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# Optimization and production of antifungal hydrolysis enzymes by *Streptomyces aureofaciens* against *Colletotrichum gloeosporioides* of mango

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## ABSTRACT

**We isolated naturally occurring actinomycetes with an ability to produce metabolites having antifungal property against, *Colletotrichum gloeosporioides*, the causal agent of mango anthracnose. One promising strain was strong antifungal activity, was selected for further studies. Based on the physiological and biochemical characteristics, the bacterial strain was identical to *Streptomyces aureofaciens*. Culture filtrate collected from the exponential and stationary phases inhibited the growth of fungus tested, indicating that growth suppression was due to extracellular antifungal metabolites present in culture filtrate. Isolate highly produced extracellular chitinase and *β*-1,3-glucanase during the exponential and late exponential phases, respectively. In order to standardize the metabolite production some cultural conditions like different incubation time in hours, pH, carbon sources and concentrations and nitrogen source were determined. During fermentation, growth, pH and hydrolysis enzymes production were monitored.Treatment with bioactive components exhibited a significantly high protective activity against development of anthracnose disease on mango trees and increased fruit yield.**

**Keywords:** Antifungal; Colletotrichum Gloeosporioides; Mango Anthracnose And Streptomyces Aureofaciens

## 1. INTRODUCTION

Mango suffers from several diseases at all stages of its life. Anthracnose disease caused by *Colletotrichum gloeosporioides* (*Glomerella cingulata* Spauld & Schrenk) is one of the most common and serious diseases of mango (*Mangifera indica* L.) in the tropics [1]. The disease occurs at any stage of fruit growth and the pathogen causes the disease on a wide range of hosts such as apple, pear, guava and mango [2]. Flower blight, fruit rot, and leaf spots are among the symptoms of this disease [3]. Severe infection destroys the entire inflorescence resulting in no setting of fruits. Young infected fruits develop black spots, shrivel and drop off. Fruits infected at mature stage carry the fungus into storage and cause considerable loss during storage, transit and marketing . The most visible evidence of disease occurs on postharvest mango fruit by latent infection which usually results in commercial losses [4]. Disease control methods include the prophylactic use of fungicides such as benomyl, prochloraz, mancozeb, carbendazim, iprodione and thiabendazol. Chemical fungicides not only may pollute the atmosphere but also can be environmentally harmful, as the chemicals spread out in the air and accumulate in the soil, level and development of pathogen resistance [5]. Therefore, microbe-based biocontrol methods are one alternative way to control diseases in place of agrochemicals. Various microbial antagonists have been investigated as potential antifungal biocontrol agents of plant diseases. Many species of actinomycetes, especially those belonging to the genus *Streptomyces* (Gram-positive, mycelia-forming soil bacteria), are well known as biocontrol agents that inhibit or lyse several soilborne and airborne plant pathogenic fungi [6,7]. The antifungal potential of extracellular metabolites of *Streptomyces* strains against some fungi was previously reported from different locations of the world. The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of extracellular hydrolytic enzymes [8]. Chitinase and *β*-1,3-glucanase are considered to be important hydrolytic enzymes in the lysis of fungal cell walls [9-12]. However, data related to the antagonistic ability of the extracellular metabolites of *Streptomyces* strains to suppress the growth of the fungal pathogens *C.*

*gloeosporioides* having a broad host range are limited. Thus ,this study investigated to study i) antifungal activity of the cell-free culture filtrate of this antagonist to determine whether the production of the extracellular hydrolytic enzymes is involved in its observed effect ii) the optimization of antifungal metabolites production and using selected antagonistic bacteria for the control of pre- and post-harvest anthracnose on mango fruit.

## 2. MATERIAL AND METHODS

### 2.1. Organisms and Media

Isolation of the pathogen from mango fruit with anthracnose symptom was performed by tissue transplanting technique using potato dextrose agar (PDA). Stock cultures of *C. gloeosporioides* was maintained on PDA slants and stored at 4˚C. Actinomycetes were isolated from the root tissues of mango trees by the surface-sterilization technique [13]. All cultures were purified by streak plate technique and confirmed by colony morphology and screened for their antifungal activity [14].

### 2.2. *In Vitro* Antifungal Activity of Extracellular Metabolites in Cell-Free Culture Filtrates

To prepare the cell-free culture filtrate, the antagonist was cultured into broth medium and incubated on an incubator shaker (150 rpm) at 28˚C. The fermentation broth was collected during the exponential and stationary phases. Cells were removed by centrifugation at 8,000 rpm for 20 min at 4˚C. Cell-free supernatant was filtered aseptically through a sterile membrane with 0.45-µm pore size and stored at 4˚C. The growth inhibitory effects of the assay as described previously by Prapagdee *et al.* [15] with some modifications. Minimum Inhibitory Concentration (MIC) of these compounds were determined.

### 2.3. Identification of Isolated Antagonist

One promising isolate which showed a unique, stable and interesting property of inhibiting only dermatophytes was selected and characterized. Identification of the isolate to species level was based on morphological, cultural, physiological and biochemical characteristics as described by Taechowisan & Lumyong [16].

### 2.4. Effect of Cultivation Conditions on Enzyme Production

**Chitinase assay.** The reaction mixture contained 1 ml of 0.1% colloidal chitin in sodium acetate buffer (0.05 M, pH 5.2) and 1 ml culture filtrate was incubated at 37˚C for 2 h in a water bath with constant shaking. Suitable substrate and enzyme blanks were included. Chitinase activity was assayed by the colorimetric method of [16]. The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetraborate, pH 9.2 to 0.5 ml of reaction mixture and then boiled in a water bath for 3 min. Then 3 ml of diluted pdimethyl amino benzaldehyde (p-DMAB, Sigma Chemicals Company, USA) reagent was added and again incubated at 37˚C for 15 min. The released product in the reaction mixture was read at 585 nm in a spectrophotometer (Hitachi, Japan). One unit of chitinase activity was defined as the amount of enzyme, which produces 1 µ mole of N-acetylglucosamine in 1 ml of reaction mixture under standard assay condition.

***β*-1,3-Glucanase activity** was assayed by colourimetric method of Nelson [17]. Reaction mixtures were incubated at 37˚C for 30 min and were stopped by boiling for 5 min. One unit of B-1,3-glucanase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar equivalents (expressed as glucose) per min.

### 2.5. Optimization of Incubation Period, PH and Carbon and Nitrogen Sources Concentration for Antifungal Metabolite Production

The optimization of composition of incubation period, and cultural conditions was carried out based on stepwise modification of the governing parameters for antifungal production. The cultures were transferred to seed broth (200 mL of Medium) contained in a 500 mL Erlenmeyer flask and incubated at 30˚C on a rotary shaker (175 rpm) for 6 - 8 hours. A 500 mL Erlenmeyer flask containing 200 mL of the same seed medium was incubated as specified above. The seed culture was transferred to a 5 liter fermenter containing each one 3.5 liter of the three liquid media

### 2.6. Assay in Liquid Cultures

Bioagents growth was estimated directly by spectrophotometric measurement of the OD600 (Amax) using a PM 2A spectrophotometer and dry biomass concentration (bmax). Changing the pH 3 to 10 in the production medium the effect of pH was observed. The effect of cultivation temperature on the antifungal production was examined at different temperatures starting from 25˚C to 60˚C with 5˚C intervals.

### 2.7. Effect of Incubation Period on Antifungal (Enzymes) Production

The effects of incubation period were evaluated by 24 h interval by checking the antifungal activity were also

done. Hydrolysis enzymes was determined as previous above. Culture optimization was carried out based on stepwise modification of the governing parameters for metabolites production and bioassay test at incubation time in hours (24, 48, 72, 96 and 120); pH (6.0, 7.0, 7.5, 8.0 and 9.0); carbon sources and concentrations (glucose, Cellulose, fructose, starch and sucrose) and nitrogen source ($KNO_3$, $NaNO_3$, $(NH_4)_2SO_4$, $NH_4NO_3$, $NH_4Cl$, urea, casein, yeast extract and peptone) in three replicates. Fermentation studies were carried out in three stages. To prepare inocula (b), a loopful spore from a well-sporulated plate added each 40.0 ml respective seed medium in 250 ml Erlenmeyer flasks and incubated at 28.0˚C for 48 h on a rotary shaker (150 rpm). After optimization of the fermentation parameters, 2.0 ml of the seed culture (5.0%, v/v) was transferred to 250 ml Erlenmeyer flask containing 40.0 ml of the production medium. The yield of the antifungal metabolite was monitored in terms of arbitrary units (AU). Antifungal metabolite production was carried out in 100 ml starch casein medium (starch 1%, casein 0.1%, KH2PO4 0.05%, 0.05%, MgSO4.7H2O, pH-7) in 500 ml Erlenmeyer flasks.

### 2.8. *In Vivo* Evaluation of Antifungal Activity

To determine the efficacy of the antifungal metabolite against anthracnose pathogen, a field experiment was conducted under natural infested conditions, using the susceptible cultivars *i.e.* Sadekia (8˚ yr-old) at Noubaria region, El Behera and Ismailia Sedekia (15˚ yr-old) Governorates. Two sprayers were applied at 30 d intervals starting from 1 st March (about one month before normal flowering) on mango trees in one year. The bioactive components at active concentration mixed with 0.1% carboxymethyl cellulase (CMC) as sticker was sprayed using a knapsack sprayer. Trees were sprayed till run-off with approximately 2˚ L of spray solution per tree. Treatments were assigned in a randomized complete block design. Trees sprayed with water and fungicide served as a check treatment. Plots consisting of three mango trees were replicated three times. Irrigation, fertilization and other cultural practices were carried out as recommended. The disease incidence was determined as percentage of infected flowers at 30 days interval during the growing season (March to July). Fruit yield (kg/tree) were determined at harvesting date for treatments.

#### 2.8.1. Statistical Analysis

The collected data were evaluated statistically using the software SPSS for Windows (release 7.5.1, 20 December 1996; SPSS Inc., Chicago, IL). Data were subjected to analyses of variance and treatment mean values were compared by a modified Duncan's multiple test ($P > 0.05$).

## 3. RESULTS

### 3.1. Antifungal Activity

Preliminary screening for antifungal production was done by streak on agar medium .Subsequent screening of 10 isolates that showed inhibitory activity against test organism (**Table 1**). In particular, *S. aureofaciens* followed by *S. griseofuscus* S2 showed significant activities against pathogen. The antifungal activity of the purified active substance against pathogen was determined. The minimum inhibitory concentration (MIC) was determined by the diffusion method. The nutrient agar plates, seeded with test organisms were used for the MIC determination. The response was observed as a clear zone (mm) around the paper discs (diameter 0.5 mm) loaded with different concentration of active compound (20 µl) of each concentration were spotted on paper discs. In most cases, purified active substance of *S. aureofaciens* showed antifungal activity against *C. gloeosporioides* growth expressed as zone inhibition. The highest reduction was recorded at 1:3 concentration. The results in **Table 1** showed the isolate *S. aureofaciens* was the best isolate produces antifungal substances than the other *Streptomyces* isolates. So, *S. aureofaciens* was chosen in this study.

### 3.2. Enzymes Assays

The general ability of tested *S. aureofaciens* to produce secondary metabolites include hydrolysis enzymes was determined (**Figure 1**). Exochitinase and *β*-1,3-glucanase appeared to be conman metabolites produced by the tested BCAs. Maximum production of chitinase and *β*-1,3-glucanase by the tested *S. aureofaciens* in shaken broth culture occurred after 150 hrs (1.98 Unit/ml) and (2.9 Unit/ml), respectively.

### 3.3. Optimization Condition for Antifungal Metabolite Production

The time course for pH shows slight increase until 72 hrs then a negligible decrease through the remaining time. The dry weight increases with time through all the incubation period. The substrate decreases radically from the beginning of incubation time till 120 hr then almost stabilize. In the inhibition zone, enzymes curves, *Colletotrichum* follow the same pattern whereas the inhibition zone, chitinase and *β*-1,3-glucanase increases with incubation period till it reaches its maximum at 69 hr. The time course for optical density shows that the maximum

**Table 1.** Antifungal activity of *Streptomyces* spp. against *Colletotrichum gloeosporioides*.

| Isolate | Spore suspension | Purified active substance | |
|---|---|---|---|
| | Inhibition zone (cm) | Inhibition zone (cm) | MIC |
| *Streptomyces aureofaciens* | 2.6a | 2.4a | 1:32 |
| *S. albidoflavu* | 1.8c | 1.6c | 1:2 |
| *S. albidoflavus* | 1.9bc | 1.7b | 1:2 |
| *S. cyanocolor* | 1.8c | 1.7b | 1:2 |
| *S. griseofuscus* S1 | 1.8c | 1.3d | 1:1 |
| *S. griseofuscus S2* | 2.1b | 1.8b | 1:16 |
| *S. nodosus*, | 1.8c | 1.5bc | 1:2 |
| *S. alanosinicus* | 2.0b | 1.7b | 1:2 |
| *S. rochei* | 1.8c | 1.3d | 1:1 |
| *S. mutabilis*, | 1.8c | 1.3d | 1:1 |

Values represent the mean percentage of six replicates. Values in each column followed by the same letter are not significantly different ($P < 0.05$)

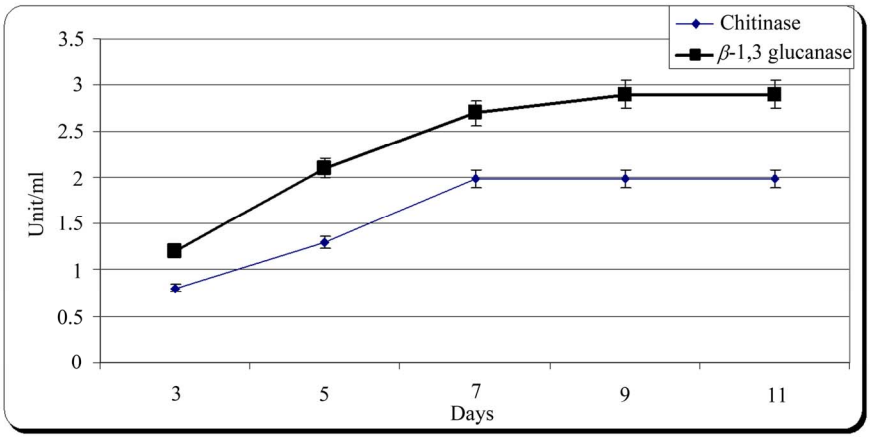



**Figure 1.** Hydrolysis enzymes activities in different days.

value is obtained at 36 hr incubation time, then a decline occurs. This is explained in the residual substrate curve as we notice that a gradual consumption of glucose occurs till 36 hrs then the glucose concentration remains constant for the rest of incubation time (**Figure 2**). From the experimental results it was noticed that by using different types of carbon source, the production of chitinase and *β*, 1-3 glucanse (unit/ml) were maximum with starch as carbon sources. Also, the zone inhibition of *Colletotrichum* was maximum after 48 h incubation with starch as carbon sources. So, the starch was chosen as an optimum carbon sources (**Figure 3**). By using different concentration of starch for the growth of *Streptomyces*, the dry weight concentration increased after 24 h with increasing the glucose and fructose concentrations till 5 g/l then decreased (**Figure 3**). Also, the production of *β*, 1-3 glucanse (unit/ml) and Chitinase increased after 48 h by increasing the stach concentration till 5 g/l then decreased again (**Figure 3**). The zone inhibition of *Colletotrichum* increased gradually by increasing the glucose concentration till 5 g/l then remained almost constant. From the previous observations, 5 g/l was chosen as the optimum glucose concentration (**Figure 4**). By using different types of nitrogen source for the growth of *Streptomyces* it was noticed that the maximum biomass growth was obtained using malt extract and soyabean as nitogen source while the maximum zone inhibition for *Colletotricum* were obtained with malt extract and CSL. The maximum production of Chitinase and *β*, 1-3 glucanse Enzymes were obtained with peptone and peptone in equal ratio in the media (**Figure 5**).

## 3.4. Field Experiments

More attention in the last decade has focused on replacing synthetic fungicides with new strategies, such as the use of biological control agents. The efficacies of the spraying mango trees *cv.* Sadekia with culture filtrate of *Streptomyces aureofaciens* on controlling of anthracnose disease were determined in season of 2010 in Boheria and Ismailia Governorates under nature infection conditions. In control treatment, the diseases incidence was higher on

leaves and flowers blossom clusters in both regions. Significant suppression of diseases incidence were achieved by applying of culture filtrate of *S. aureofaciens* compared to the fungicide and untreated control (**Table 2**). Application of bioactive components of *S. aureofaciens* gave completely reduced in all diseases, increased in flowering and yield of both regions (**Table 2**).

## 5. DISCUSSION

In recent years, anthracnose has become a major challenge to both the pathologists working with the pathogen and to mango researchers in general. The routine control measure involving chemical pesticide application leads to toxicity, residual effect and resistance development by pathogens. The current situation is mainly focused on biological control. Since all the commercial mango varieties are susceptible to the disease, biological control provides an effective, persistent and durable protection *Streptomyces aureofaciens* had the ability to exhibit high [18].

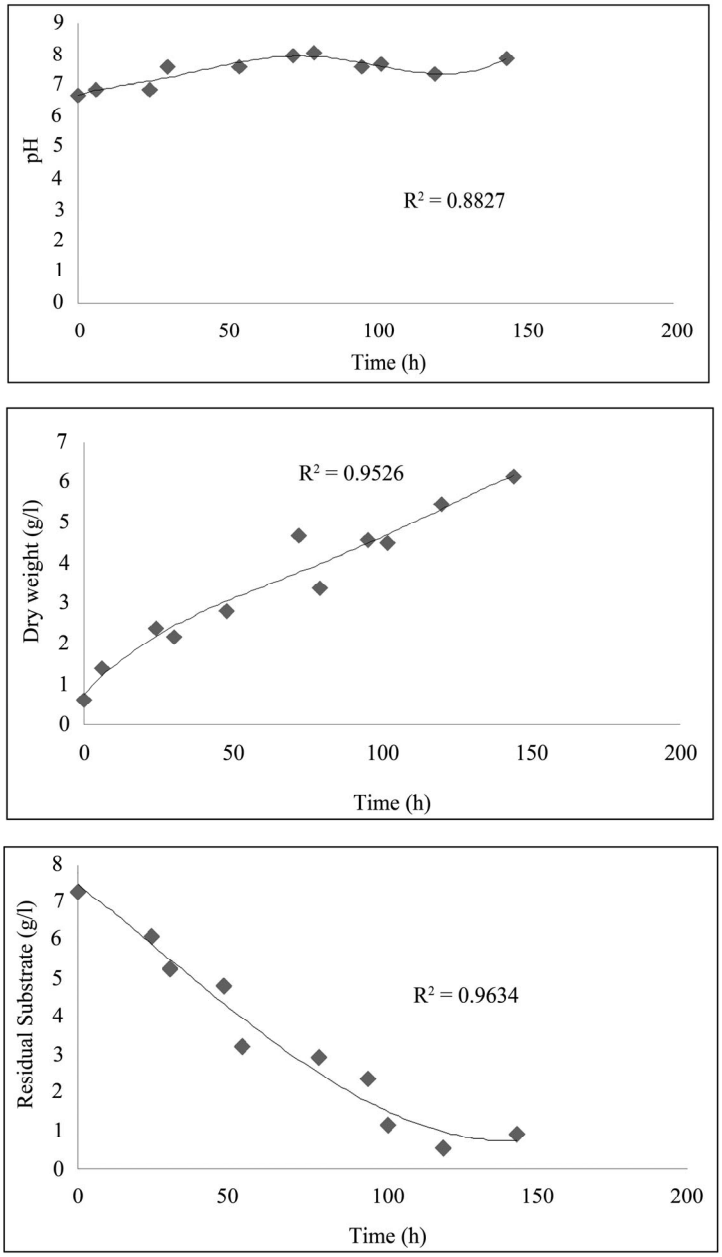



**Figure 2.** Effect of different nitrogen sources on the growth, inhibition activity and production of hydrolysis enzymes by *S. aureofaciens.*

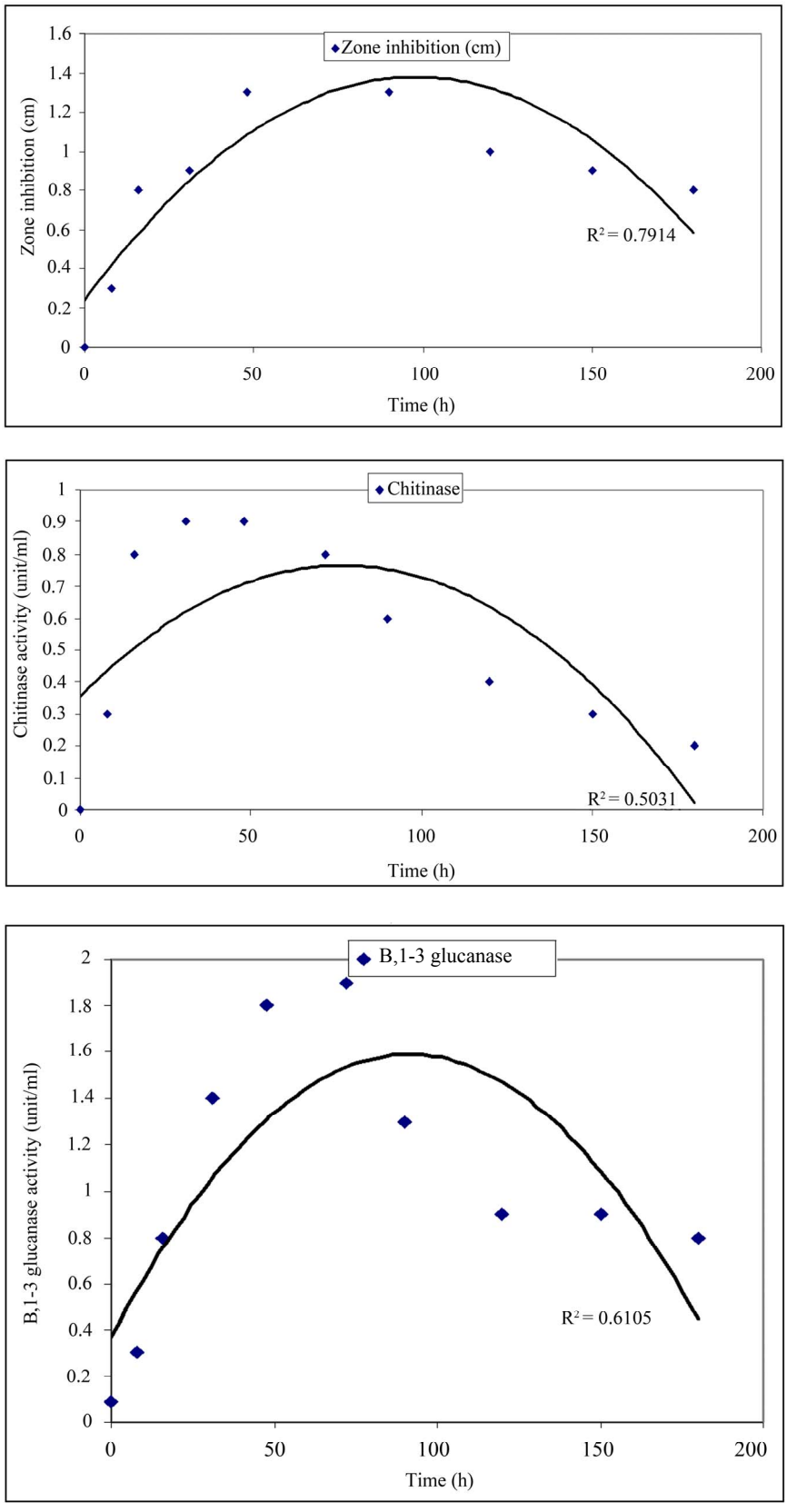



**Figure 2.** Cont.

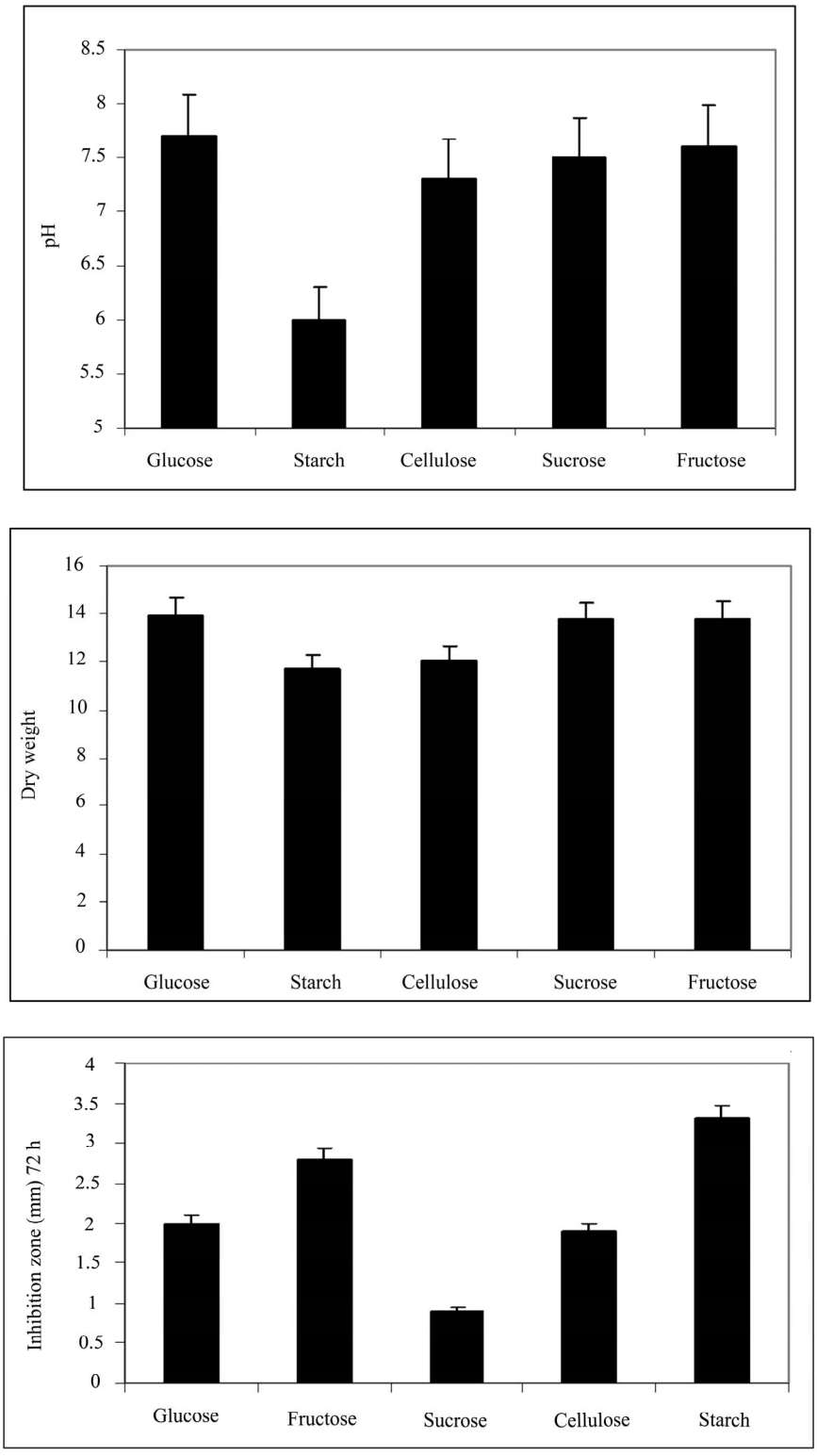
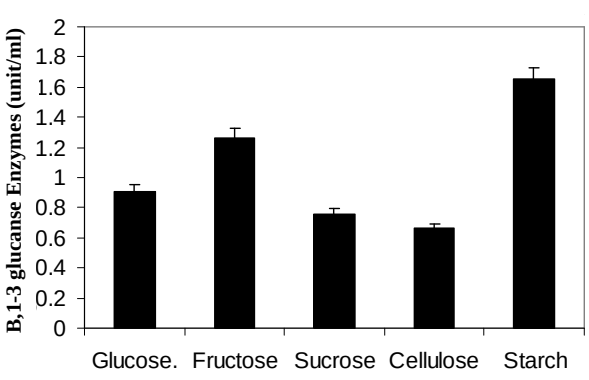
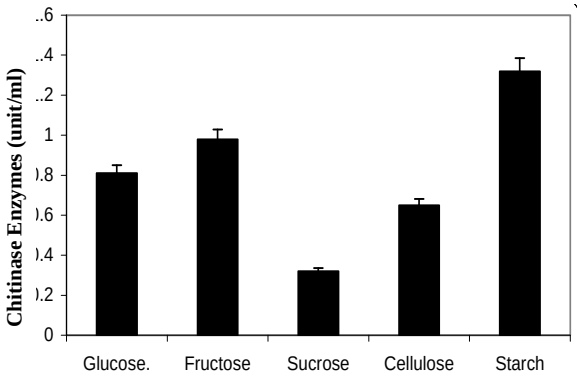



**Figure 3.** Effect of different carbon sources on the growth, inhibition activity and production of hydrolysis enzymes by *S. aureofaciens.*

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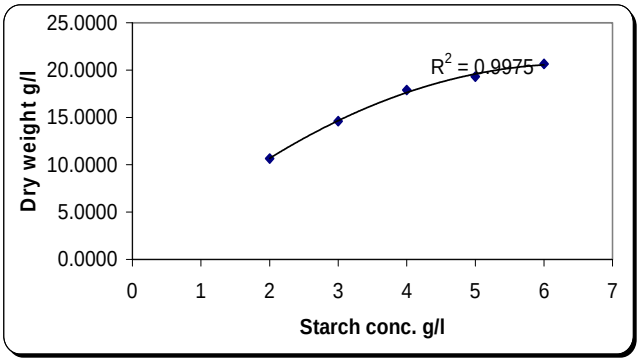


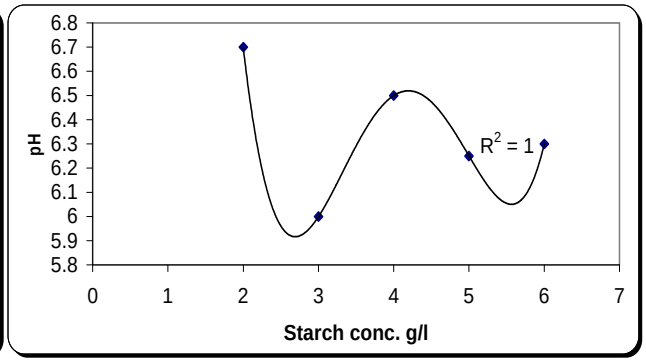
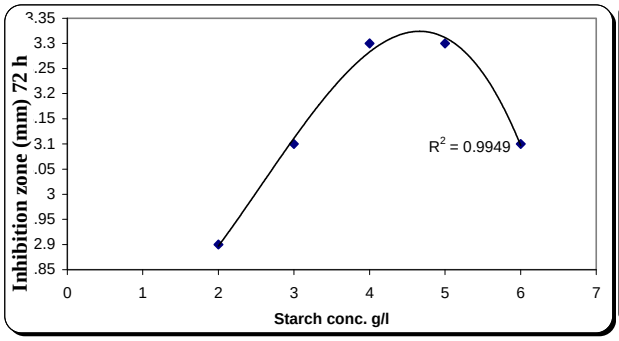
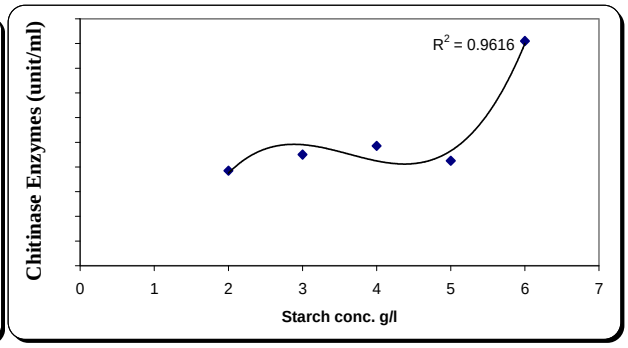




**Figure 4.** Effect of different carbon concentration on the growth, inhibition activity and production of hydrolysis enzymes by *S. aureofaciens.*



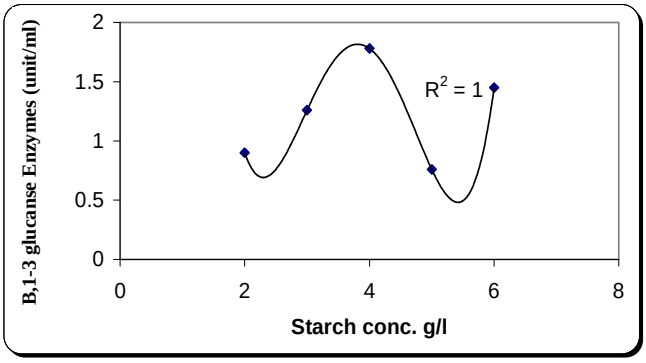




**Figure 4.** Cont.

antifungal activity *in vitro* against *Colletotrichum gloeosporioides*. The culture filtrate of this strain had also the ability to *in vivo* suppress infection of *Colletotrichum gloeosporioides* on mango trees. Many species of actinomycetes, especially those belonging to the genus *Streptomyces* , are well known as biocontrol agents that inhibit or lyse several soilborne and airborne plant pathogenic fungi [7]. It is well known that *Streptomyces* sp. can produce industrially useful compounds, notably wide spectrum of antibiotics, as secondary metabolites, and continues to be screened for new bioactive compounds [12]. Optimization of fermentation media ingredients and environmental factors for enzyme production is a more convenient and effective strategy, compared to other recent approaches like molecular techniques, to manifest the physiological characteristics to synthesis enzymes. Biological control of plant pathogens could reduce of this concern. More recently an increased number of researches focused on the potential of some types of bacteria, yeast, and actinomycetes as a biocontrol agent [9,11,6]. Regardless of the fermentation process that is used to grow cells, it is necessary to monitor and control parameters starting from the selection of optimum carbon and nitrogen sources and including inoculum volume, moisture content, pH, temperature, incubation period etc. Changes in one of these parameters can have a dramatic effect on the yield of cells and the stability of protein product. The meaning of optimization in this context needs careful consideration of the environmental and nutritional parameters for growth and production. Medium formulation is the foremost step for designing successful laboratory experiments for yield enhancement.

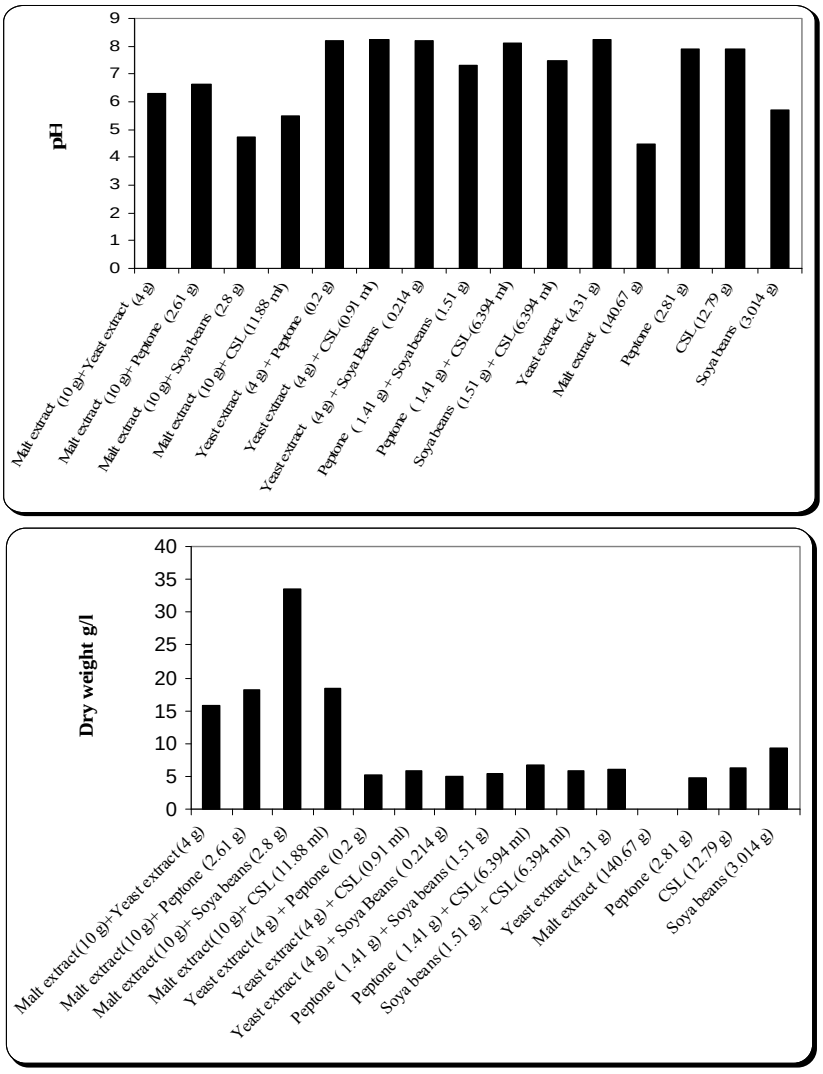



**Figure 5.** Effect of different nitrogen sources on the growth, inhibition activity and production of hydrolysis enzymes by *S. aureofaciens.*

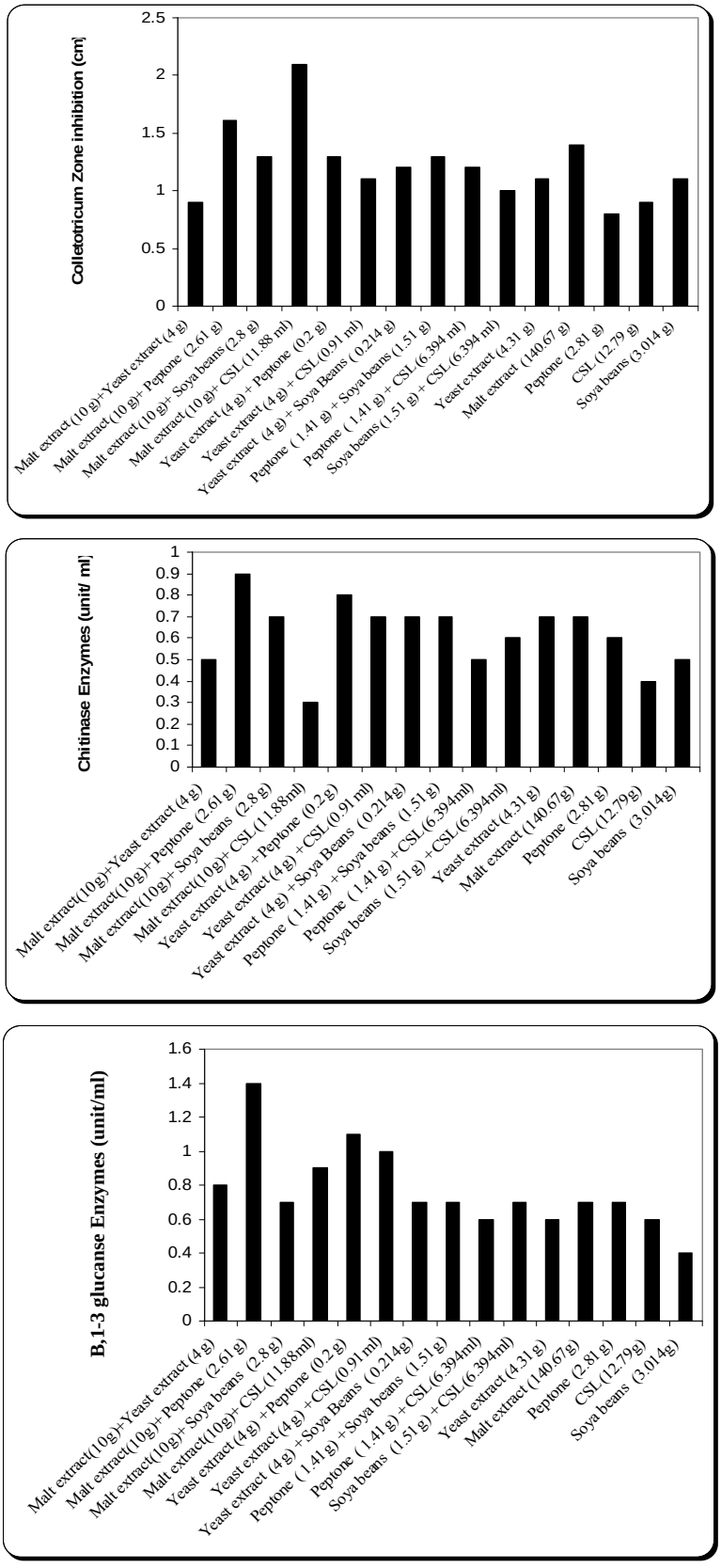



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**Table 2.** Efficacy of foliar sprays with bioactive substance of *Streptomyces aureofaciens* on the incidence of anthracnose disease and yield of Sadika and Ewais mango cultivars.

| Treatment | Percentage of disease reduction % | | | | | | | | Fruit yield | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Noubaria | | | | Ismailia | | | | Noubaria | | Ismailia | |
| | Leaves | | Fruits | | Leaves | | Fruits | | | | | |
| | Ewais | Sadikka | Ewais | Sadikka | Ewais | Sadikka | Ewais | Sadikka | Ewais | Sadikka | Ewais | Sadikka |
| **Control** | -- | -- | -- | -- | -- | -- | -- | -- | 8.5d | 12.9d | 11.5d | 21.3c |
| **Culture filtrate of** *Streptomyces aureofaciens* | 98.8a | 99.7a | 100a | 100a | 97.9a | 98.8a | 99.8a | 100a | 29.6a | 33.6a | 36.8a | 42.7a |
| **Fungicide (Benomyl)** | 76.9b | 74.6b | 75.7b | 74,6b | 66.8b | 73.9b | 77.6b | 75.7b | 18.5b | 21.5b | 20.7b | 26.4b |
| **LSD** | 8.87c | 6.46c | 8.54c | 6.46c | 8.38c | 6.54c | 7.76c | 7.43c | 4.65c | 6.54e | 5.41e | 6.49d |

Values represent the mean percentage of six replicates. Values in each column followed by the same letter are not significantly different ($P < 0.05$)

The medium components play a vital role in the production of antibiotic s and enzymes. In general, the productivity of microbial metabolites is closely related to the fermentation process used. In addition to physiological and genetic characteristics of strain, the medium composition plays an important role in the improvement of productivity. The changes of nutrients and their concentrations have different effects on the accumulation of different metabolites, which are controlled by intracellular effectors. Where, the carbon and nitrogen source can dramatically influence antibiotic formation [19].

Spray application of bacterial filtrate on mango treesprovided greater efficacy for controlling anthracnose disease suggested that the bacterial produce some antifungal enzymes for protecting the fruit against the pathogen. This strain is promising for industrial application since they grow quickly in broth condition in simple and of a low cost process to enhance production yield, and the excreted enzymes are frequently required for industrial applications. Therefore, it is thought to be considered as potential industrial candidate for effective saccharification process.

## 6. ACKNOWLEDGEMENTS

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