# SCREENING Of XYLANASE PRODUCER FROM SOIL

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# DECLARATION

I declare that this thesis entitled “Screening of xylanase producer from soil.” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

Signature :…….

Name of Candidate : Lim Ting Chin

Date : November 20$^{th}$ , 2006

# DEDICATION

*Special Dedication to my family members that always love me,*
*My friends, my fellow colleague*
*and all faculty members*

*For all your Care, Support and Believe in me.*

*Sincerely*
*Lim Ting Chin*

## ACKNOWLEDGEMENTS

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My fellow colleagues should be noted for their support. Thank you for the time sacrificed to accompany me when I'm down and the time we share our University life.

## ABSTRACT

The purpose of this study is to screen and to characterize the bacterial produce xylanase. The scopes are included screening, characterization of bacterial and xylanase and to study the effect of carbon and nitrogen source on the enzyme production ezyme. This research is start from the collect soil sample and screens the best xylanase producer from sample. After this, characterize xylanase producer in morphology. Besides this, need to characterize enzyme xylanase in the different temperature and pH ambient in term xylanase activity and protein activity. At the end, study the xylanase activity in various carbon and nitrogen source in volume fraction using Lowry method.The optimum xylanase activity.was obtained pH 7and 70$^{o}$C is 13 U.And xylan is the best carbon source and organic nitrogen source is the best nitrogen source for production xylanase using this xylanase producer.

# ABSTRAK

Tujuan utama untuk kajian ini ialah memencilkan dan mencirikan bakteria yang menghasilkan enzim xylanase. Dan skop termasuk pemencilan, pencirian bakteria dan enzim xylanase serta kajian kesan sumber karbon dan sumber nitrogen. Kajian ini dimulakan dengan pengumpulan sampel yang diambil daripada tanah dan memencilkan sampel untuk mendapat bakteria yang terbaik menghasilkan enzim xylaanse. Selepas ini, kaijian ini diteruskan dengan eksperimen pencirian terhadap hasil pemencilan. Di samping itu, kajian ini juga memerlukan pencirian enzim xylanase dalam pelbagai suhu dan pH berdasarkan aktiviti enzim xylanase dan aktiviti protein. Akhirnya, kajian ini diteruskan dengan mengkaji bakteria tersebut dalam pelbagai sumber karbon dan sumber nitrogen.dengan pecahan composis. Keputusan kajian ini ialah mendapati aktiviti enzmi xylanase tertinggi pada suhu 70$^{o}$C dan pH 7 (13U). Dan sumber karbon dan nitrogen terbaik untuk penghasilan enzim xylanase adalah xylan dan organik sumber nitrogen.

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| | | |
|---|---|---|
| $^{o}C$ | | degree Celsius - |
| pH | - | potential hydrogen |
| kDa | | kilo Dalton |
| m | - | Micro |
| ml | | milliliters |
| U | | amount of enzyme release 1 mol xylose /minutes/milliliters |
| % | | percentage |
| rpm | - | radius per minutes |
| w | | weight |
| v | | volume |
| h | | hours |
| min | | minutes |
| g | | gram |
| M | | molar |
| L | | liter |

## LIST OF APPENDICES

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

### 1.1.1 Screening Xylanase Producer From Soil

Xylan is the most abundant hemicellulose , ranking second only to cellulose and constitutes up to 35% of the total dry weight of higher plants.(Dekker, RFH, 1985) Xylan has a complex structure consisting of β-1,4-linked xylose residues in the backbone (Coughlan *et al.*, 1993). Endoxylanases (1,4-b-D-xylan xylohydrolase; EC 3.2.1.8)are the main enzymes involved in xylan hydrolysis. Endoxylanase degrade plant cell wall polysaccharides by cleaving internal glucosidic bonds of xylan. (Coughlan *et al.*, 1993). From the biotechnological standpoint, xylanases have applications in production of ethanol, aroma, fruit juices, animal feed and in baking, textile, paper and pulp industries (Bhat, 2000).

Dye-labeled method substrates enable the direct selection of appropriate activities by incorporating them into the growth media.(Fulop *et al.*, 1997) When the substrate is hydrolyzed, dye labeled diffuse from the colony zone that becomes visible as a halo in which the label is (nearly) absent. The solubilization of substrates particle and the formation of haloes, represent the activities of enzymes (Leonid N.Ten *et al.*, 2004) As a result, the plate assay is simple, rapid and well adapted for screening of large number of samples.(Ten *et al.*, 2004)

There are a few organisms that have been reported such as *B.Circulans* (Heck *et al.*, 2005), *Enterobacter* sp (Khandeparkar *et al.*, 2004) and *Streptomyces* spp. (Rawashdeh *et al.*, 2005) are xylanase producer. *B.Circulans* and *Enterobacter* sp are isolate from aquatic ambient but *Streptomyces* spp is isolate from soil samples..

Generally, polysaccharides degrading enzymes are assayed by determining the amount of reducing sugars produced during enzymic digestion(Miller,G.L,1959).Dye labeled substrates can also be used for the analysis of cellulases, xylanase and mannanase (Biely *et al.*,1985)

## 1.2 Problem Statement

Economically feasible xylanase production is can be achieve using microorganisms that are capable of utilizing inexpensive carbon source.

## 1.3 Objectives

The objective of this study is to screen and to characterize the bacterial produces xylanase.

## 1.4 Scopes

1) Screening of xylanase producer.
2) Characterization xylanase and microorganisms
3) To study the effect various carbon and nitrogen source for xylanase production.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Hemicelluloses

Hemicelluloses have the property of being soluble in dilute alkali. They are usually classified according to the sugar residues present xylan, mannans, arabinans and galactans. Most hemicelluloses do not occur as homopolysaccharides but as heteroploysaccarides, containing different types of sugar in the backbone chain and in the side chain or appendages. These may be D-xylose, L-arabinose, D-mannose acid, O-acetyl groups or feruloyl and coumaryl ester linked via L-arabinose residues to the backbones.( Coughlan)

### 2.1.1 Xylan

Xylan is the most abundant hemicelluloses, ranking second only to cellulose and constitutes up to 35% of the total dry weight plants. Xylans consist of a homopolymeric backbone of 1,-linked α-D-xylopyranose units depending on its origin the backbone may be substituted (Coughlan) The xylans are the major hemicelluloses of many plant materials where they often contribute to the rigidity of plant cell walls. Wood xylans are either O-accetyl-4-O-methylglucuronoxylans (in hardwoods) or arabino-4-O-methylglucuronoxylans (in softwoods). The degree of

polymerization hardwoods xylan (150-200) is higher than that of softwoods (70-130)(Gray F)

### 2.1.2 Xylanase

Xylanase are typical endo acting enzyme and attack the xylan chain in a random manner, causing a decrease degree polymerization of the substrate and liberating shorter oligomers, xyloboise and even xylose. The mode of action of different xylanase and hydrolysis products vary according to the source of the enzyme.( Coughlan) Xylanase consists of 190 amino acids and has a molecular weight of 21 kD. Xylanases belong to the glucanase enzyme family and are characterized by their ability to break down various xylans to produce short-chain xylo-oligosaccharides. Xylanase readily crystallizes in ammonium sulfate and sodium potassium phosphate across pH 3.5 to 9.0. Xylanase can also be crystallized with other salts, polymers, and organic solvents. Xylanase solubility increases with increasing temperature in moderate concentrations of ammonium sulfate. Xylanase solubility in phosphate buffer (pH 9) decreases in the temperature range of 0 to 10 $^{o}$C but remains constant in the range of 10 through 37 $^{o}$C. Xylanase has been extracted from many different fungi and bacteria. It is commonly used in animal feeds, paper production, and food production.(Greatvista, 2006)

### 2.1.3 Xylan hydrolysis reaction

Xylanase effectively convert xylan into xylooligosaccharides (mainly xylobiose, which is a major product of xylan degradation). Xylobiose is the smallest molecule to induce the production of xylanolytic enzymes ( Mensfield *et al.*) Xylanase attacks the main chain , most easily and therefore most rapidly at non-substituted regions, generating non-substituted and branched or esterified oligosaccharides. During xylan hydrolysis, xylanase acts synergistically with accessory enzyme. Xylanase release substituted xylooligoaccharide which are more

readily diffusible and more favorable substrates for accessory enzyme. On the other hand, the removal of side chains substituents by accessory enzyme creates new sites on the main chain for productive binding with xylanase. (ShamDMensfield *et al.*) Xylanase activity is highly dependent on the presence of debranching enzymes. The highest affinity was found for the triouronic acid ( 4-O-Me-Glc A)$X_2$, the nature intermediate product of xylanase hydrolysis of many fungal and bacterial xylanase.

**2.2 Xylanase producer**

Actinomycetes are gram positive bacterial found in large number of habitats. Soil, composts and decaying plants are their natural environmental, where they play an important role in the degradation of lignocellulosic materials. Since hemicellulose represent an important substrate in these habitats, actinomycetes can be considered as a potential source of hemicellulolytic enzyme. Strains with hemicellulolytic activities have been isolated from different soil compost, animal manure, hay or decaying woods. Hemicellulose degrading enzyme are produced by both mesophilic, thermotolerant or thermophilic actinomycetes. Most of the information available on hemicellulose degrading enzyme from actinomycetes has been obtained from studies with Streptomycetes (Coughlan) Table 2-1shows example some actinomycetes produce xylanase. Table 2-2 shows properties of actinomycete xylanase**.** Table 2-3 shows example of microorganisms Xylanase producer.

**Table 2-1: Example some actinomycetes produce xylanase(Coughlan)**

| Producing Strain | Reference |
|---|---|
| *Actinomadura sp.* | Zimmermann *et al.*(1988) |
| *Cellulomonas sp.* | Peiris *et al.*(1982) |
| *Cellulomonas flavigena* | Rajoka & Mal.ik (1984) |
| *Cellulomonas uda* | Rapp & Wagner(1986) |
| *Chainia sp.* | Srinivasan *et al.*(1984) |
| *Microbispora rosea* | Kusakabe *et al.*(1969) |
| *Microbispora bispora* | Bal.l& McCarthy(1988) |
| *Micromonospora chal.cea* | Sorensen(1957) |
| *Micromonospora sp.* | Ball &McCarthy(1988) |
| *Pseudonocardia thermophila* | Zimmermann *et.*(1988) |
| *Saccharomonospora viridis* | McCarthy *et al.*(1985) |
| *Stmy. albus* | Sorensen(1957) |
| *Stmy. xylophagus* | Iizuka & Kawaminami (1965) |
| *Streoptomyces spp.* | Kusakabe *et al.*(1969) |
| *Stmy. viridochromogenes* | Kusakabe *et al.*(1969) |
| *Stmy. mistakaensis* | Kusakabe *et al.*(1969) |
| *Stmy. albogriseolus* | Kusakabe *et al.*(1969) |
| *Stmy. coelicolor* | Kusakabe *et al.*(1969) |
| *Stmy. purpurascens* | Kusakabe *et al.*(1969) |
| *Stmy. scabies* | Kusakabe *et al.*(1969) |
| *Stmy. netropsis* | Kusakabe *et al.*(1969) |
| *Stmy. shindenensis* | Kusakabe *et al.*(1969) |
| *Stmy. olivaceus* | Iizuka & Kawaminami (1969) |
| *Stmy. olivochromogenes* | Kusakabe *et al.*(1969) |
| *Stmy. afghaniensis* | Loginova *et al.*(1981) |
| *Stmy. flavogriseus* | Ishaque & Kluepfel(1981) |
| *Stmy. exfoliatus* | Sreenanth & Joseph(1982) |
| *Stmy. osteogriseus* | Park & Toma (1982) |
| *Stmy. albosporus* | Sreenath & Joseph (1983) |
| *Stmy. cyaneus* | Sreenath & Joseph (1983) |
| *Stmy. caiusiae* | Sreenath & Joseph (1983) |
| *Streptomyces sp.* | Marui *et al.*(1985) |
| *Stmy. viridosporous* | Deobald & Crawford (1987) |
| *Streptomyces spp.* | Yasui *et al.*(1988) |
| *Streptomyces spp.* | Ball & McCarthy (1988) |
| *Stmy. lividans* | Kluepfel *et al.*(1986) |
| *Stmy. cyaneus* | Zimmermann *et al.* (1988) |
| *Stmy. rubiginosus* | Taiana *et al.*(1989) |
| *Streptosporangium roseum* | Kusakabe *et al.*(1969) |
| *Temo. fusca* | McCarthy *et al.*(1985) |
| *Temo. curvata* | McCarthy *et al.*(1985) |
| *Temo. chromogena* | McCarthy *et al.*(1985) |
| *Thermomonospora sp.* | Ristroph & Humphrey (1985) |

**Table 2-2: Properties of actinomycete xylanase(Coughlan)**

| Producing Strain | pH opt. | Temp opt. | pI | MW (kD) | Ref. |
|---|---|---|---|---|---|
| Chainia sp. | 5.0 | 65 | 8.0 | - | 1 |
| Saccharomonospora viridis | 5.0-8.0 | 60 | - | - | 2 |
| Stmy. xylophagus | 6.2 | 55-60 | - | - | 3 |
| Stmy. flavogriseus(CD45-2) | 6.5 | 50 | - | - | 4 |
| Stmy. exfoliates | 5.5 | 50 | - | - | 5 |
| | 7.0 | 55 | - | - | 5 |
| | 5.5 | 55 | - | - | 5 |
| Streptomyces sp.(KT23) | 5.5 | 55 | 6.9 | 43 | 6 |
| Streptomyces sp.(3137) | 5.5-6.5 | 60-65 | 7.1 | 50 | 7 |
| | 5.0-6.0 | 60-65 | 10.1 | 25 | 7 |
| | 5.0-6.0 | 60-65 | 10.3 | 25 | 7 |
| Stmy. lividans(1326) | 5.5-6.5 | 55-65 | 5.2 | - | 8 |
| Strepomyces sp.(E86) | 5.5-6.2 | 55-60 | 7.3 | 40 | 9 |
| Thermomonospora sp. | 5.5-7.7 | 65-80 | - | - | 10 |
| Temo. curvata | 5.0-8.0 | 60 | - | - | 2 |
| Temo. fusca | 5.0-8.0 | 70 | - | - | 2 |
| Temo. chromogena | 5.0-8.0 | 75 | - | - | 2 |

**Table 2-3: Example of microorganisms Xylanase producer**

| Microorganisms | Source | Xylanase Molecular Weight | Optimum Condition | Optimum Activity | References |
|---|---|---|---|---|---|
| *B. circulans BL53* | Aquatic ambient | - | 80ºC at pH4–7 | 0.95 U/mg protein | MaesJu'lio Xandro Heck *et al.*, 2005. |
| *Arthrobacter* sp. | Aquatic ambient | ~20 kDa | 100 ◦ºC at pH 9 | 240 U/ml in 2 days. | Khandepaprkar *et al.*, 2006. |
| *Bacillus circulans D1* | Aquatic ambient | - | 37 ºC | 0.437U/mg protein in 5 days. | Bocchini *et al.*, 2005. |
| *Enterobacter* sp. *MTCC 5112* | Aquatic ambient | ~43 kDa. - | 50°C at pH 9 | 49 U/ml | Rakhee Khandeparkar *et al.*, March 2005. |
| *Streptomyces* sp. *(strain Ib 24D)* | Soil | 25-50 kDa | 60°C at pH 6.5 | 1447 U/ml in 3 days | Rifaat Rawashdeh, Ismail Saadoun et.al., March 2005. |
| *Paecilomyces themophila J18* | Soil | 25.8 kDa | 75 ºC at pH 7 | 998U/ml in 5days | Lite Li, Hongmei Tian et .al., 2005. |
| *Streptomyces sp. AMT-3 strain* | Soil | - | 55 to 65 ºC at pH 6 | 70.0U/ml in 10 days | Nascimento, R.R.R. Coelho et. al., 2002. |

### 2.2.1 Bacterial

*Streptomyces* bacteria are gram positive, soil inhabiting, and filamentous, with a high G+C content in their DNA (Tsujibo *et al.*, 2004) *Streptomycetes* grew most in pore spaces which were humid and air-filled, and growth was reduced in water-logged pores. Spores of *Streptomycetes* survived for long periods in dry soil.( Williams *et al.*, 1972) There are 3 different journals which show bacterial *B.Circulans* (Heck *et al.*, 2005), *Enterobacter* sp(Khandeparkar *et al.*, 2004) and *Streptomyces* spp. (Rawashdeh *et al.*, 2005) are produce xylanase. *B.Circulans* and *Enterobacter* sp are isolate from aquatic ambient but *Streptomyces* spp is isolate from soil samples.(Rawashdeh *et al.*, 2005). Table 2-4 shows survey of Xylanase producer.

**Table 2-4: Survey of Xylanase producer (bacterial)**

| Microorganisms | Condition | Media | Basic liquid medium | References: |
|---|---|---|---|---|
| B. circulans BL53 | 18 h, 37 ºC and 125 rpm. | basal salt solution (BSS) supplemented with xylan | In (g /L) $MgSO_4$ 0.2, $KH_2PO_4$ 1.0, $K_2HPO_4$ 1.0, $NH_4NO_3$ 1.0, $CaCl_2$ 0.02, and $FeCl_2$ 0.05. | MaesJu' lio Xandro Heck *et al.*, 2005 |
| *Enterobacter* sp. *MTCC 5112* | At 50 °C and pH 9.0 for 48 h | basal salt solution (BSS) supplemented with xylan (0.5%) | In (w/v) NaCl, 30.00 g; KCl, 0.75 g; $MgSO_4$, 7.00 g; $NH_4Cl$, 1.00 g; $KH_2HPO_4$ (10%) 7.00 ml; $KH_2PO_4$ (10%) 3.00 ml | Rakhee Khandeparkar *et al.*, 2004 |
| *Streptomyces sp*. (strain Ib 24D) | At 28°C for 4 days | oat spelt xylan agar medium | 0.1 ml trace salt solution :1 ml/L: $FeSO_4$. $7H_2O$, 0.1 g; $MnCl_2$. $4H_2O$, 0.1 g; $ZnSO_2.7H_2O$, 0.1 g; distilled water, 100 ml | Rifaat Rawashdeh *et al.*, March 2005. |

## 2.3 Effect of carbon source and nitrogen source on the Xylanase production

### 2.3.1 Effect of carbon source

Organisms can be categorized into two broad groups based on their source. Organisms that utilize an inorganic carbon source as their carbon called autotrophs. More precisely, autotrophs make organic compounds from $CO_2$ and thus not need feed on organic compounds from other organisms to acquire carbon. In contrast, organisms called heterotrophs catabolize reduced organic molecules they acquire from other organisms.(Bauman, 2006) Some of the carbon source reported for the Xylanase productions are glucose, xylose, arabinose, xylan, and carboxymethyl cellulose (CMC)( Rawashdeh *et al.*, 2005). The activity response of *Streptomyces albus* and *Streptomyces chromofuscus* grown on different carbohydrate sources is shown in Table 2-5. The highest xylanase production was obtained with 20.0 gram xylan as sole carbon sources for both *Streptomyces strains*.( Rifaat *et al.*) Table 2-6 shows survey of effect of carbon source

**Table 2-5: Example of experimental result effect carbon source (Rifaat *et al.*)**

| Carbon sources 2 % | Maximum enzyme activity U/ml | |
|---|---|---|
| | *Sterptomyces albus* | *Streptomyces chromofuscus* |
| Xylan | 49.92 | 60.49 |
| Cellulose | 15.45 | 16.45 |
| Dextrin | 16.31 | 18.71 |
| Sucrose | 12.22 | 15.52 |
| Maltose | 12.23 | 15.42 |
| Xylose | 10.05 | 12.31 |
| Glucose | 9.01 | 10.26 |

**Table 2-6: Survey of effect of carbon source**

| Microorganisms | Carbon source | Xylanase Activity | References: |
|---|---|---|---|
| *Arthrobacter sp. MTCC 5214* | On birchwood xylan | 121.3 micro U/ml | Khandeparker *et al.*, 2006. |
| *Streptomyces sp. AMT-3 strain* | Larchwood xylan 1% (w/v), | 70.0U/ml | Nascimento *et al.*, 2002. |
| *Bacillus circulans D1* | Bagasse hydrolysates | 8.4 U/mL | Bocchini *et al.*, 2005. |
| *Streptomyces strain Ib 24D* | Larchwood medium. | 1447 U/ml | Rifaat Rawashdeh *et al.*, March 2005. |
| *Aspergillus niger GCBMX-45* | Starch | 2300 U/ml | Ikram-ul-Haq *et al.*, 2002. |
| *Aspergillus niger* | Xylose 0.75% (w/w) | 25.40 U/g | Pang Pei Kheng and Ibrahim, 2005. |

### 2.3.2 Effect of nitrogen source

Another essential element for enzyme production is nitrogen, which contained in many organic compounds, including the amine group of amino acids and as part of nucleotide base. Nitrogen makes up about 14% of dry weight of microbial cells. Nitrogen is often a growth limiting nutrient for many organisms; that is their anabolism cease because they do not have sufficient nitrogen to build proteins and nucleotides.(Robert W.Bauman, 2006) Example of nitrogen source like peptone, urea, $NaNO_3$, yeast extractand $NH_4NO_3$ , and 0.3% , $(NH_4)_2SO_4$. (Pang Pei Kheng *et al.*, 2005) Concerning the inorganic nitrogen sources used as shown in Table 3.2, a slight increase in enzyme activity was observed with $KNO_3$ in both *Streptomyces* species. In case of organic nitrogen sources, a maximum increase was observed with yeast extract rather than with peptone and casein (Table 2-7). In addition, yeast extract showed a maximum production comparable with different inorganic nitrogen sources. ( Rifaat *et al.*) Table2-8 shows survey of effect nitrogen source.

**Table2-7: Example of experimental result effect nitrogen source (Rifaat *et al.*)**

| Nitrogen sources | Maximum enzyme activity U/ml | |
|---|---|---|
| | *Sterptomyces albus* | *Streptomyces chromofuscus* |
| Inorganic nitrogen sources | | |
| $KNO_3$ | 78.78 | 89.27 |
| $NaNO_3$ | 74.49 | 84.26 |
| $NH_4NO_3$ | 69.50 | 80.45 |
| $(NH_4)_2SO_4$ | 76.56 | 87.31 |
| $NH_4H_2PO_4$ | 76.61 | 86.52 |
| Organic nitrogen sources | | |
| Peptone | 63.31 | 74.14 |
| Casein | 40.02 | 51.23 |
| Yeast extract | 79.21 | 90.01 |

**Table2-8: Survey of effect nitrogen source**

| Microorganisms | Nitrogen source | Xylanase Activity | References |
|---|---|---|---|
| *Aspergillus niger* GCBMX-45 | $(NH_4)_2SO_4$ | 2480 U/g | Ikram-ul-Haq *et al.*, 2002. |
| *Aspergillus niger* | $NaNO_3$ | 33.99 U/g | Pang Pei Kheng and Ibrahim, 2005. |

# CHAPTER 3

# METHODOLOGY

## Flowchart

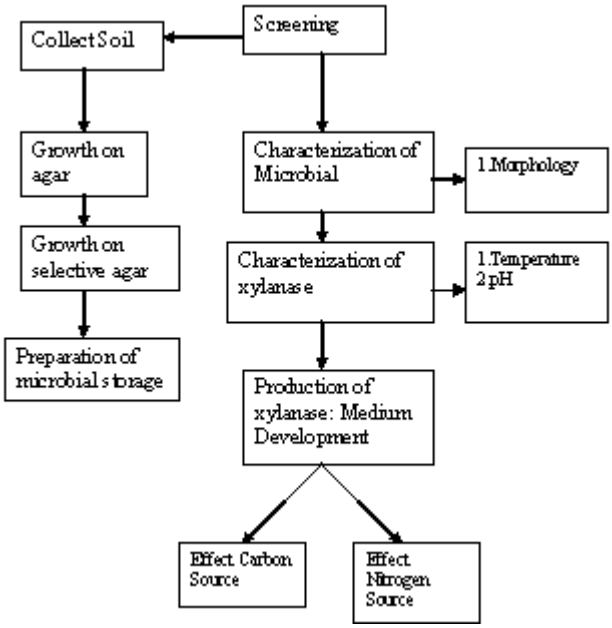


**Figure 3-1: Flow chart for research**

## 3.1 Chemical

All chemicals are obtained from Fluka Company (Aldrich Chemical). All chemicals are in the analytical grade.

## 3.2 Screening

### 3.2.1 Collect soil

Five soil samples are collected from around KUKTEM. After removing approximate. 3 cm of soil from the surface, samples were taken to a depth of 10 cm. Each soil sample was crushed, mixed thoroughly and sieved through a 2 mm sieve to get rid of large debris, and the sieved soil used (Rawashdeh *et al.*, 2005)

### 3.2.2 Primary screening plate

Sub samples of 1 g were suspended in 100 ml sterile distilled water then incubated in an orbital shaker incubator at 28°C with shaking at 140 rpm for 30 min. Mixtures were allowed to settle then serial dilutions up to $10^{-6}$ were prepared. From each dilution, 0.1 ml was taken and spread evenly over the surface of nutrient agar with sterile L-shaped glass rod then incubated at 27°C for 3 days. (Saadoun *et al.*, 2003)

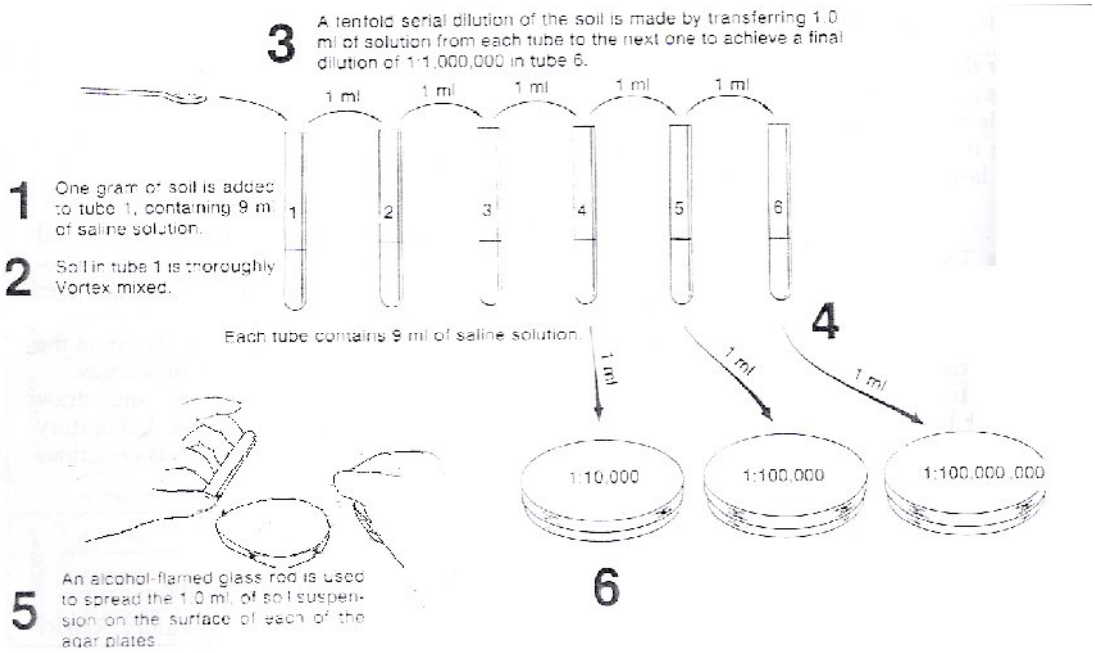


**Figure 3-2: Example Sample dilution and primary screening(J.Benson, 1994)**

### 3.3 Screening

#### 3.3.1 Preparation of Selective agar plate

Prepare the selective agar plate containing $KNO_3$ 2g/l, $K_2HPO_4$ 1g/l, Mg $SO_4$ 0.5g/l, $CaCO_3$ 3g/l, $FeSO_4$ 0.01g/l, xylan 0.80 g/l and agar powder 20g/l. ( Rifaat *et al.*) Then, the colonies were transfer from nutrient agar to the selective agar and incubate for 3 days in 33$^{o}$C.

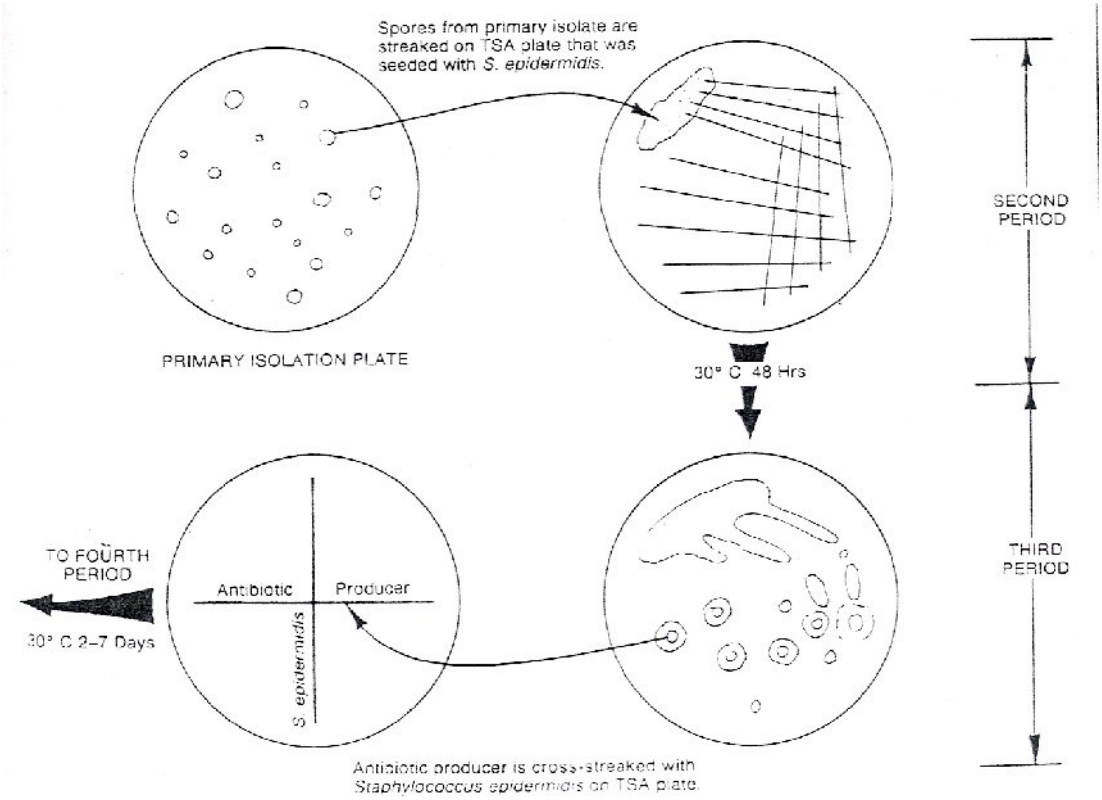


**Figure 3-3: Example second and third screening (J.Benson, 1994)**

### 3.4 Storage of microbial

#### 3.4.1 Frozen agar plugs

Actinomycetes may preserved as agar plugs under 10% glycerol for short term to intermediate term preservation.(Khandeparkar *et al.*, 2004)

10 % (w/w) solution of glycerol is prepared and 2 ml is dispensed into 4 ml capacity Wheaton borosilicate glass vials fitted with rubber lined caps. The vials are autoclaved twice for 45min each. Transfer tubes are autoclaved for 25min. Several plugs are cut and deposited into each of the vials, which are then frozen and stored at -70$^{o}$C.(L.Demain *et al.*, 1999)

### 3.5 Growth medium

#### 3.5.1 Medium for seed culture

The colony from storage suspended in the 250ml Erlenmeyer flask containing 30 ml of a medium composed of nutrient broth (8g/l). The flask were grown at 33$^{o}$C for 36h with rotary shaking 200 rpm.

#### 3.5.2 Production medium

Growth the colony with seed culture from agar plate in the 250ml Erlenmeyer flask containing 30 ml of a medium composed of $KNO_3$ 2g/l, $K_2HPO_4$ 1g/l, Mg $SO_4$ 0.5g/l, $CaCO_3$ 3g/l, $FeSO_4$ 0.01g/l and xylan 0.80 g/l. The flask were grown at 33$^{o}$C for 36h with rotary shaking 200 rpm .

### 3.6 Characterization bacterial

#### 3.6.1 Morphological Study

#### 3.6.2 Morphological colony

Observe the morphology colony in term of physical structure and colour in process incubation on the surface of agar plate about 1 day.

### 3.6.3 Morphological cell

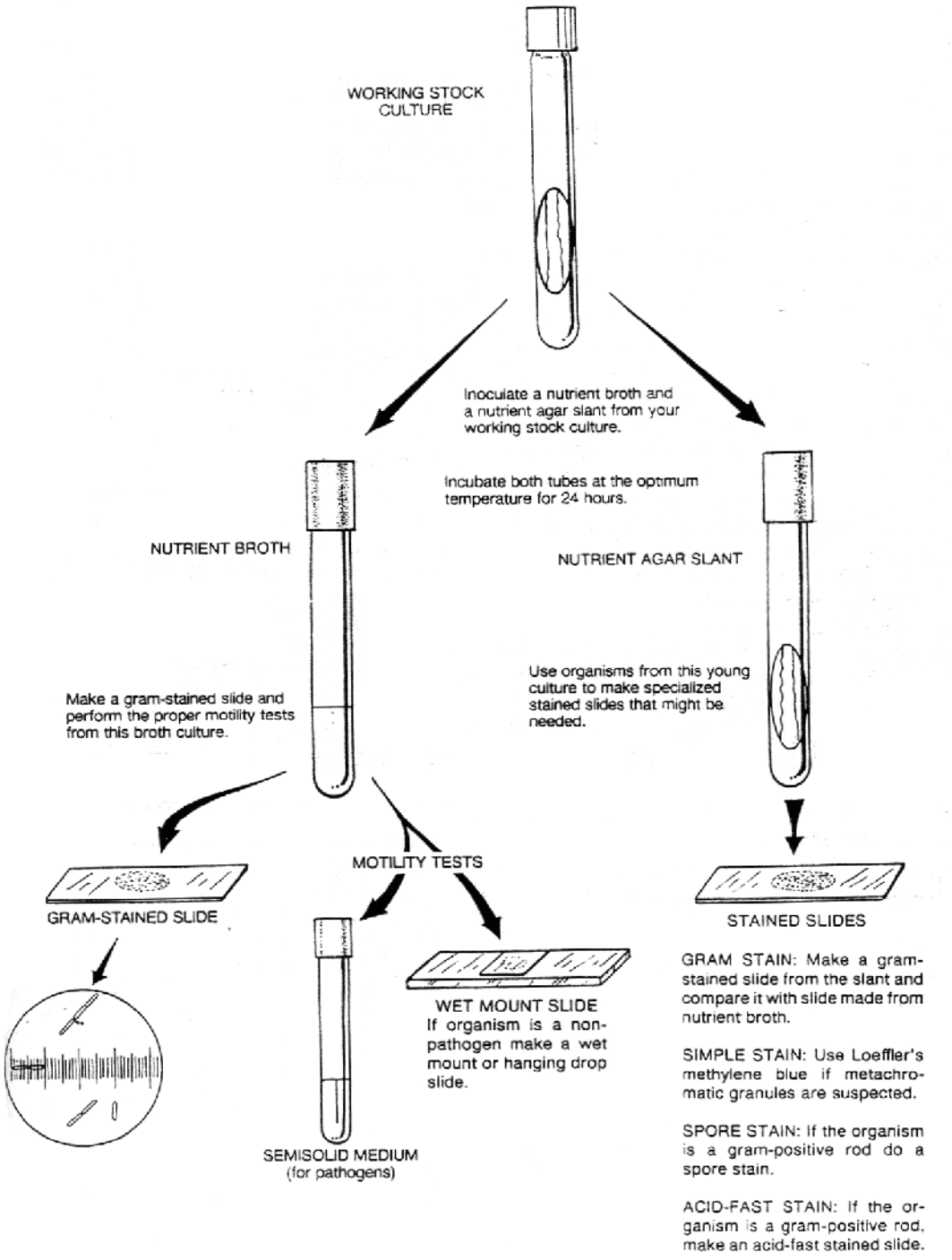


**Figure 3-4: Example Morphology Study (Benson, 1994)**

### 3.6.3.1 Simple Stain

Simple stain are composed of a single basic dye such as crystal violet, safranin, or methylene blue. They are simple because they involve no more than rinsing off the slide with water. The smear observed under the microscope after carefully blotting the slide dry. Size, shape and arrangement of cell determined.(Bauman, 2006)

### 3.6.3.2 Gram Staining

Gram staining procedure is based on the ability of microorganisms to retain the purple colour of crystal violet during decolorized alcohol. Gram positive bacterial are not decolorized and remain purple The crystal violet the primary stain, cause both gram positive and gram negative organisms to become purple after 20 seconds of staining. When gram iodine , the mordant is applied to the cells for one minutes , the colour of gram-positive and gram negative bacteria remains the same. The function of the mordant here is to combine with crystal violet to form a relatively insoluble compound I the gram positive bacteria. When the decolorizing agent ,95% ethanol is added to the cell for 10-20 seconds the gram-positive remain purple. In the final step a counterstain, safranin, a pink color add to the decolorized gram negative bacteria without affecting the colour of the purple gram positive bacterial (Benson, 1994)

### 3.6.3.3 Acid –Fast Stain

The acid fast stain is another important differential the stain. (Bauman, 2006) Bacterial that are not readily decolorized with acid-alcohol after staining with hot carbolfuchsin are said to be acid fast. These bacterial contain considerable quantities of waxlike lipoidal material which combine tenaciously with this red dye.

This strain is used primarily in the identification of the tuberculosis bacillus, Mycobacterium tuberadosis and leoprosy organism, Mycobacterium leprae. After decolorization,the methylene blue is added to the organisms to counterstain any material that is not acid fast; thus, a propely stained slide of a mixture of acid fast organisms, tissue cell and bacterial(Benson, 1994)

### 3.6.3.4 Spore Staining

Species of bacterial belonging to *Bacillus* and *Clostridium* produce extremely heat-resistant structure called endo-spores. In Schaeffer-Fulton method, malachite green utilized to stain the endo spore and safranin to stain the vegetative portion of the cell. That is a properly stained spore-former will have a green endo spore contained in a pink sporangium.

The smear covered with small piece of paper toweling and saturated it with malachite green. The smear steamed over boiling water for 5 minutes. After the slide has cooled sufficiently, removed the paper toweling and rinse with water for 30 seconds. Counterstain with safranin for about 20 seconds. The smear rinsed with water to remove salfranin. Blot dry with bibulous paper and examined slide under oil immersion.(Benson, 1994)

## 3.7 Characterization of enzyme

An attempt was also made to determine enzyme stability pH and temperature in incubation period requirements for their maximum growth and activities.

### 3.7.1 Medium pH

The activity of an enzyme depend s strongly on the pH of the medium for 2 major reasons: the presence of essential proton accepting groups in the catalytic center and maintenance of overall structure of the enzyme. To determine the optimum medium pH, for maximum enzyme production, selected medium of different pH 5, pH6 and pH7 was inoculated with the crude enzyme at 70 $^{o}$C about 15 minutes and the effects of medium pH on xylanase activity were recorded.

### 3.7.2 Temperature

When tested at various temperatures, enzyme activity does show a maximum, resembling the pH optimum. The determination of the optimum temperature for xylanase activity was done at temperature ranges from 30-70$^{o}$C and was at optimum pH (3.7.1) in 15 minutes. The effects of temperature on xylanase activity recorded.

## 3.8 Analysis procedure

### 3.8.1 Enzyme assay

Assays for crude xylanase were performed using 0.5% (w/v) soluble oat spelt xylan (Sigma) in 50 mM sodium phosphate buffer, pH 7.0.The reaction mixture was composed of 1.8 ml substrate and 0.2 ml crude enzyme.

#### 3.8.1.1 3,5- Dinitrosalicylic acid (DNSA) method

The mixture was incubated in a water bath at 60ºC for 15 min. The released reducing sugar was measured by the 3,5- dinitrosalicylic acid (DNSA) method in which the reaction was stopped by incubated in ice cubes and reddish brown colour developed after placing the reaction tubes in a boiling water bath for 5 min. After cooling the reaction tubes to room temperature, the O.D. was measured at 575 nm

with xylose as the standard, where one unit (U) of xylanase activity is defined as the amount of enzyme that releases 1 mol xylose/min/ml under the above mentioned conditions.(Rawashded *et al.*, 2005)

### 3.8.1.2 Lowry Method

The method of Lowry is one of the most sensitive protein assays. Preparation solution A( Carbonate buffer) dissolved 0.4 g Sodium tartrate and 20g sodium carbonate 1 N $Na_2$ $CO_3$ in 100ml alkaline and dilute with water to 200ml. For solution B(alkaline copper tartrate solution) dissolved 2g sodium potassium tartrate, 1g $CuSO_4.5H_2O$ in 90ml water add 10ml 1 N NaOH.Solution C diluted Folin – Ciocalteau reagent is 1 part Folin-Ciocalteau reagent and 15 part water. Solution D is 1 mg/ml bovine serum albumine in water for the standard calibration curve. Solution A and B stable at room temperature and solution C must prepare daily, freeze BSA at -20 ºC. Protein solution contain 4-40micro g protein; bring to 0.3ml with water add 0.3ml solution A incubated for 10 min at 50 ºC cool at the room temperature and 33 micro L solution B, let for 10mins at room temperature .1ml solution C added and mixed immediately incubate for 10 min at 50 ºC cool at room temperature and measure the absorption at 650nm.

## 3.9 Production of xylanase : Medium Development

### 3.9.1 The effect of carbon source

To define the effect of various carbon-sources on xylanase production 250 ml Erlenmeyer flasks were prepared containing 30 ml of mineral source.

The medium supplemented with 0.16g of one of the following carbon sources: glucose, xylose and carboxymethyl cellulose (CMC). Xylanase assays were performed after 24 hours.( Rawashdeh *et al.*, 2005)

### 3.9.2 The effect of nitrogen source

To define the effect of various nitrogen-sources on xylanase production 250 ml Erlenmeyer flasks were prepared containing 30 ml of mineral salts medium supplemented with 0.4g of one of the following nitrogen sources: peptone, urea and yeast extractand. Xylanase assays were performed after 24 hours .(Rawashdeh *et al.*, 2005).

# CHAPTER 4

# RESULTS AND DISSICUSION

## 4.1 Screening results

The screening process is the procedure which uses the selective xylan agar plate to screen colonies from the nutrient agar. 10 best colonies had been chosen from the nutrient agar plate and transfer to selective xylan agar plate. The selective agar plates incubated about 5 days .Only 5 colonies from 10 samples gave the positive results to the selective agar and shows some remarked activities of xylanase enzyme.

**Figure 4-1: Positive Result**

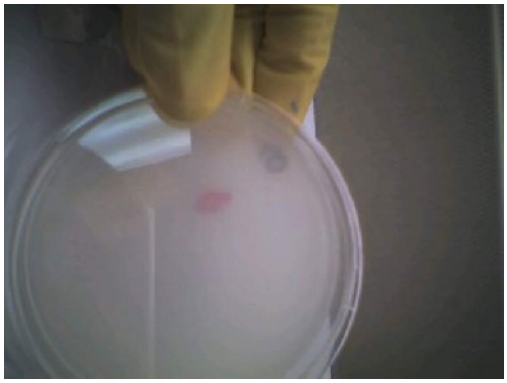

**Figure 4-2: Negative Result**