among pellets and eddies.
2. Impacts among pellets and impellers or baffles.
3. Collision among pellets.

The density of the fungal pellets is very close to that of the medium, so pellets will follow the stream lines of liquid flow. Therefore, it can be concluded that the interaction of eddies with pellets is the most important mechanism of fragmentation. The others are negligible (**Cui *et al.*, 1997; Nielsen *et al.*, 1995**).

There are also several types of damage, which vary from simple chipping off of hyphae from the pellet surface to the complete rupture of whole pellets. For modelling fungal breakup, the most common approach is to calculate the hyphal strength and magnitude of mechanical forces by turbulent flow (Cui *et al.*, 1997; Cui *et al.*, 1998c; Cui *et al.*, 1998a).

Product formation of filamentous fungi and pellet growth is influenced by pellet size and structure (Cui *et al.*, 1997; El-Enshasy *et al.*, 1999; Metz and Kossen, 1977; Papagianni *et al.*, 1998; Wongwicharn *et al.*, 1999; Zetelaki and Vas, 1968).

The maximum enzyme activity occurs at a low energy dissipation and at a relatively high pellet concentration. With higher energy dissipations, the enzyme activity decreases. This seems very plausible because of the lower amount of active biomass in the case of fewer pellets. Biomass activity depends on substrate availability, which itself depends on transport processes inside the pellets (Cui *et al.*, 1998c; Cui *et al.*, 1998a; Hellendoorn *et al.*, 1998; Paul *et al.*, 1999). If only a few pellets occur with low activity because of inner nutrient limitation, the production rate has to be lower than at cultivations with a higher amount of active pellets.

Papagianni *et al.* (2001) found that the enzyme production was strongly affected by the agitation. At the higher agitation rates the enzyme production dropped.

Purkarthofer *et al.* (1993b) reported that 120 rpm was optimal for xylanase production by *Thermomyces lanuginosus* strain **DSM 5826** between agitation speeds tested 100-250 rpm. They described the lower xylanase activity at the lower shaking speed to poor oxygen transfer within the medium, whereas at higher shaking speeds the xylanase production was lower, contributed to an increase in hyphal branching damage.

Hoq *et al.* (1994) found that xylanase production in bioreactor by *Thermomyces lanuginosus* strain RT9 had an optimum agitation rate of 200 rpm.

Oxygen supply into the broths constitutes one of the decisive factors of cultivated microorganism growth and can play an important role in the scale-up and economy of aerobic biosynthesis systems (**Galaction *et al.*, 2004**).

Sufficient oxygen supply for biological cultures is essential for efficient aerobic bioprocess development. According to **Hilton (1999)**, often, in liquid batch processes, the critical parameter is gas exchange or balance between the oxygen demand of the culture (OUR, oxygen uptake rate) and oxygen transfer to the culture (OTR, oxygen transfer rate).

OUR is defined as the quantity of oxygen consumed by cells per unit of volume and per unit of time. OUR is constant for resting cells.

OTR in the reactor is defined as the quantity of oxygen transferred from the gas to the liquid phase per unit of volume and per unit of time (**Kilani and Lebeault, 2007**).

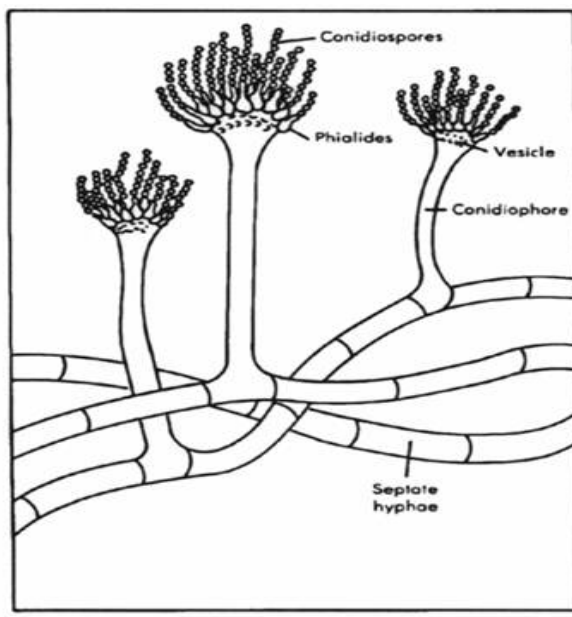
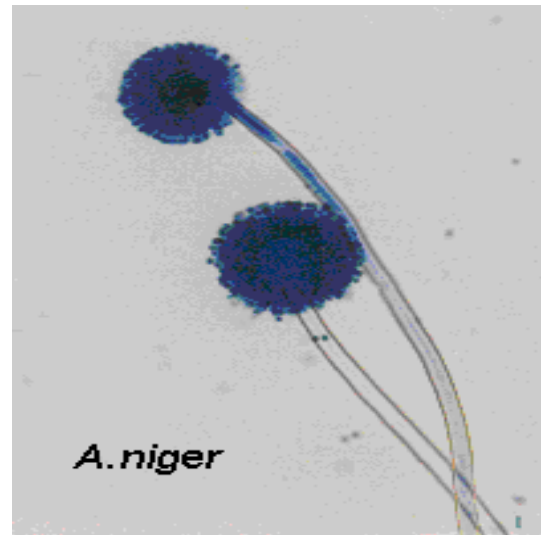
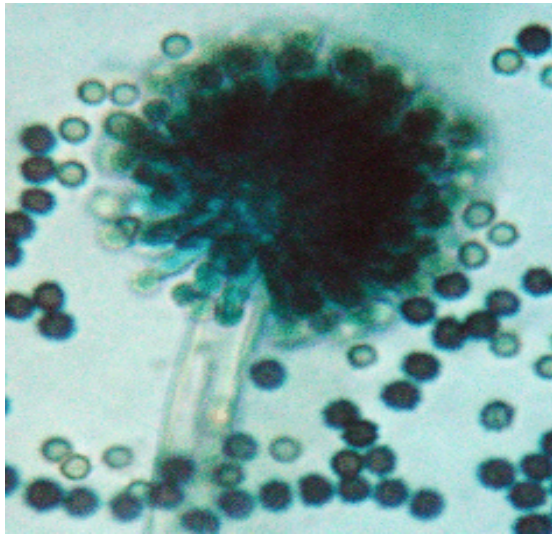
Diffusion and consumption rates are affected by many factors such as geometrical and operational characteristics of the vessels, media composition, type, concentration and microorganism morphology, biocatalysts properties (particle size, porosity, etc.) (**Galaction *et al.*, 2004**). One of the essential conditions for optimization of oxygen transfer is to reduce the length of the oxygen diffusion route through microorganisms, i.e., to reduce the thickness of the film layer around the bubbles and microbial cells. Generally, this could be easily obtained by means of an intense mixing, but the sensitivity of cultivated microorganisms to shear stress is rather high (**Galaction *et al.*, 2004**).

Agitation and aeration in a stirred tank bioreactor always cause foaming. Excess foaming will force the broth out of the fermenter and contaminate the system. Antifoam which destroys the foam by reducing sulfate tension is always applied in fermentations. However, antifoam also affects the rate of oxygen transfer and cell growth. Therefore, the addition of antifoam is detrimental to fermentation operations (**Liu *et al.*, 1994**).

1.5. *Aspergillus*

The genus *Aspergillus* is a group of filamentous fungi with a large number of species. The first record of this fungus can be found in Micheli's *Nova Plantarum Genera*, but a more detailed description of the aspergilli did not appear until the middle of the 19th century. In 1926 a first classification of these fungi was proposed describing 11 groups within the genus. A reexamination of the genus was published by Thom and Raper, identifying 14 distinct groups. Some of these groups consist of pathogenic fungi (e.g., *A. fumigatus*, *A. flavus*, and *A. parasiticus*), but the most important for industrial applications are some members of the black aspergilli group (*A. niger* and *A. tubingensis*) (Parenicova *et al.*, 1997). In addition to the morphological techniques traditionally applied, new molecular and biochemical techniques have been used in the reclassification of this group of aspergilli (Hamari *et al.*, 1997). These analyses resulted in the clear distinction of eight groups of black aspergilli (*A. niger*, *A. tubingensis*, *A. foetidus*, *A. carbonarius*, *A. japonicus*, *A. aculeatus*, *A. heteromorphus*, and *A. ellipticus*). Products of several of these species have obtained a GRAS (Generally Regarded As Safe) status, which allows them to be used in food and feed applications. The black aspergilli have a number of characteristics which make them ideal organisms for industrial applications, such as good fermentation capabilities and high levels of protein secretion. In particular, the wide range of enzymes produced by *Aspergillus* for the degradation of plant cell wall polysaccharides is of major importance to the food and feed industry.

Recently, several *Aspergillus* spp. have received increased interest as hosts for heterologous protein production (Vreis and Visser, 2001).



1.6. Objectives

This work is considered the first step to optimize enzyme production in bioreactor in Syria.

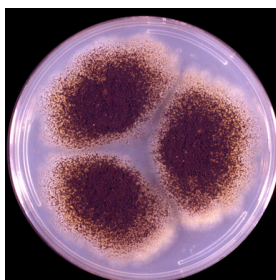
For the development of scale-up strategies of a bioprocess, it is necessary to investigate the optimal conditions of cultivation providing the highest rate of product formation.

This work aimed to study the influence of aeration and agitation rates on xylanase production by *Aspergillus niger* isolated from Syrian soil in bioreactor.

2. Materials and Methods:

2.1. Microorganism:

Aspergillus niger SS7 was isolated from local soil, and identified by the Paris Natural History Museum (PNHM), Paris, France. It was previously reported as xylanase producing strain in sub-merged culture (Bakri et al., 2008). In The laboratory, stock cultures were maintained on potato-dextrose-agar (PDA) at 4 °C.



Aspergillus niger SS7

2.2. Culture Medium:

3.2.1. Submerged culture:

The culture medium used for xylanase production in submerged culture composed of 30 g/l of corn cob hulls, in mineral salt medium. The mineral salt medium contained: K_2HPO_4 (0.15% w/v), $MgSO_4 \cdot 7H_2O$ (0.05% w/v), Peptone (0.2% w/v) and Yeast extract (0.4% w/v).

3.2.2. Liquid culture:

The alternative to use insoluble hemicelluloses was using soluble sugars extract from corn cob hulls to be used in batch and continuous liquid culture. In order to prepare the hydrolysate, 10% (w/v) of milled corn cob hulls was soaked in distilled water at 30°C for 24 hours. The liquid hydrolysate obtained after centrifugation was used as a carbon source for xylanase production. The nitrogen source and mineral salts were used as the same in submerged culture. The pH in all experiments was adjusted to 6.5 before sterilization.

2.3. Bioreactor:

In this investigation, a 3-L Electro-lab fermenter (Electro-lab limited, UK, figure 9) was used as the base mechanical vessel for xylanase production from local isolate *Aspergillus niger* SS7 in submerged and liquid culture. The agitation system is made of Rushton turbine DT6 (Figure 10). The diameters of those turbines were: $d = 50$ mm. The clearance above the base of the bioreactor and the distance between the two impellers were equal to turbine diameter (d) according to geometry standards of bioreactor (**Bruxelmane, 1993**). The bioreactor was filled with 1.5 L of culture medium and then sterilized at 121°C for 20 min. The fermentor provided by a thermostate, pH-meter and adjustant. Aeration rate flow control, dissolved oxygen measurement and control, sampling system, air pumps, 4 peristaltic pumps, tubing, reagent bottles with filters and silicon tubes. The fermentor was connected to PC station-based control software to control and permeate to automatic storage for all parameter data and profiles.

A regulation system was used to control the temperature at 30°C throughout of the experiment after sterilization. Tego antifoam KS911 (Goldschmidt, Essen, Germany) 0.05% (v/v) was added to culture medium into the bioreactor.

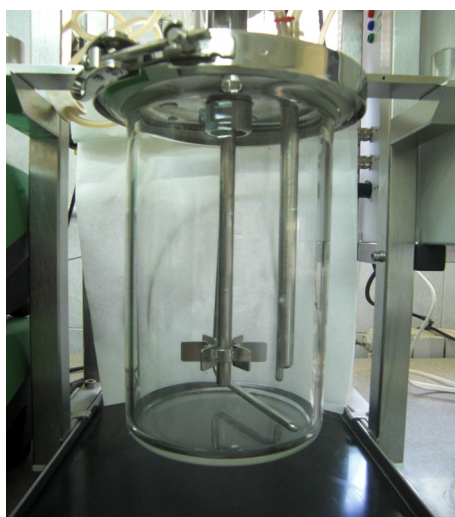
The air flow rates studied were 0.5 , 1, and 1.5 vvm, the agitation speeds were 100, 200, 300, 400, and 500 rpm, which correspond the following peripheral speeds (πND) 0.26, 0.52, 0.79, 1.05 and 1.31 m/s respectively.

The bioreactor was inoculated with a 5% vol of inoculum under aseptic conditions. The inoculum was prepared in a 250-mL Erlenmeyer flask in the medium containing 2% glucose, 1% yeast extract and 1% peptone. The inoculums medium was inoculated after sterilization with a spore suspension to supply a spore concentration of 10^6 spores/ml and incubated at 30°C under shaking (150 rpm) for 48 h. Two systems of culture were carried out batch and continuous culture (Figure 11).



Figure 9: 3-L Electro-lab fermenter (Electro-lab limited, UK).

A



B



Figure 10: A. fermenter vessel mounted with one turbine DT6. B. fermenter vessel mounted with two turbines DT6 – DT6.



Figure11: xylanase production during continuous culture of *Aspergillus niger* in a 3-L bioreactor fitted with two DT6.

2.4. Spectrophotometer:

The absorbance was measured at 550 nanometer using a spectrophotometer (CECIL CE 2502-England) (Figure 12).



Figure 12: spectrophotometer CECIL CE 2502-England.

2.5. Enzymatic Assays:

2.5.1. Substrate preparation:

The substrate was 1% of xylan from Birchwood in 500mM citrate-phosphate buffer at pH equalled 5.

2.5.2. Hydrolyze reaction:

Xylanase activity was determined according to **Bailey *et al.* (1992)**. The reaction mixture total volume was 2ml consisting of 1.8 ml of substrate and 0.2 ml of appropriate dilution of enzyme solution. The incubation time was 5 min at 55 °C , then 3ml of DNS reagent was added to stop the reaction. The color was developed by boiling the samples for 15 min and then by placing them immediately on cold water prior to taking an aliquot of 1 ml into a disposable plastic cuvette. The absorbance was measured at 550 nm against a blank was prepared in the same way but with 0.2 ml citrate - phosphate buffer pH 5. The xylanase activity related to the amount of reducing sugars (xylose) presented by comparison with a standard curve.

Enzyme activity (IU) represented the amount of enzyme that released (1) micromole of reducing sugar (expressed as xylose equivalents) per minute under those assay conditions.

2.6. Statistic analysis:

Effects of agitation speeds and aeration rates on xylanase activity were analyzed according to statistic analysis program (SPSS v15).

3. Results and Discussion

Oxygen transfer is often a rate-limiting step for bioprocesses. Where aerobic processes were carried out in aqueous medium with ionic salts, the rate of oxygen transfer is limited due to the low oxygen solubility. The rate of oxygen utilization by microorganisms for growth and activity is considerably high and any shortage of oxygen drastically affects the performance (**Gogate *et al.*, 2000**). On the other hand, efficient break-up of gas bubbles for conventional oxygenation cannot be directly used in shear-sensitive biological systems (**Wang and Zhong, 1996**). Hence, the design of bioreactor which gives the maximum potential of microorganisms productivity and product concentration achievable is one of the most challenging tasks in the biochemical industry. In such processes, oxygen availability depend upon mixing and mass transfer which in turn, primarily are governed by vessel design, impeller type, and fluid properties (**Amanullah *et al.*, 1998**).

Traditionally, the radial flow disc turbines, typically Rushton disc turbine of a diameter d of one third of the reactor diameter D are the commonly used mixers and considered to be good for gas dispersion in the processes of chemical industries (**Nienow, 1990**).

In the design of a fermentation process, many operation parameters, such as agitation and aeration, pH, dissolved oxygen, and inoculum levels have to be investigated (**Jüsten *et al.*, 1996**). Among them, agitation and aeration rates are the most critical parameters used for process scale-up (**Felse and Panda, 2000**) and play significant roles in determining the productivity of the process (**Jüsten *et al.*, 1996**). Aeration of growing microbial culture is undertaken primarily to supply its oxygen requirements and at the same time to remove waste products.

The necessary oxygen for the growth and production of fungal culture can be ensured by agitation and aeration of the culture. The efficiency of aeration can be improved by agitation, resulting in an increased interface between gas and liquid. It is known that the intensive flow of liquid, caused by agitation, forces the air bubbles to disintegrate into a large number of small bubbles. An additional beneficial effect of agitation is to diminish the size of mycelial aggregates, making oxygen more easily accessible to the cell (Jafari *et al.*, 2007).

In this study, the effect of agitation and aeration on xylanase production by *Aspergillus niger* SS7 in 3L bioreactor in single and dual turbines systems were investigated to increase xylanase production.

3.1. Xylanase production in mono-agitator conditions

The effects of aeration rate and agitation speed on xylanase production were studied in fermenter mounted with one turbine DT6.

The following aeration rates were investigated: 0.5, 1 and 1.5 vvm. Different agitation speeds were tested: 100, 200, 300, 400, and 500 rpm which correspond the following peripheral speeds (πND) 0.26, 0.52, 0.79, 1.05 and 1.31 m/s respectively. The results obtained after 96 hours of culture at different agitation speeds for aeration rates of 0.5, 1 and 1.5 vvm shown in (Figures 13, 14 and 15).

Table 2: Effect of agitation rates on xylanase production. The aeration rate was 0.5 vvm.

Every value in these tables represents 3 replicates.	Peripheral speed (m/s)	Activity (IU/ml)
	0.26	108.32
	0.52	136.4
	0.79	94.89
	1.05	52.23
	1.31	44.21

$$L.S.D_{0,05} = 6.58$$

$$L.S.D_{0,01} = 9.35$$

Table 3: Effect of agitation rates on xylanase production. The aeration rate was 1 vvm.

Peripheral speed (m/s)	Activity (IU/ml)
0.26	158.9
0.52	162.16
0.79	139.25
1.05	59.68
1.31	54.48

$$L.S.D_{0,05} = 8.19$$

$$L.S.D_{0,01} = 11.65$$

Table 4: Effect of agitation rates on xylanase production. The aeration rate was 1.5 vvm.

Peripheral speed (m/s)	Activity (IU/ml)
0.26	154.72
0.52	140.7
0.79	116.82
1.05	101.36
1.31	53.2

$$L.S.D_{0,05} = 7.96$$

$$L.S.D_{0,01} = 11.32$$

The results of statistic analysis (**Table 2, Table 3, Table 4**) showed that xylanase production decreased significantly when agitation rates increased. Agitation rates(0.26-0.52m/s) gave a high production of xylanase for aeration rates 0.5,1 and1.5vvm

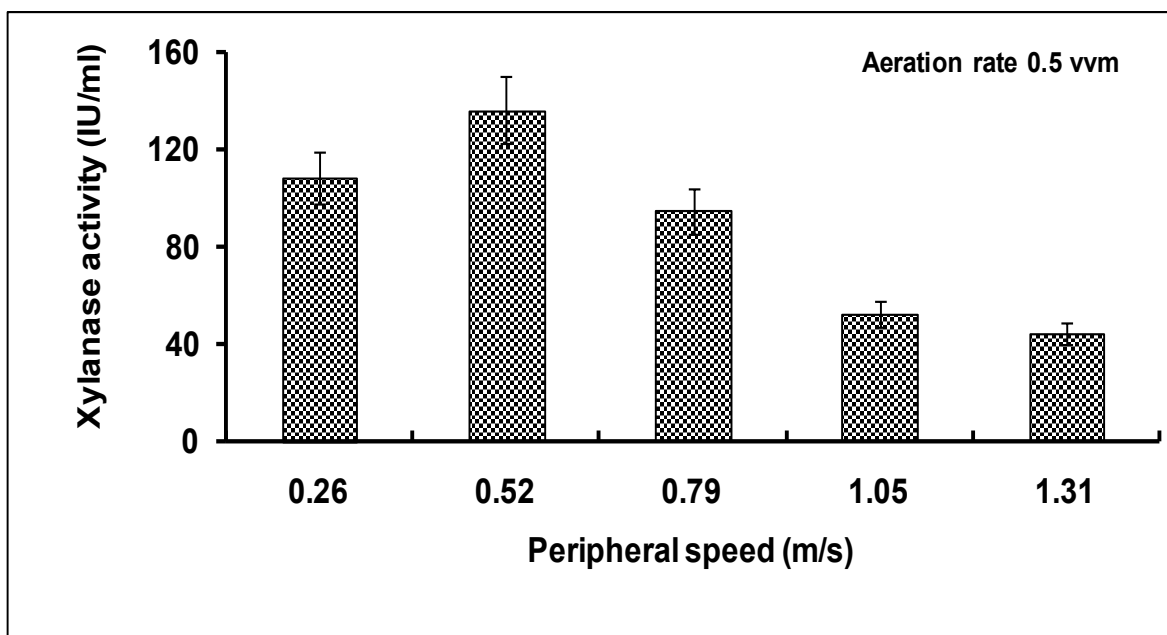


Fig.13. Xylanase production (IU/ml) after 96 h in batch cultivation of *Aspergillus niger* SS7 in a 3-L bioreactor fitted with DT6, at different peripheral speeds. The aeration rate was 0.5 vvm.

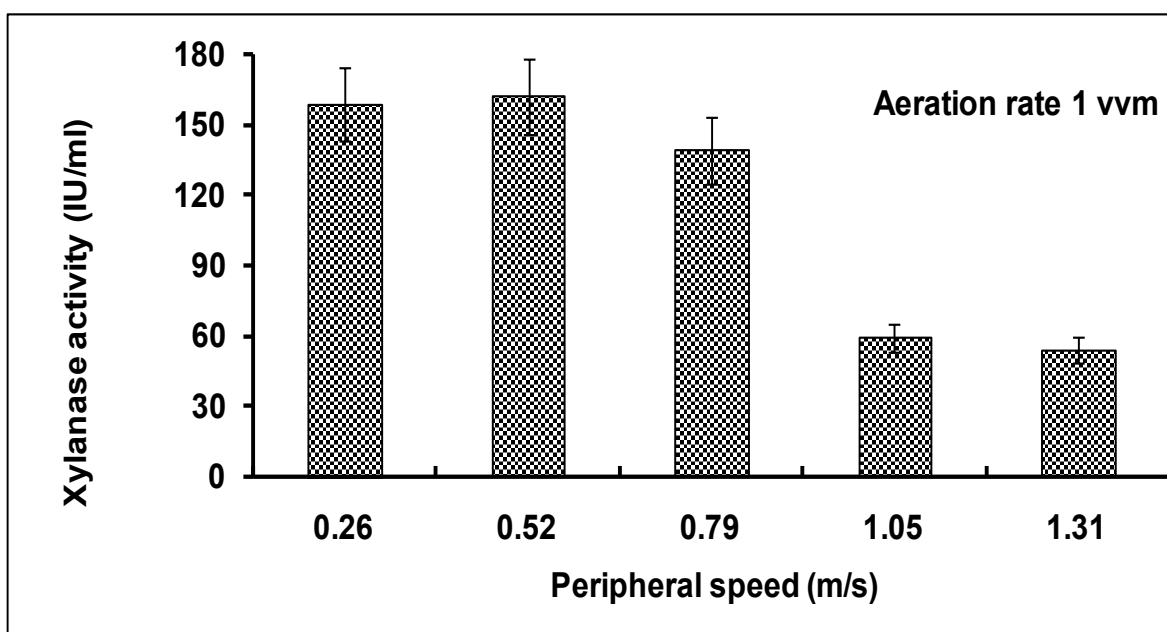


Fig.14. Xylanase production (IU/ml) after 96 h of culture of *Aspergillus niger* in a 3-L bioreactor fitted with DT6, at different peripheral speeds. The aeration rate was 1 vvm.

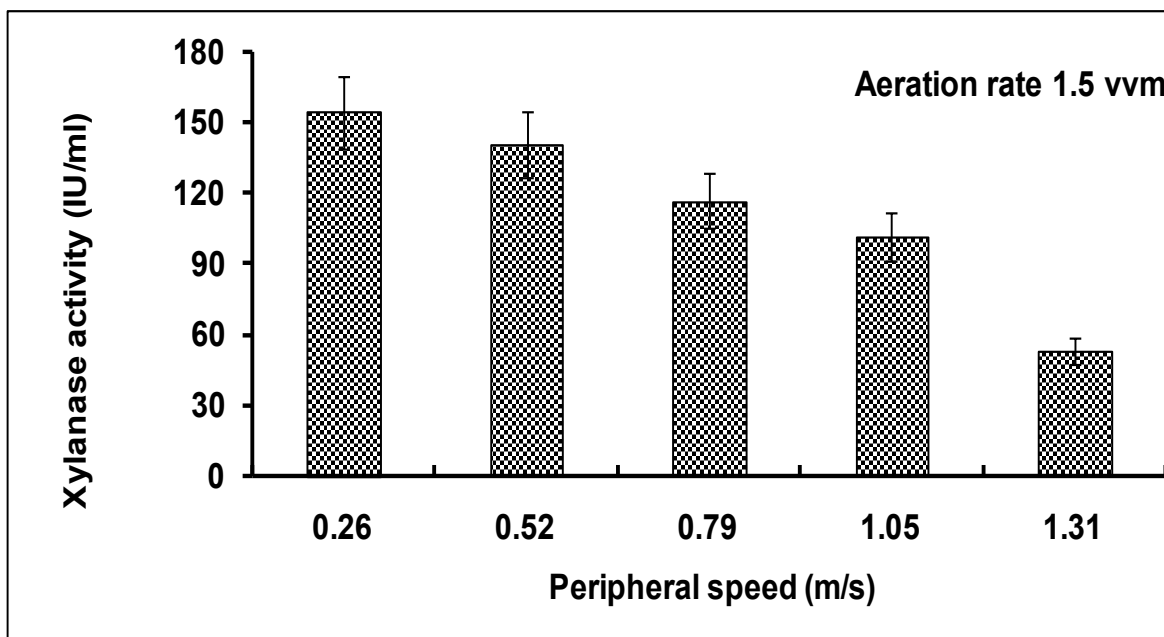


Fig.15. Xylanase production (IU/ml) after 96 h of culture of *Aspergillus niger* in a 3-L bioreactor fitted with DT6, at different peripheral speeds. The aeration rate was 1.5 vvm.

The optimum xylanase production of 162 (IU/ml) was obtained in 1 vvm aeration rate at 0.52 m/s peripheral speed.

The decrease in xylanase production at high stirrer speeds has been attributed to the effect of hydrodynamic stress, which may cause hyphal disruption and leakage of intracellular compounds (**Chipeta *et al.*, 2008**). The harmful effect of the shear forces as a result of agitation intensity has also been reported to cause morphological as well as physiological changes in some filamentous fungi, resulting in decreased enzyme production (**Palma *et al.*, 1996**; **Lenartovicz *et al.*, 2002**; **Cho *et al.*, 2002**; **Techapun *et al.*, 2003**). That damage probably occurred in the regions of high shear, near the tips of the impeller blades (**Amanullah *et al.*, 2000**).

At all peripheral speeds tested, the xylanase production in 1.5 vvm was higher than 0.5 vvm aeration rate. That indicates that low aeration rate has more negative effect on xylanase production than high aeration rate. However, it has been reported that improving aeration rate had a positive effect on enzyme production from aerobic microorganism (**Bakri *et al.*,**

2002; Jafari *et al.*, 2007; El-Enshasy *et al.*, 2008).

At 0.26 and 0.52 m/s peripheral speeds, improvement in xylanase production was observed when aeration rates increased from 0.5 vvm to 1 vvm as shown in (Figures 16 and 17). Whereas the increase in the aeration rates to 1.5 vvm caused a reduction in enzyme production.

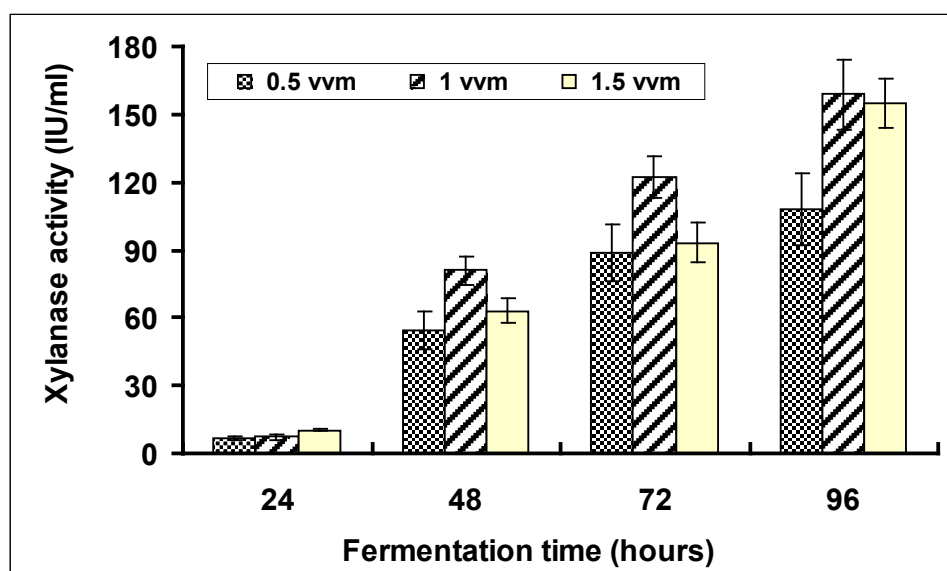


Fig.16. Evolution of xylanase production (IU/ml) during batch culture of *Aspergillus niger* in a 3-L bioreactor fitted with DT6. The peripheral speed was 0.26 m/s, and the aeration rates were 0.5, 1 and 1.5 vvm.

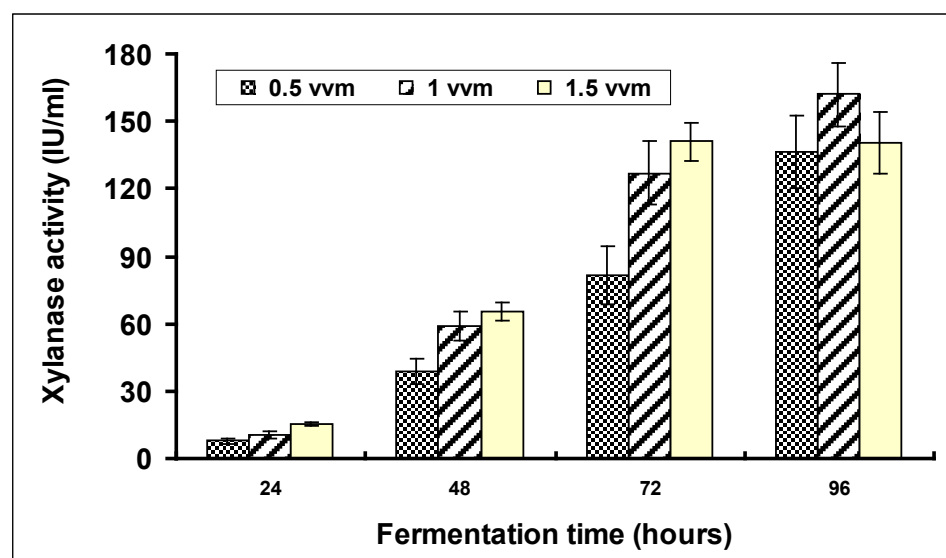


Fig.17. Evolution of xylanase production (IU/ml) during batch culture of *Aspergillus niger* in a 3-L bioreactor fitted with DT6. The peripheral speed was 0.52 m/s, and the aeration rates were 0.5, 1 and 1.5 vvm.

The results of statistic analysis (**Table 5**) showed that xylanase production increased when aeration rates increased from 0.5 vvm to 1 vvm. Whereas the increase in the aeration rates to 1.5 vvm caused a insignificant reduction in enzyme production at peripheral speed 0.26 m/s. But, at peripheral speed 0.52 m/s, the reduction was significant.

Table 5: Effect of aeration rates on xylanase production at 0.26 and 0.52 m/s peripheral speeds after 96h.

Peripheral speed (m/s)	Aeration (vvm)	Activity (IU/ml)	L.S.D	
			5%	1%
0.26	0.5	108.32	6.42	9.73
	1	158.9		
	1.5	154.72		
0.52	0.5	136.4	5.55	8.41
	1	162.16		
	1.5	140.7		

These results agree well with the results reported by (**Reddy *et al.*, 2002**), whereby, at an aeration rate higher than 0.75 vvm, there was a decreased xylanase production by *thermomyces lanuginosus* SSBP.

Similar results were obtained with *Thermomyces lanuginosus* RT9 by **Hoq *et al.*, (1994)** who observed an increase in β -xylanase production with increasing aeration, where obtained highest xylanase production at 1vvm. However, when aeration was increased to 1.5 vvm, β -xylanase titre dropped 1.5-fold.

Techapun *et al.*, (2003) have also found that an increase in the aeration rate from 0 to 1 vvm increased the xylanase production from *Streptomyces sp.* Ab106. However, when the aeration rate was increased to more than 1 vvm, xylanase production gradually decreased.

At 0.26 m/s, xylanase production for 1vvm was higher than that for 1.5vvm after 48 h of culture as shown in (Figure 16). Whereas at 0.52 m/s, xylanase production for 1.5vvm was higher than that for 1vvm until 72 h of culture. After that, xylanase production increased for 1vvm and decreased for 1.5vvm.

In the presence of oxygen in excess, an alkoxyl radical is obtained which leads to the peptide bond cleavage (**El Enshasy *et al.*, 2008**). Similar effect of high aeration rate on xylanase activity has been reported by (**Cabisco *et al.*, 2000**) who found that the higher enzyme inactivation in highly aerated culture may be due to irreversible oxidation of amino acid residues of the enzyme structure.

3.2. Xylanase production in bi-agitator conditions

The effects of aeration rate and agitation speed on xylanase production were studied in fermenter mounted with two turbines DT6 – DT6.

Xylanase production was determined at 0.26 and 0.52 m/s peripheral speeds which were the best for xylanase production in mono agitator system. The following aeration rates studied are 0.5, 1 and 1.5vvm. The results are shown in (Figure 18).

The results of statistic analysis (Table 6, Table 7) showed that xylanase production increased when aeration rates increased from 0.5 vvm to 1 vvm. Whereas the increase in the aeration rates to 1.5 vvm caused a reduction in enzyme production.

Table 6: Effect of aeration rates on xylanase production. The peripheral speed was 0.26m/s.

Aeration rate (vvm)	Activity (IU/ml)
0.5	162.71
1	182.11
1.5	165.34

$$L.S.D_{0,05} = 4.64$$

$$L.S.D_{0,01} = 7.03$$

Table 7: Effect of aeration rates on xylanase production. The peripheral speed was 0.52 m/s.

Aeration rate (vvm)	Activity (IU/ml)
0.5	119.7
1	146.5
1.5	131.37

$$L.S.D_{0,05} = 3.43$$

$$L.S.D_{0,01} = 5.19$$

In bi- agitator conditions, xylanase production was higher at 0.26 m/s than 0.52 m/s for 0.5, 1 and 1.5 vvm. The maximum xylanase production was obtained at 0.26 m/s for 1vvm (182 IU/ml) after 96 h of culture. At 0.52 m/s, higher shear force caused lower xylanase production (Figure 18).

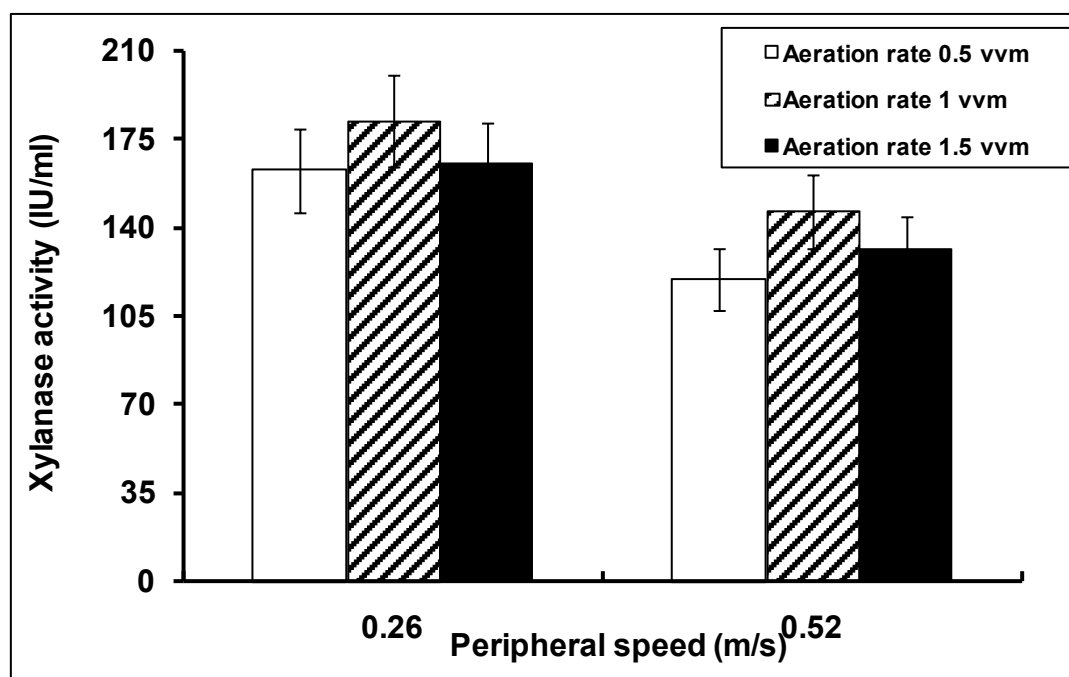


Fig.18. Evolution of xylanase production (IU/ml) during batch culture of *Aspergillus niger* in a 3-L bioreactor fitted with two DT6 at different aeration rates. The peripheral speeds were 0.26 and 0.52 m/s.

In bi-agitator system, at low peripheral speed 0.26 m/s, the xylanase production was enhanced by 13% in comparison with mono agitator systems for an aeration rate of 1 vvm (Figure 19).

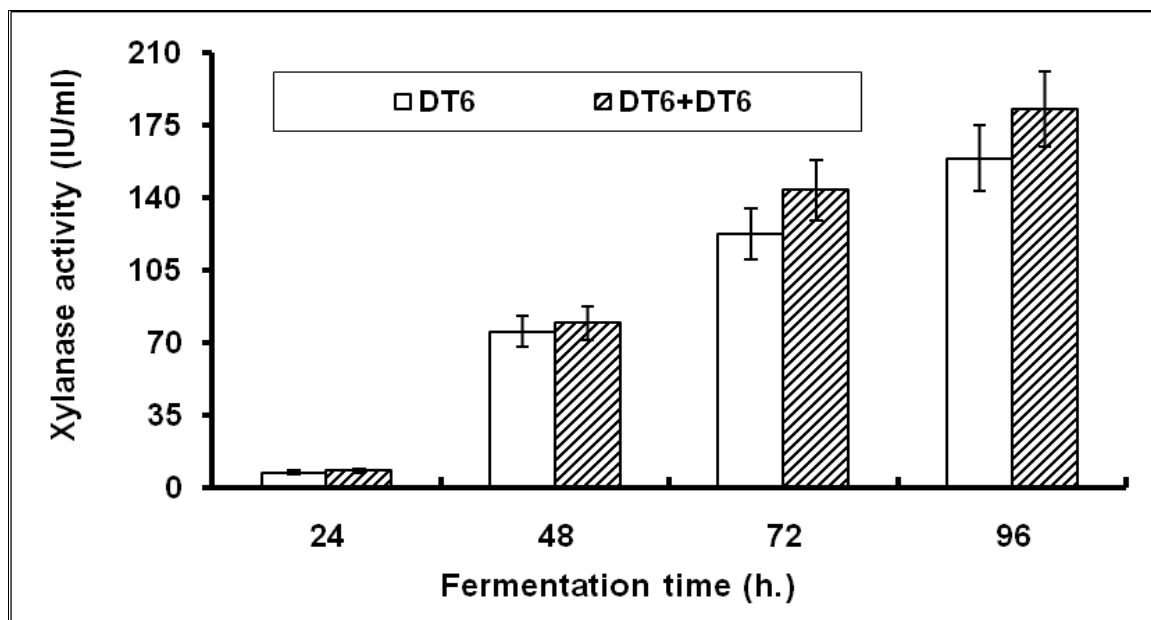


Fig.19. Evolution of xylanase production (IU/ml) during batch culture of *Aspergillus niger* in a 3-L bioreactor fitted with one or two DT6 at 1 vvm and 0.26 m/s peripheral speed.

The results of statistic analysis(**Table 8**) showed that xylanase production in bi agitator was better than mono agitator.

Table 8: Comparison between mono agitator and bi agitator system at 1 vvm and 0.26 m/s peripheral speed.

Time (h)	System	Activity (IU/ml)	L.S.D	
			5%	1%
24	mono agitator	7.2	0.92	0.93
	bi agitator	7.8		
48	mono agitator	75.3	8.25	8.26
	bi agitator	79.25		
72	mono agitator	122.46	9.32	14.12
	bi agitator	143.6		
96	mono agitator	158.9	4.26	6.44
	bi agitator	182.11		

Multiple impeller systems are found to be more efficient in mass transfer than the single-impeller. Single-impeller stirred tanks are often criticized for the uneven distribution of shear and energy dissipation which are known to be harmful, especially to the microorganisms in the bioreactors. Hence, multiple impeller systems will be favored where microorganism is sensitive to shear effect (**Gogate *et al.* 2000**).

3.3. Xylanase production in batch and continuous culture

In this study, we tested batch and continuous culture for evaluation the xylanase production from *Aspergillus niger* SS7 using the optimum conditions of agitation and aeration selected from pervious experiments.

Continuous culture systems have been widely used to culture microbes for industrial and research purposes (Toda *et al.*, 1979; Kim *et al.*, 2004; Yousten and Wallis, 1987; Baptisa-Neto *et al.*, 2005).

Very few studies have been reported on continuous production of xylanases with bacteria and fungi; mainly because of the problems associated with media containing insoluble xylan substrates (Röthlisberger *et al.*, 1992).

Therefore, Corn cob hulls hyrolysates were used as a soluble inducer for xylanase production in batch and continuous cultures. The corn cob hulls extract obtained from soaking them in water at 30°C, as a carbon source for xylanase induction.

Batch and continuous culture were carried out on a corn cob hulls hyrolysates medium and extra cellular xylanase activity were determined during the both culture processes and the results are shown in (Figures 20 and 21).

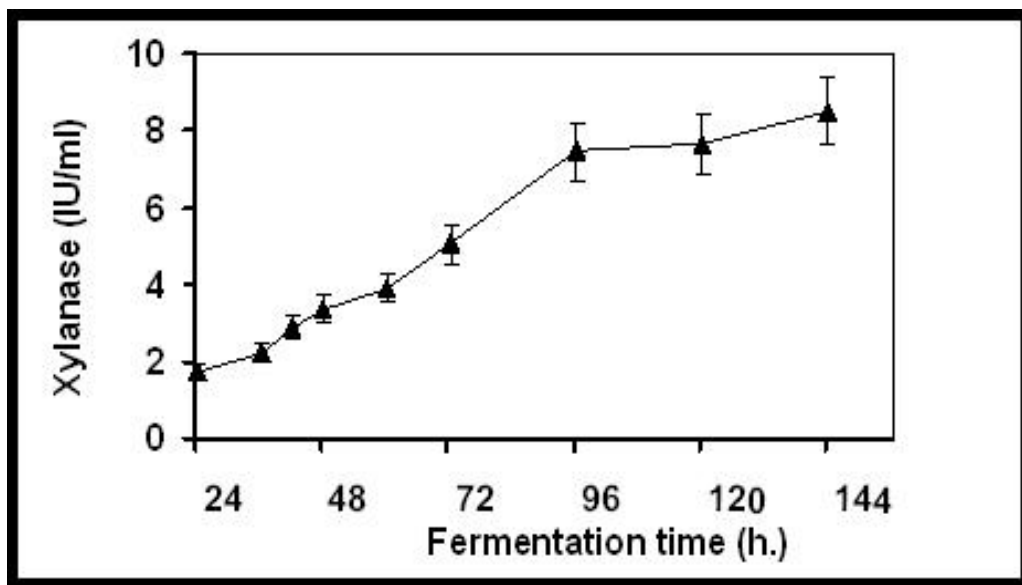


Fig.20. Evolution of xylanase production (IU/ml) during batch liquid culture of *Aspergillus niger* in a 3-L bioreactor fitted with two DT6 at 1 vvm and 0.26 m/s peripheral speed.

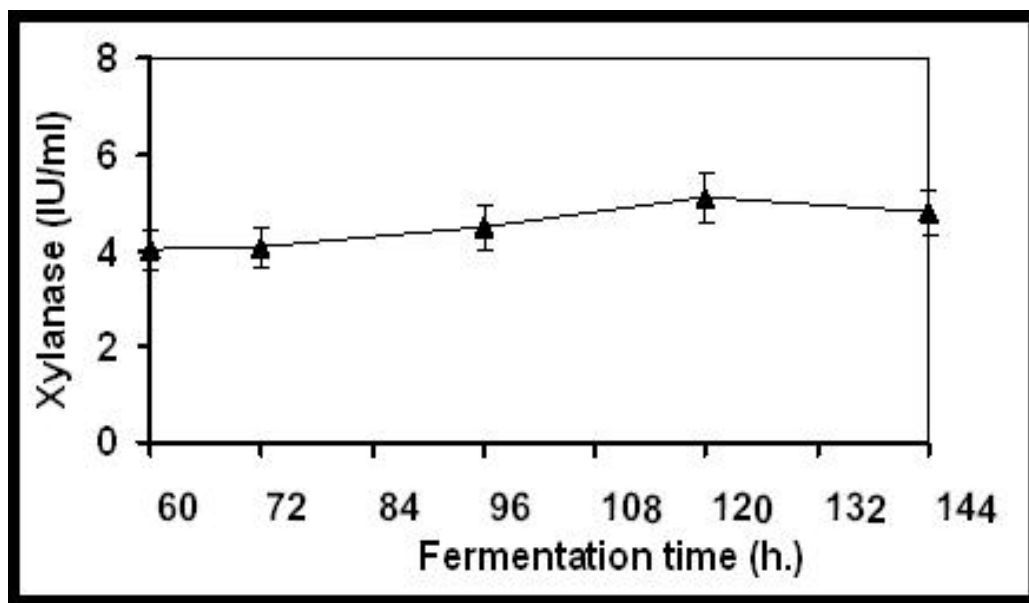


Fig.21. Evolution of xylanase production (IU/ml) during continuous liquid culture of *Aspergillus niger* in a 3-L bioreactor fitted with two DT6 at 1 vvm and 0.26 m/s peripheral speed.

In batch culture, xylanase production after 144h of culture was 8, 53 IU/ml using corn hulls hydrolysate as shown in (Figure 18) Whereas, in continuous culture, the xylanase production during stationary phase was 5,1 IU/ml using dilution rate of 0.05 h^{-1} as shown in (Figure 21).

The results of statistic analysis(**Table 9**) showed that xylanase productivity in continuous culture was better than batch one.

Table 9: Comparison between continuous and batch culture at 1 vvm and 0.26 m/s peripheral speed.

Time (h)	System	Productivity (IU/ml/h)	L.S.D	
			5%	1%
60	batch	0.065	0.01	0.011
	continuous	0.202		
72	batch	0.070	0.01	0.015
	continuous	0.205		
96	batch	0.078	0.004	0.006
	continuous	0.225		
120	batch	0.064	0.007	0.01
	continuous	0.255		
144	batch	0.059	0.008	0.012
	continuous	0.240		

In comparison between batch and continuous processing to produce xylanase from *Aspergillus niger* SS7 as shown in (Figure 22). It was observed that, in spite of, xylanase production in batch culture was higher than that in continuous culture, the enzyme productivity obtained in continuous culture 0.255 IU/ml/h was significantly greater than that obtained in batch culture 0.077 IU/ml/h by approximately 3.5 times. Many researchs have reported the increase of the enzyme productivity in the continuous culture compared with the batch one (Mamo and Gessesse, 2000; Cheng *et al.*, 1989; Montesinos *et al.*, 1996).

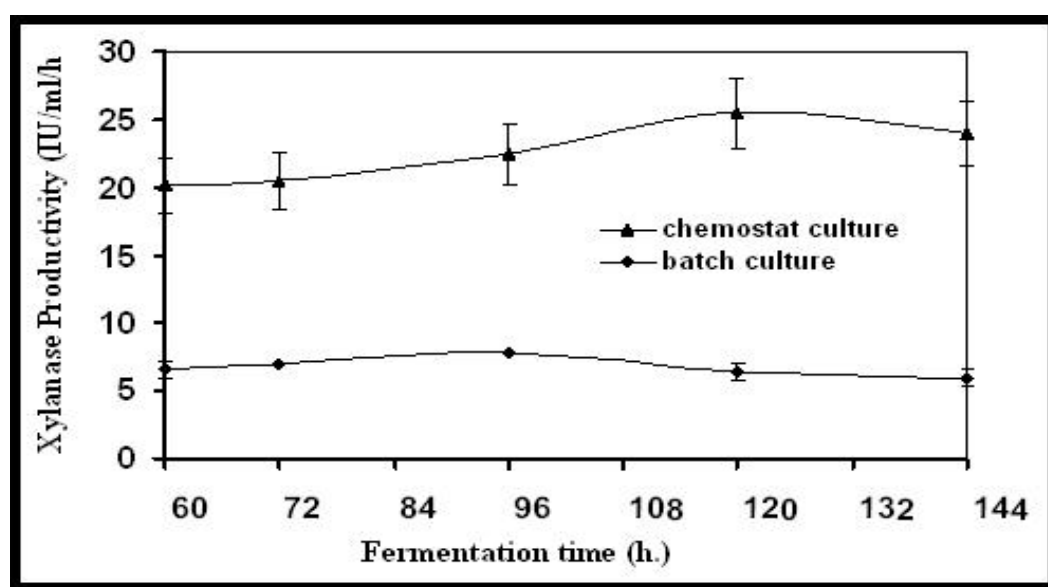


Fig.22. Evolution of xylanase productivity (IU/ml/h) during batch and continuous liquid culture of *Aspergillus niger* in a 3-L bioreactor fitted with two DT6 at 1 vvm and 0.26 m/s peripheral speed. The xylanase productivity values presented in figure equal to obtained values multiple 100.

Conclusions

The effects of aeration rate and agitation speed on xylanase production were studied as single and dual-impeller DT6 systems.

Mixing is very crucial for the maximum productivity in microbial fermentation and it could be achieved by means of aeration and agitation. But agitation at higher stirring speeds may cause disruption of free cells in the reactor by shear forces and formation of vortex which may result in poor mass transfer (oxygen/substrate). Therefore, it is important to provide optimum combination of aeration and agitation in free cell batch bioreactor operation. From the results of present investigation, it could be concluded that the agitation and aeration have significant effects on xylanase production.

- 1- Submerged culture:** The maximum xylanase production of *Aspergillus niger* SS7 in submerged culture was obtained at 1vvm and 0.26 m/s speed peripheral using two turbines (DT6-DT6). In bi-agitator system, at low peripheral speed 0.26 m/s, the xylanase production was enhanced by 13% in comparison with mono agitator systems for an aeration rate of 1 vvm.
 - multiple impeller systems will be favored where microorganism is sensitive to shear effect.
- 2- Liquid culture:** The results obtained from liquid culture showed that batch liquid culture gave xylanase activity higher than continuous culture after 144h. But, the xylanase productivity of *Aspergillus niger* SS7 was approximately 3.5 times higher in chemostat culture than in batch culture on the same medium.
- 3-** From all results, we observed that submerged culture was better than liquid culture and gave a significant improvement to xylanase production.

Recommendations

- 1-** Improving the process of extracting hydrolysate by using other temperature degrees.
- 2-** Optimizing the continuous culture system by using different dilution rates.
- 3-** Producing enzymes in large bioreactors according to the optimal conditions we obtained it.
- 4-** Testing other types of impellers at different rates of aeration and agitation and comparing them with the results obtained by using Rushton impellers.
- 5-** Showing interest in enzyme production on the industrial level in Syria.

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