Int. J. Adv. Res. Biol. Sci. (2016). 3(2): 170-180

International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
Coden: IJARQG(USA)

Research Article

SOI: http://s-o-i.org/1.15/ijarbs-2016-3-2-23

# Isolation and Identification of Bacterial Strains for Saccharification of Agriculture Wastes for Bioethanol Production

**Mervat Aly Abo-State[1*], Hussein Hosny El-Sheikh [2], Seham Aly El-Temtamy[3], Mohamed Hosny[3]**

[(1)]Department of Radiation Microbiology, National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Cairo, Egypt.
[(2)] Faculty of Science, Al-Azhar University, Cairo, Egypt.
[(3)] Egyptian Petroleum Research Institute (EPRI), Cairo, Egypt.
***Corresponding author: Mervat Aly Abo-State,** (NCRRT) 3Ahmed El Zomar, Nasr City, Cairo, Egypt
E-mail: *abostatem@yahoo.com/moh_hosny2020@yahoo.com*

## Abstract

Agriculture wastes represent a great problem all over the world and especially in Egypt. The present study deals with agricultural wastes disposal by eco-friendly manner to the environment and production of valuable products from a cheap and renewable lignocellulosic wastes. Twenty bacterial strains were isolated from different agriculture wastes [(Rice straw (RS), Wheat straw (WS), Wheat bran(WB), Bagasse (BA) Corn stover (CS), Potato peal(PP) and Saw dust (SD)]. Bacterial isolates MH2, MH3, MH5, MH11 and MH15 were selected for their ability to saccharifying agriculture wastes, the highest reducing sugar (7423.26, 3224.82, 2279.34, 1 174.34, and 1581.08µg/l) were produced from the agriculture waste BA, PP, RS, SD and WS respectively. "Extracellular protein, Carboxy methyl cellulase (CMCase), Filter paperase (FPase), avicelase and xylanase production were studied for each bacterial isolates with selected substrates, The isolated strain MH5 gave the highest CMCase (726.9U/ml) and xylanase (1248.9 U/ml) on bagasse , However the isolated strain MH11gave the highest extracellular protein(633.5µg/ml)on rice straw, while the highest FPase (282.90U/ml)on bagasse, the highest avicelase (169.41 U/ml) were produced by MH3 on rice straw. The highest cellulases were produced on the agriculture wastes in the order BA>RS>WS>PP>SD. The most potent strain MH5 was identified as *Bacillus flexus.*

**Keywords:** Isolation of bacteria, Agriculture wastes, Enzymes, Identification, Bioethanol production.

## 1. Introduction

Large-scale production of biofuels offers an opportunity for certain developing countries to reduce their dependence on oil imports. In developed countries there is a growing trend towards employing modern technologies and efficient bioenergy conversion using a range of biofuels, which are becoming cost-wise competitive with fossil fuels (**Demirbas, 2008).**

Bioethanol can be produced from different kinds of raw materials. The raw materials are classified into three categories of agricultural raw materials: sucrose-containing feed stocks (e.g. sugar cane, sugar beet, sweet sorghum and fruits), starch materials (e.g. corn, milo, wheat, rice, potatoes, cassava, sweet potatoes and barley) which represent first generation of bioethanol production and lignocellulosic materials (e.g. wood, straw and grasses) which represent second generation of bioethanol production. Currently, a focus is on bioethanol production from crops, such as corn, wheat, sugar cane, as well as on highly abundant agricultural Wastes **(Mojovic *et al*., 2009).**

However is an increase in the price of the raw materials, which can highly affect the production costs of the bioethanol **(Yoosin and Sorapipatana, 2007)**. Lignocellulosic materials serve as a cheap and abundant feedstock, which is required to produce fuel bioethanol from renewable resources at reasonable costs **(Slade *et al*., 2009).**

Lignocellulosic materials are one of the most abundant natural complex organic carbons in form of plant biomass, which is highly renewable natural resource in the World, reaching annually over 150 billion tons on the earth **(Zhu *et al*., 2006).**

Lignocellulose wastes are accumulated every year in large quantities, causing environmental problems. However, due to their chemical composition based on sugars and other compounds of interest, they could be utilized for the production of a number of value added products, such as ethanol, food additives, organic acids, enzymes, and others. Therefore, besides the environmental problems caused by their accumulation in the nature, the nonuse of these materials constitutes a loss of potentially valuable sources **(Mishra, and Thakur, 2015).**

Egypt is the largest rice producer in the Near East region, where rice cultivation area occupies over 1,080,000 feddan with an average farm yield of 4.76 tons/feddan and an approximate straw production of 2.4 tons/feddan **(Sabaa and Sharaf, 2000)** Currently, the major practice to eliminate such massive amounts of post-harvest rice residues is field open air burning. Although field burning provides effective destruction. Of weed seeds and pathogenic microbial spores, the produced black smoke represents a threat to public health. The burning results in respirator particles of <10 mm size (PM10. Particulate matter less than 10 mm in diameter), which are a major cause of respiratory ailments such as asthma emphysema; it also introduces carbon monoxide and some nitrogen dioxide, which has statistically significant effect on asthma morbidity **(Schwartz *et al*., 1993)**.

Chemical composition of lignocellulosic materials is a key factor affecting efficiency of biofuel production during conversion processes. The structural and composition of lignocellulosic materials is highly variable because of genetic and environmental influences and their interactions **(Lee *et al*., 2007).** A typical chemical composition of lingo-cellulosic materials is 48% wt. C, 6% wt. H, and 45% wt. O, the inorganic matter being a minor component **(Molina-Sabio, and Rodriguez-Reinoso, 2007).**

Lignocelluloses consist mainly of cellulose, hemicellulose and lignin; these components build up about 90% of dry matter in lignocelluloses, with the rest consisting of e.g. extractive and ash **(Dehkhoda, 2008)**. The basic structure of all woody biomass consists of three basic polymers: cellulose (C6Hl0O5)x , hemicelluloses such as xylan $(C_5H_8O_4)$m, and lignin [C9H10O3(OCH3)0.9-I.7]n. The proportion of these wood constituents varies between species, and there are distinct differences between hardwoods and softwoods. Cellulose and hemicellulose contents are more in hardwoods (78.8%) than softwoods (70.3%), but lignin is more in softwoods (29.2%) than hardwoods (21.7%) **(Balat, 2011).**

The carbohydrate polymers in lignocellulosic materials need to be converted to simple sugars before fermentation, through a process called hydrolysis **(Taherzadeh and Karimi, 2007).**

Biochemical conversion of lignocellulosic materials through saccharification and fermentation is a major pathway for bioethanol production from biomass. Bioconversion of lignocellosics to bioethanol is difficult due to: (1) the resistant nature of biomass to breakdown; (2) the variety of sugars which are released when the hemicellulose and cellulose polymers are broken and the need to find or genetically engineer organisms to efficiently ferment these sugars; (3) costs for collection and. storage of low density lignocellosic materials **(Cardona Alzate, and Sanchez Toro, 2006)**. The basic process steps in producing bioethanol from lignoeellulosic materials are: pretreatment, hydrolysis, fermentation and product separation/distillation **(Abo-state *et al*., 2014)**.

Most of our understanding about cellulose degradation comes from the study of the mesophilic fungi and anaerobic thermophilic cellulolytic bacteria. Relatively few aerobic cellulolytic, thermophilic bacteria have been reported in comparison with anaerobic thermophilic cellulolytic bacteria **(Sukumaran et al., 2005).** Members of the genus *Bacillus* are producers of extracellular enzymes including CMCase activity **(Roboson and Chambliss, 1984; Fukumori *et al*., 1985; Chan and Au, 1987 and Abo-state *et al*., 2013a, b)**

The bacterial cellulases have very high activities against crystalline celluloses like cotton or Avicel **(Johnson *et al*., 1981)** and are also more thermostable in comparison to fungal cellulases **(Rani *et al*., 2013).**

Cellulases produced by *Bacillus subtilis* for the saccharification of wheat straw, rice straw and bagasse were used**(Akhtar *et al*., 2001).**

The present study aimed to isolate bacterial strains having the ability to saccharifying agriculture wastes efficiently and determining their enzymes that hydrolyzing the agriculture wastes to be used for bioethanol production.

## 2. Materials and Methods

### 2.1. Collection and preparation of substrates

Rice straw (RS), wheat straw (WS), wheat bran (WB), corn stover (CS), potato peal (PP) and saw dust (SD) was collected from agriculture areas, Elmansoura, Egypt, while bagasse (BA)was collected from Upper Egypt . Wastes were dried, cut to 3-5 mm lengths, grind in an electric grinder and passed through a sieve to get uniform particles. Substrates were stored in plastic bags at room temperature until analysis and treatment were performed.

### 2.2. Isolation and purification of bacterial isolates.

According to **Abo-State *et al*. (2010),** ten grams of lignocellulosic wastes (RS),(WS), (WB), (BA),(CS), (PP) and (SD) were added under aseptic conditions to 90 ml sterile saline (8.5 g/L NaCl) in 250 ml conical flasks. The flasks were shaken at 200 rpm for 60 min. The agriculture waste suspension was then subjected to serial dilutions (10 fold dilutions) and cultured on L.B agar plates **(Kirimura *et al*. 2001).**Then the plates were incubated at 30$^{o}$C for 48 h. The well growing colonies were picked up, streaked on sterile L.B agar plates for purification. Pure separated single colonies were maintained on sterile L.B agar slants at 4$^{o}$C for further investigation, and these slants were sub-cultured monthly. The isolates were preserved in micro-tubes containing 1:1(v/v glycerol: pure broth culture of each isolate in L.B. medium) at -4$^{o}$C.

### 2.3. Screening for the ability of Bacterial isolates to saccharify of different agriculture wastes.

According to **Abo-State *et al*. (2013a, b)** the agriculture wastes (RS, WS, WB, B, CS, PP and SD) were used as cheap substrate to test the ability of the bacterial isolates for saccharification of different agriculture wastes. Five grams of (RS, WS, WB, B, CS, PP and SD) were used as substrate. Five grams of each substrate were put in 250 ml conical flasks and moisted with 10 ml of distilled water. The moisted flasks were sterilized by autoclaving at 121°C for 30 minutes. The sterilized flasks were inoculated with different bacterial isolates 10.0 ml ($3.0\times10^{7}$CFU/ml). Three replicates were used for each substrate. The inoculated flasks were incubated at 30°C for 48 h. After incubation period, 50 ml of distilled water were added to each flask and shaked for 60 minutes on shaker (200 rpm). All the content of the flask was filtered in a clean dry flask through muslin cloth on a glass funnel. The filtrates were centrifuged by cooling centrifuge (Sigma, model, 3k30, Germany) at 8000 rpm for 10 minutes. The supernatants were used to determine the total reducing sugars (TRS) and enzymes assay.

### 2.4. Determination of reducing sugar (Miller, 1959).

According to **Abo-State *et al*. (2010a,b)** an aliquot (1 ml) of DNS reagent was added to the tubes containing 1ml of sugar solution and the tube containing 1ml of water as a blank. The tubes were placed in boiling water bath for 10 minutes and then cooled to room temperature. Four ml Distilled water was added to each tube. The change in intensity of yellow to orange color was determined at 540 nm. Glucose was used as a standard.

### 2.5. CMCase assay.

Endoglucanase, carboxymethyl cellulose(CMCase) activity was determined according to **Wang, *et al.* (1988)** one gram of Carboxmethyl Cellulose (CMC) (Sigma, St. Loius, USA) was added to 100 ml sodium acetate buffer, pH 5.0. In a clean dry tube an aliquot of 1ml of the supernatant (enzyme) was mixed with 1 ml of 1% CMC in acetate buffer. The tube was incubated at 63$^{0}$C for 30 min., then the librated reducing sugar was measured by DNS method described by **Miller (1959)**, the absorbance was measured at 540 nm. The blank was 1ml of distilled water instead of 1ml of supernatant (enzyme).

The concentration of the resulted reducing sugar was determined using glucose standard curve. One unit of CMCase, is the micromole of glucose librated per ml of culture filtrate per minute.

### 2.6. FPase assay.

The total cellulose (FPase) activity was determined according to **Gadgil, *et al.* (1995)** In a clean dry tube an aliquot of 1ml of the supernatant (enzyme) was mixed with 2ml of 0.1M citrate buffer pH 4.8

containing 0.05g. Filter paper (Whatman No.1).The tube was incubated at $50^0$C for 1hour. Then the librated reducing sugar was measured by DNS method, described by **Miller (1959).** The absorbance was determined at 540 nm. The blank was 1ml of distilled water instead of enzyme. The concentration of the resulted reducing sugar was determined using glucose standard curve. One unit of FP ase, is the micromole of glucose librated per ml of culture filtrate per minute.

### 2.7. Avicellase assay.

Avicellase activity was determined according to **Li and Gao (1997)** Avicel (2.0 grams)(Sigma / Aldrich, USA) was added to 100ml phosphate buffer pH 6.6 In a clean dry tube an aliquot of 1ml of the supernatant (enzyme) mixed with 1ml of 2% Avicel in phosphate buffer. The tube was incubated at $40^0$C for 2hours. Then the librated reducing sugar was measured by DNS method, described by **Miller (1959),** the absorbance was determined at 540 nm. The blank was 1ml of distilled water. The concentration of the resulted reducing sugar was determined using glucose standard curve. One unit of Avicelase is the micromole of glucose librated per ml of culture filtrate per minute.

### 2.8. Xylanase assay.

Xylanase activity was determined according to **Chaplin (1996)** Birch wood xylan (2.0 grams) (Sigma, St. Loius, USA). was added to 100ml acetate buffer pH 5.5. In a clean dry tube an aliquot of 1ml of the supernatant (enzyme) mixed with 1ml of 2% xylan in acetate buffer. The tube was incubated at $50^0$C for 30 min. Then the librated reducing sugar was measured by DNS method, described by **Miller (1959),** the absorbance was determined at 540 nm. The blank was 1ml of distilled water. The concentration of the resulted reducing sugar was determined using xylose standard curve. One unit of xylanase is the micromole of xylose librated per ml of culture filtrate per minute.

### 2.9. Protein determination.

Extracellular protein have been determined according to **Lowry, *et al.* (1951)** five ml of the reaction solution was added to 1ml of supernatant of the fungal filtrate and 1ml distilled water was used as a blank. The mixture was allowed to stand at room temperature for 10 min.

After that 0.5 ml of Folin reagent (Sigma / Aldrich, USA) was added. The reaction tubes were incubated at room temperature for 20 min. The absorbance was determined at 720 nm. To determine the concentration of the protein in the samples, a standard curve of Bovin serum albumin (BSA)(Sigma, St. Loius, USA) was determined.

### 2.10. Molecular identification of the most promising bacterial strain.

An analysis of 16S rRNA was performed to taxonomically characterize the isolate strain (Sigma Scientific Services Co., Egypt).The cells of the bacterial strain were harvested through the enrichment medium up to $2x10^9$ bacterial cells. DNA was extracted using protocol of Gene Jet genomic DNA purification Kit (Thermo K0721) (Sigma Scientific Services Co., Egypt). To amplify the16S rDNA genes, a polymerase chain reaction (PCR) was performed using (5'-AGA GTT TGA TCC TGG CTCAG-3')(5'-GGT TAC CTT GTTACG ACTT-3') as forward and reverse primer respectively.

PCR was cleaned up to the PCR product using Gene JET™ PCR Purification Kit. A 45µl of Binding Buffer was added to the completed PCR mixture. The mix was then thoroughly transferred from step 1 to the Gene JET™ purification column. The mixture was then centrifuged for 30–60 s at > 12,000xg, then the flow was discarded. A 100µl wash buffer was added to the Gene JET™ purification column, centrifuged for 30–60s, discarded the flow-through and place the purification column back into the collection tube. The mixture was centrifuged at empty purification column for an additional 1 min to completely remove any residual wash buffer. The purification column was transferred to a clean 1.5 ml micro centrifuge tube. A 25µlof elution buffer were then added to the center of the column membrane which then centrifuged for 1 min, discard the column and store the purified DNA at 20°C.

Following purification of the PCR products, the DNA sequence of the positive clone was subjected to a similarity search BLAST on the NCBI web-site (http://www.ncbi.nlm.nih.gov), and deposited into GenBank. Many relevant 16S rRNA gene sequences with validly published names were selected as references from the Gen-Ban**k**. The ladder used is (1 kbp plus). Also, the obtained sequence used to building phylogenetic tree with closely related strains with sequence MH5 in gene bank, bioinformatics program is Mega 4 software 2015.

## 3. Results and Discussion

### 3.1. Isolation of Different Microbial Isolates

The aim of this study was to isolate strains of bacteria with improved cellulases production and agriculture waste hydrolysis, Twenty bacterial isolates were isolated from different agriculture wastes (RS), (WS), (WB), (BA), (CS),(PP) and(SD).The purified bacterial isolates were used on different agriculture wastes using solid state fermentation.

### 3.2 Screening for the ability of Bacterial isolates to saccharify different agriculture wastes.

Bacterial isolates showed different ability to saccharify (RS), (BA) and (WS) as indicated in Table (1) and (SD), (CS), (WB) and (PP) as indicated in Table (2). Bacterial isolates (MH2), (MH3), (MH5), (MH11) and (MH15) were selected for further study for their ability to saccharify different agriculture wastes, the highest reducing sugar (7423.26,3224.82, 2279.34, 1174.34,and 1581.08µg/l) were produced on the agriculture waste BA. PP, RS, SD and WS respectively.

**Table (1):** Total reducing sugars (TRS) from rice straw, bagasse and wheat straw saccharify by different bacterial isolates.

| Isolate code | Total reducing sugars (µg/l) | | |
|---|---|---|---|
| | Rice straw | Bagasse | Wheat straw |
| MH1 | 2117.21 | 7150.37 | 963.37 |
| MH2 | 2250.05 | 7026.68 | 928.06 |
| MH3 | 1346.11 | 7022.94 | 710.26 |
| MH4 | 1186.37 | 7051.29 | 619.14 |
| MH5 | 2279.34 | 6861.5 | 963.28 |
| MH6 | 533.99 | 1984.34 | 228.62 |
| MH7 | 696.25 | 2532.52 | 210.69 |
| MH8 | 1302.72 | 2863.06 | 577.28 |
| MH9 | 779.09 | 3591.7 | 290.06 |
| MH10 | 1001.78 | 3494.96 | 482.31 |
| MH11 | 1862.13 | 5638.93 | 1174.34 |
| MH12 | 955.59 | 6710.6 | 1022.41 |
| MH13 | 978.14 | 7243.62 | 585.37 |
| MH14 | 1272.4 | 6470.24 | 781.67 |
| MH15 | 1740 | 7423.26 | 749.91 |
| MH16 | 818.07 | 5847.16 | 350.1 |
| MH17 | 748.7 | 6550.34 | 353.75 |
| MH18 | 1023.5 | 7163.16 | 400.48 |
| MH19 | 834.11 | 7090.12 | 733.44 |
| MH20 | 1522.71 | 7142.62 | 805.52 |

**Table (2):** Total reducing sugars (TRS) from saw dust, corn stover, wheat bran and potato peel saccharify by different bacterial isolates.

| Isolate code | Total reducing sugars (µg/l) | | | |
|---|---|---|---|---|
| | Saw dust | Corn stover | Wheat bran | Potato peel |
| MH1 | 840.8 | 493.79 | 2146.16 | 1532.86 |
| MH2 | 915.53 | 463.32 | 2692.28 | 1670.66 |
| MH3 | 874.37 | 515.15 | 4426.21 | 3224.82 |
| MH4 | 613.69 | 449.37 | 718.25 | 464.65 |
| MH5 | 702.94 | 513.62 | 1613.9 | 2963.69 |
| MH6 | 261.21 | 45.12 | 1210.11 | 383.5 |
| MH7 | 272.86 | 197.54 | 776.75 | 307.2 |
| MH8 | 648.7 | 0 | 3108.81 | 271.37 |
| MH9 | 513.91 | 0 | 2840.9 | 759.79 |
| MH10 | 535.7 | 5.38 | 3571.98 | 1652.34 |
| MH11 | 1581.08 | 449.19 | 4138.22 | 894.46 |
| MH12 | 743.32 | 223.09 | 1508.98 | 515.18 |
| MH13 | 449.58 | 276.08 | 1268.14 | 284.44 |
| MH14 | 719.22 | 270.46 | 4234.69 | 1871.3 |
| MH15 | 587.02 | 239.46 | 1459.45 | 604 |
| MH16 | 364.09 | 149.95 | 1749.94 | 140.59 |
| MH17 | 454.17 | 161.04 | 1213.57 | 135.3 |
| MH18 | 603.55 | 283.19 | 1137.73 | 112.24 |
| MH19 | 952.8 | 145.67 | 3890.63 | 284.13 |
| MH20 | 753.32 | 383.62 | 3124.34 | 640.98 |

### 3.3. Determination the best substrate for ccllulases production by solid- state fermentation.

Extracellular protein as indicated in Figure (1), Carboxy methyle cellulase (CMCase) Figure (2), Filter paperase (FPase), Figure (3), Avicelase, Figure (4) and Xylanase production ,Figure (5) were studied for each bacterial isolates with selected substrates, the isolated strain MH5 gave the highest CMCase (726.9U/ml) and xylanase (1248.9 U/ml) on bagasse , However the isolated strain MH l1 gave the highest extracellular protein (633.5 µg/ml) on rice straw, while the highest FPase (282.90U/ml) on bagasse ,the highest avicelase (169.41 U/ml) were produced by MH3 on rice straw, So, the highest cellulases were produced on the agriculture wastes in the order BA>RS> WS> PP> SD.

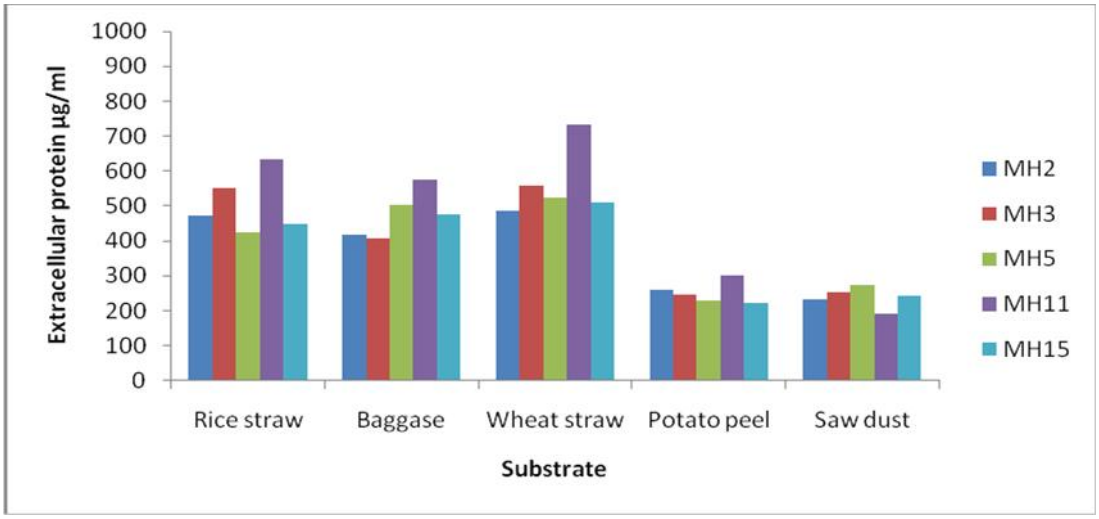


**Figure (1):** Extracellular protein produced by different bacterial isolates on different agriculture wastes.

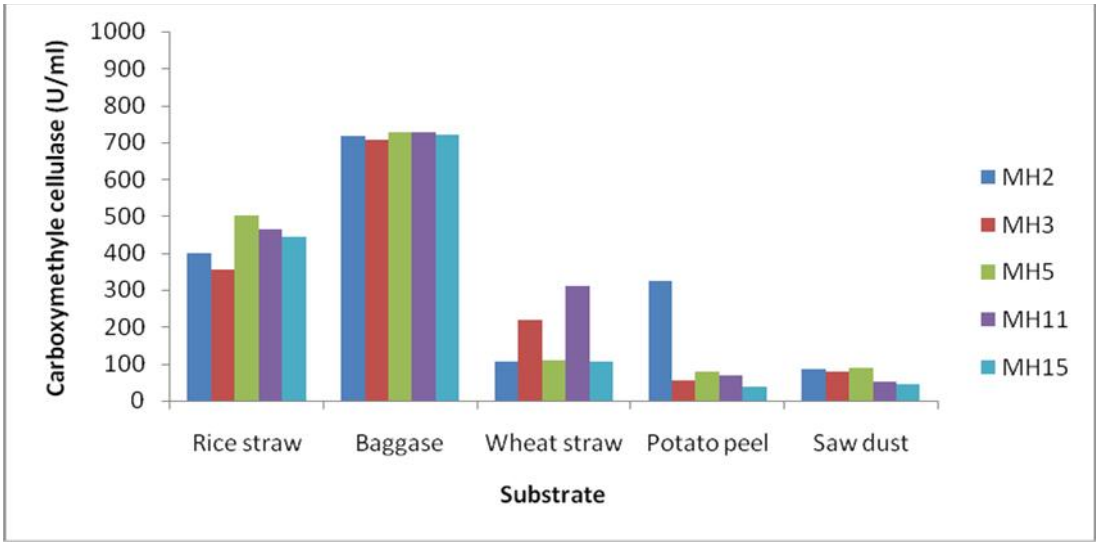


**Figure (2):** Carboxymethyle cellulase produced by different bacterial isolates on different agriculture wastes.

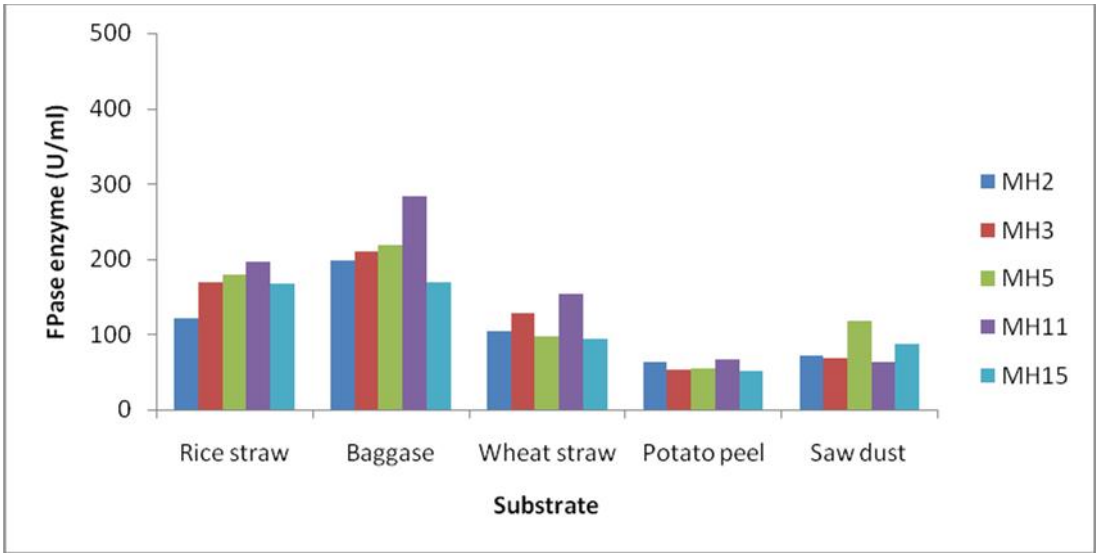


**Figure (3):** FPase enzyme produced by different bacterial isolates on different agriculture wastes.

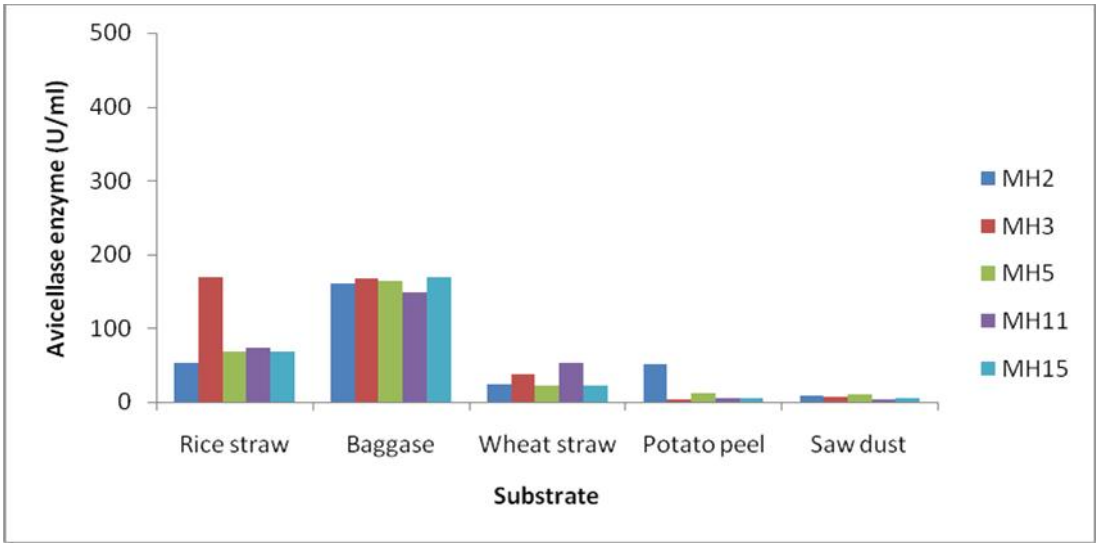


**Figure (4):** Avicellase enzyme produced by different bacterial isolates on different agriculture wastes.

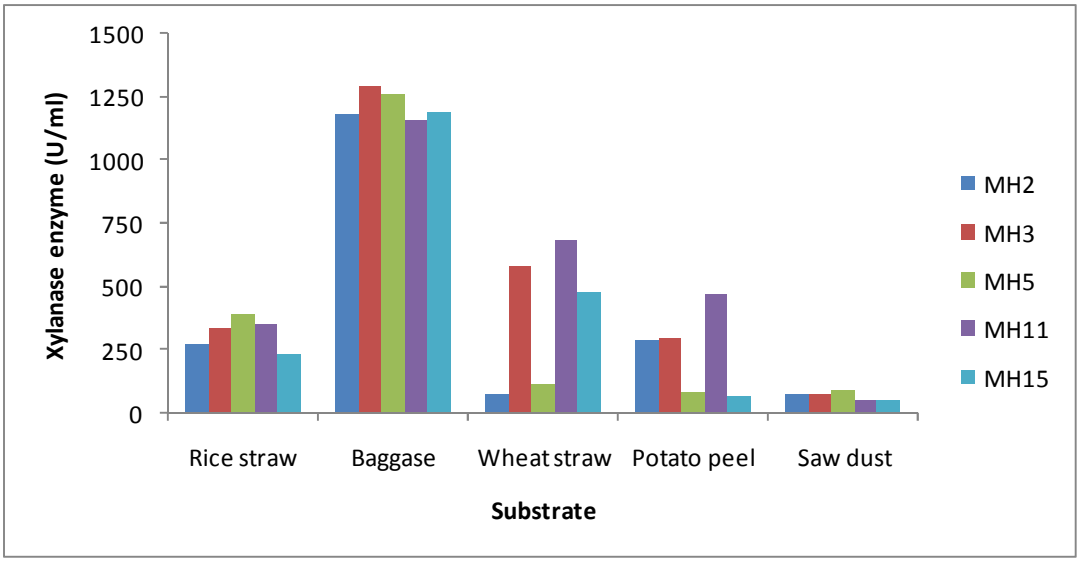


**Figure (5):** Xylanase enzyme produced by different bacterial isolates on different agriculture wastes.

From the previous results, it was found that bagasse supported the production of different cellulases followed by rice straw followed by wheat straw followed by potato peel and at the last was saw dust. The ability of microorganisms to produce cellulases considered to be critical step in the microbial hydrolysis of agriculture wastes.

In fact, the comparisons of cellulase activities produced by different laboratories is not readily made in quantitative manner as no standard conditions of cellulase activity assay have been adopted by **(Gao *et. al*., 2008)**. Also the difficulty in comparison between cellulose(s) activities depends on the difference between strains used in production, condition of production (SmF or SSF), assay determination and other physical factors.

**(Sangkharak, *et. al*., 2011)** report that Five novel cellulase-producing bacteria were isolated and identified through 16S rRNA sequence as *Cellulomonas* sp. The activity of enzymes (mainly xylanase and endoglucanase) produced from isolated strains was almost present extracellularly and the production of enzyme was dependent on cellulosic substrate (xylan, rice straw and wastepaper) used for growth. *Cellulomonas* sp. Strain TSU-03 produced the highest activity of xylanase and endoglucanase at 1860.1 and 388.5 U mg-1 protein, respectively.

.

Five *Aspergillus* spp. and standard strain *Trichoderma viride* were grown on the agriculture wastes and CMCase. FPase, Avicelasc and soluble protein were determined. *T. viride* produces the highest CMCase on WS (555U/ml).while the highest FPase (141U/ml) and Avicelase (46U/ml) were produced on WB. The isolated strain *Aspergillus* MAM-F35 gave the highest CMCase (487U/ml), FPase (701 ml) and Avicelase (35U/ml) on WS. However, the isolated strain *Aspergillus* MAM-F23 gave the highest CMCase (309U/ml) on RS, while the highest Avicelase (45U/ml) on WS. So, the highest cellulases were produced on the agriculture wastes in the order WS> WB> RS> CC. **(Abo state *el. al*, 2010a)**.

### 3.4. Identification of the most potent bacterial isolate.

Agarose gel electrophoresis of PCR product indicated a sharp band in 1500 bparea as shown in figure (6) The most potent bacterial isolate (MH5) was identified using molecular identification performed by amplifying and sequencing the 16S rRNA gene sequences, Figure (7) showed that the (MH5) bacterial isolate belong to the *Bacillus flexus* with in similarity 98% comparing with others *Bacillus* strains as showed in alignment with gene bank. Depending on the above discussion phylogenetic tree established (construct phylogenetic tree using maximum parsimony) using Mega 4 program software. That is proof isolate MH5 is *Bacillus* species specifically subspecies *flexus*

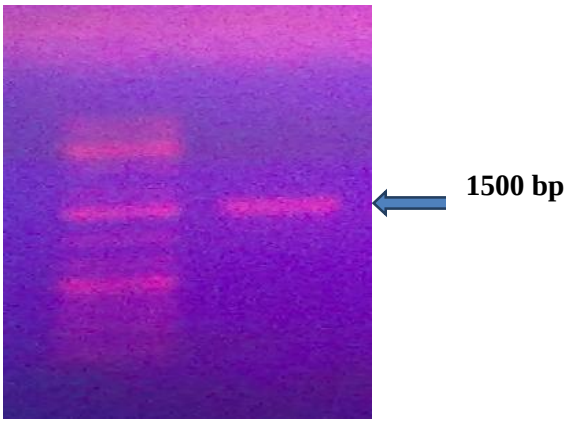


**Figure (6):** DNA agarose gel electrophoresis of PCR amplified 16S rDNA of bacterial strain MH5.

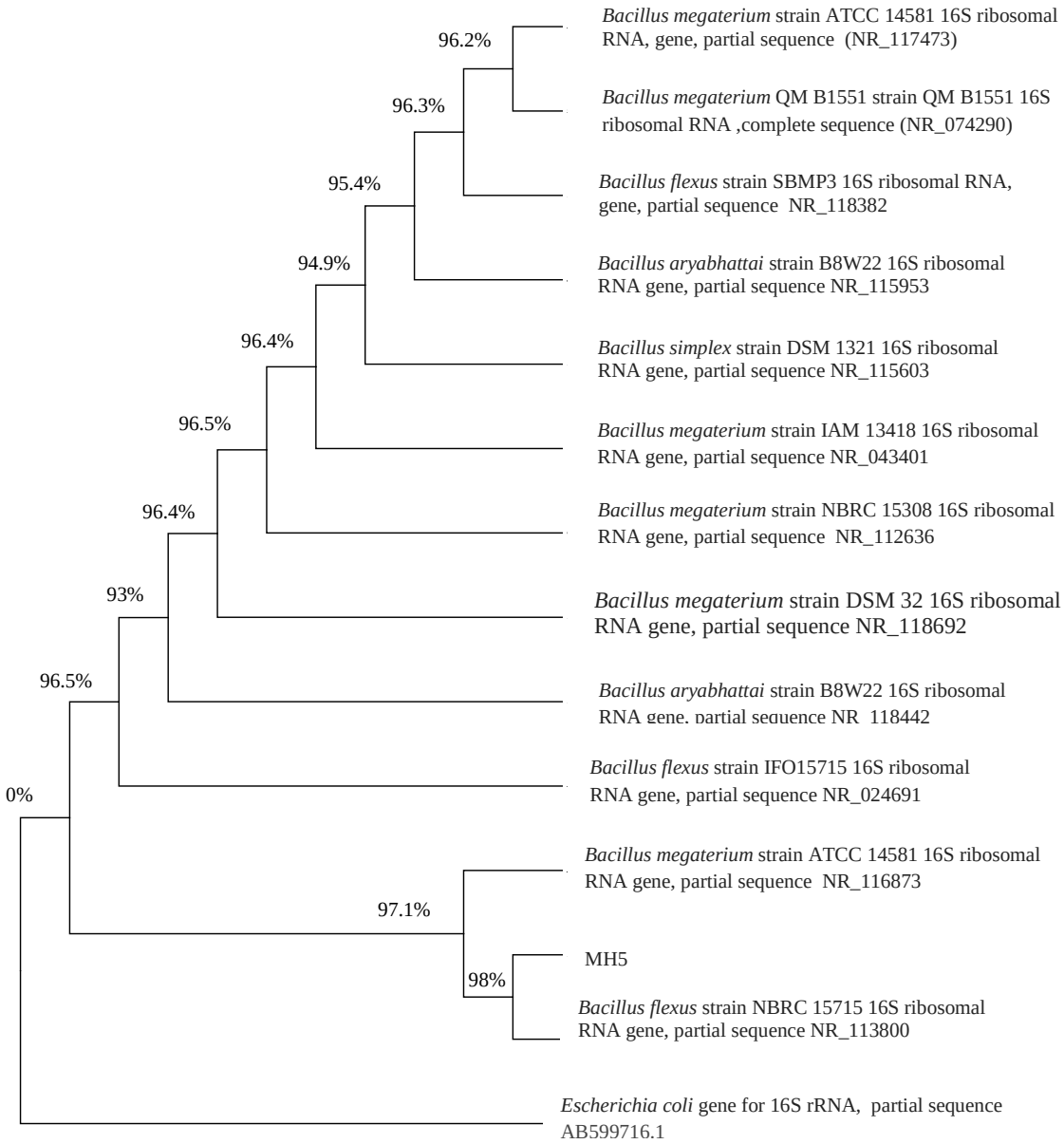


**Figure (7):** Phylogenetic tree of bacterial strain MH5.

**Akhtar *et al*. (2001)** report that Cellulases produced by *Bacillus (B.) subtilis* were used for the saccharification of wheat straw, rice straw and bagasse. Pretreatment of these substrates with 2% NaOH was found to be more effective for increasing the saccharification. The saccharification rates of 33.0, 25.5 and 35.5% were obtained with 2% NaOH pretreated wheat straw, rice straw and bagasse, respectively. The saccharification of wheat straw was 33, 26 and 16.9% after 20 h at 50ºC when 4, 6 and 10% substrate was used, respectively.

Bacterial strain *Bacillus subtilis* (CBTK 106), isolated from banana waste is able to production of filter paper enzyme, carboxymethyle cellulose and cellobiase. The optimal filter paper activity (FP Ase) of 2.8 $IUgds^{-1}$, CMCase activity of 9.6 $IUgds^{-1}$ and cellobiase activity of 4.5 $IUgds^{-1}$ were obtained at 72 h incubation with media containing banana fruit stalk (autoclaved at 121°C for 60 min, particles of 400 µm size), with optimal moisture content of 70%, pH of 7.0, incubation temperature of 35°C, with minerals, and additional nutrients of (NH4)2SO4 or NaNO3 or glucose at 1.0% (w/w) and an inoculum to substrate ratio of 15% (v/w) (**Shah *et. al*., 2015).**

Generally sacchaification step is carried out by commercially available cellulase enzymes which are very expensive. Bacterial isolates (MH2, MH3, MH5, MH1land MH15) in this study offer a good prospect for cellulolytic enzyme production and this method can be applied as a part of bioethanol process using a cheap and renewable source such as agriculture wastes (bagasse, rice straw, wheat straw and saw dust). Five cellulolytic bacterial strains were isolated from agriculture wastes. All isolated strains showed the great ability to hydrolyzed cellulosic compound and produced endoglucanase (CMCase). total cellulase (FPase), exoglucanase (Avicelase), extracellular xylanase and protein by solid state fermentation.

## Acknowledgments

The authors are thankful to the Academy of Scientific Research and Technology for financial support of this work.

## References

**Abo-State, M.A.M.; Hammad, M.A.M.; Swelim, M. and Gannam. R.H (2010a):** Enhanced Production of cellulase (S) By *Aspergillus* spp. Isolated from Agriculture Wastes by Solid State Fermentation. American-Eurasian J. Agric. Environ. Sci., 8(4): 402-410.

**Abo-State, M.A.M.; Swelim, M.; Hammad, M.A.M.; and Gannam. R.H (2010b):** Some critical factors affecting cellulose(s) Production by *Aspergillus terreus* MAM-23 and *Asperigillus flavus* MAM-35 under solid state fermentation of wheat straw. World Appl. Sci.J.9:1171-1179.

**Abo-State, M.A.M.; Ghaly,M.F. and Abdellah,E.A.(2013a):** Production of cellulase and xylanase by thermophilic and alkaliphilic bacteril strains isolated from agriculture wastes. World App. Sci. J. 22:1603-1612.

**Abo-State, M.A.M.; Ghaly,M.F. and Abdellah,E.A.(2013b):** Optimization of cellulose(s) and xylanase production by thermophilic and alkaliphilic *Bacillus* isolates. Am. Eurasian J. Agric. Environ. Sci. 13:553-564.

**Abo-State, M.A.M.; Ragab, A. M.; EL-Gendy, N. S.; Farahat, L. A., and Madian, H. R. (2014):** Bioethanol production from rice straw enzymatically saccharified by fungal isolates *Trichoderma viride* F94 and *Aspergillus terreus* F98.Scientific Research Publishing. soft. 3:19-29.

**Akhtar, M. S.; Saleem, M., and Akhtar, M. W. (2001):** Saccharification of Lignocellulosic Materials by the cellulases of *Bacillus subtilis*. Int.. J. Agri. Biol., 3(2), 199-202.

**Balat, M. (2011):**Production of bioethanol from lignocellulosic materials via the biochemical pathway: "a review." Energ. Convers. Manage. 52.2: 858-875.

**Cardona Alzate, C.A.and Sánchez Toro, O.J. (2006):** Energy consumption analysis of integrated flowsheets for production of fuel ethanol from lignocellulosic biomass. Energy 31: 2447-2459.

**Chan, K.Y. and Au, C.K. (1987):** Studies on cellulase production by a *Bacillus subtilis*. Antonie van Leeuwenhoek J. of Microbil.53, 125–136.

**Chaplin, M.F. (1996):** Monosaccharides. In: chaplin, M.F., Kenndey, J.F. (Eds), Carbohydrate Analysis Oxford, IRL press, pp 1-3.

**Demirbas, A. (2008):** The importance of bioethanol and biodiesel from biomass. Energ. Sourc. Part B;3:177-185.

**Dehkhoda A. (2008):** Concentrating lignocellulosic hydrolysate by evaporation and its fermentation by repeated fedbatch using flocculating *Saccharomyces cerevisiae*. M.Sc Thesis, Industrial Biotechnology Boras University and SEKAB E-Technology, Sweden.

**Fukumori, F.; Kudo, T. and Horikoshi, K. (1985):** Purification and properties of a cellulase from alkalophilic *Bacillus* sp. No. 1139.J. of Gen. Microbil.131, 3339–3345.

**Gadgil, N.J.; Daginawala, H.F.; Chakakrabarti,T. and Khanna.P. (1995):** Enhanced cellulose production by mutant of *Trichoderma reesei*. Enzyme Microb. Tcchnol.17:942-946.

**Gao, j.H.; Weng, D.; Zhu, M.; Yuan, F. and Guan Y. Xi. (2008):** Production and characterization of cellulolytic enzymes from thermoacidophilic fungal *Aspergillus terreus* M1l under solid-state cultivation of corn stover. Biores. Technol., 99:7623-7629.

**Johnson, E.A.; Madia, A. and Demain, A.C. (1981):** Chemically defined minimal medium for growth of Feedstocks. South Dakota State University Publication, SGINC1-07, Brookings, SD; June.

**Lee, J.S.; Parameswaran, B.; Lee, J.P. and Park, S.C. (2008):** Recent developments of key technologies on cellulosic ethanol production. J. of Sci. and Indust. Research 67; 865-873.

**Li, X. and Gao, P. (1997):** Isolation and partial properties of cellulose decomposing strain of *Cytophaga* sp. LX- 7 from soil. J. Appl. Microbil. 82:73-80.

**Lowry, O. H.; Rosebrough, N. J.; Farr, A. L. and Randall, R. J. (1951):** Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265-275.

**Miller, G.L. (1959):** Use of dinitrosalysilic acid reagent for the determination of reducing sugars. Anal. Chem. 31:426-428.

**Mishra, M. and Thakur, I. S. (2015):** Biodegradation of Lignocellulosic Waste in the Environment. Advances in Biodegradation and Bioremediation of Industrial Waste, 155.

**Mojovic, L.; Pejin, D.; Grujic, O.; Markov, S.; Pejin, J.; Rakin, M. and Vukasinovic, M. Nikolić, S. and Savić, D. (2009):** Progress in the production of bioethanol on starch-based feedstock. Chem. Ind. Chem. Eng. Q.; 15:211–26.

**Molina-Sabio, M. and Rodriguez-Reinoso, F. (2007):** Role of chemical activation in the development of carbon porosity. Colloids and Surfaces A: Physicochem Eng. Aspects; 24:15-25.

**Rani, S. R.; Patel, A. K.; Sukumaran, R. K.; Larroche, C., and Pandey, A. (2013):** Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Biores. Technol., 127, 500-507.

**Robson, L.M. and Chamblis, G.H. (1984):** Characterization of the cellulolytic activity of a *Bacillus* isolate. Appl. Environ. Microbil. 47, 1039–1046.

**Sabaa, M.F. and Sharaf, M.F. (2000):** Egyptian Policies for Rice Development. Cahiers Options the anaerobic cellulolytic thermophile *Clostridium thermocellum.* Appl. Environ. Microbiol.41, 1060–1062.

**Kirimura, K.; Furuya, T.; Nishii, Y.; Ishii, Y.; Kino, K. and Usami, S. (2001):** Biodesulfurization of dibenzothiophene and its derivatives through the selective cleavage of carbon-sulfur bonds by a moderately thermophilic bacterim *Bacillus subtilis* WU-S2B. J. Biosci. and Bioen. 91(3), 262-266.

**Lee, D.; Owens, V.N.; Boe, A. and Jeranyama, P. (2007):** Composition of Herbaceous Biomass

Mediterraneennes, 40, 25-36.

**Sangkharak, K.; Vangsirikul, P. and Janthachat, S. (2011):** Isolation of novel cellulose from agriculture soil and application for ethanol production. Ini. Lot' Adv. Biotechnol. and Resear. 2 (2): 230-239.

**Schwartz, J. L.; Garneau, L.; Savaria, D.; Masson, L.; Brousseau, R. and Rousseau, E. (1993):** Lepidopteran-specific crystal toxins from *Bacillus thuringiensis* form cation- and anion-selective channels in planar lipid bilayers. J. Membr. Biol. 132, 53-62.

**Shah, S. P.; Kalia, K. S. and Patel, J. S. (2015):** Optimization of cellulase production by *Penicillium oxalicum* using banana agrowaste as a substrate. J. General and Appl. Microbiol., 61(2), 35-43.

**Slade, R.; Bauen, A. and Shah, N. (2009):** The greenhouse gas emissions performance of cellulosic ethanol supply chains in Europe. Biotechnol Biofuels; 2:1–19.

**Sukumaran, R. K.; Singhania, R. R., and Pandey, A. (2005):** Microbial cellulases-production, applications and challenges. J. Sci. Indust. Resea., 64(11), 832.

**Taherzadeh, M.J. and Karimi, K. (2007):** Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a review. Bio Resources 2:707-738.

**Wang, c; Hseu, T. and Huang, C. (1988):** Induction of cellulose by cello- oligosaccharides in *Trichodcrma konigii* G-39. .J. Biotechnol.9:47-60.

**Yoosin, S. and Sorapipatana, C. (2007):** A Study of ethanol production cost for gasoline substitution in Thailand and its competitiveness. Thammasat Int. J. Sci. Technol. 12(1): 69-80.

**Zhu, S.Y.; Wu, Z.; Yu, X.; Zhang, H. Li. and Gao, M. (2006):** The effect of microwave irradiation on enzymatic hydrolysis of rice straw. Biores. Technol., 97: 1964-1968.