Metabolomics Analysis on Antifungal Activities Produced by *Penicillium oxalicum* T3.3 Grown on Different Types of Carbon Sources

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Abstract*—In-vitro antagonist tests such as disc diffusion and minimum inhibition concentration (MIC) were conducted against C. gloeosporioides.* ^{1}*H-NMR coupled with multivariate statistical analysis was carried out to identify possible compounds produced. Glucose crude extract exhibited the highest percent inhibition of radial growth (PIRG) with 75% and the lowest MIC value with 78 µg mL*$^{-1}$*. For metabolomics, different metabolites produced were clustered according to the carbon sources used and gave a representative impression of the metabolites produced by P. oxalicum T3.3. The study has shown the potential of using a combination of* ^{1}*H-NMR spectroscopy and multivariate statistical analysis and their correlation with MIC in differentiating the effect of carbon sources used based on the identification of possible metabolites contributing to their differences. Findings from this work may potentially provide the basis for further studies on both antimicrobial activities against plant pathogen and elucidation of the metabolite compounds produced by P. oxalicum T3.3.*

***Keywords*—*Colletotrichum gloeosporioides, metabolomics, Partial Least Square (PLS), Penicillium oxalicum*.**

I. INTRODUCTION

Colletotrichum gloeosporioides, known as one of the world's most plant pathogenic fungi can cause a serious damage to most parts of plants including stems, fruits, roots, leaves, and flowers but are often highly specific to individual tissues (Bailey *et al.* 1992). This pathogenic fungus attacked an extremely wide range of plants growing in both temperate and tropical environments. In Korea, *C. gloeosproriodes* had been identified as the cause of anthracnose disease that attack tulip trees as the necrotic lesions became black as the spots expanded on the leaves of that trees (Choi *et al.* 2012). The first report of anthracnose of *Pisonia alba,* commonly called lettuce tree was reported in India described the *C. gloeosproriodes* as the fungus that produced white mycelia, which became dark grey with later formation of numerous salmon pink colored spore masses (Vidyalakshmi and Divya, 2013).

In Malaysia, the first report on the occurrence of anthracnose disease in dragon fruit (*Hylocereus* spp.) caused by this fungus was reported by Masyahit *et al.* (2009). The infected stem and fruit had reddish-brown lesions or black spots symptoms where it can expand and merge to cover the whole affected area. At present, the great potential health benefit (Ching and Yusof, 2005), physic-chemical characteristics (Chuah *et al.* 2008) and nutritional value (Ariffin *et al.* 2009; Rebecca *et al.* 2008) of dragon fruit had been a great interest among the researchers, however the exploitation of natural organism as biological control and the potentialities of these microorganisms in production of bioactive metabolites and bio-control agents in controlling the pathogenic fungi has not receive any further investigations yet.

There is no agreement on which media are the optimal for metabolite production. However, according to Mathan *et al.* (2013), the growth media and incubation conditions have a very great influence on secondary metabolites production. Some of the physical and chemical parameters like pH, temperature, carbon and nitrogen sources play a major role on fungal growth and production of bioactive compounds and antimicrobial agents (Gunasekaran and Poorniammal, 2008; Mathan *et al.* 2013). The availability and type of carbon and nitrogen source gives effect on polyketide production whereby carbon

source such as glucose and sucrose have been found to increase the fungal growth and sporulation along with the high aflatoxin production (Keller *et al.* 2002).

Metabolomics can be described as a comprehensive quantitative and qualitative analysis of holistic metabolites present in a biological organism, which are the end-products of its gene expression (Van der kooy *et al*. 2009). There are many tools that can be used to analyze large number metabolites simultaneously such as nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and Fourier transform infrared spectroscopy (FTIR). Some of them rely on chromatographic separation step and others do not require any in which represent a global view of the sample (Ulrich-Merzenich *et al*. 2007). Among these tools, NMR method is able to provide a wide range of many molecular classes including sugars, amino acids, organic acids, alcohols or polyols, amine and ketones (Wishart, 2008).

P. oxalicum T3.3 had shown a promising antagonism in producing antifungal secondary metabolites that could inhibit the growth of *C. gloeosporioides*. Thus, the aim of this study was to determine the effect of different types of carbon sources for the production of antifungal compounds from *P. oxalicum* T3.3 against *C. gloeosporiodes*. Metabolomics approach was employed in identifying the possible compounds produced by theT3.3 strain exposed to the different carbon sources.

II. MATERIAL AND METHOD

2.1 Fungal strains and culture conditions

P. oxalicum T3.3 was obtained from the Industrial Biotechnology Laboratory culture collection. *C. gloeosporiodes*, a plant pathogenic fungus tested in this study was obtained from MARDI Serdang, Selangor. Stock cultures were maintained on potato dextrose agar (PDA) slant and stored at 18^{o}C. *P. oxalicum* and pathogenic fungi *C. gloeosporioides* were cultured on PDA plates and incubated at 30^{o}C.

2.2 Preparation of fungal inocula

To produce spores of *P. oxalicum,* a volume of 10 mL sterile distilled water was added to a 5-day culture of strain T3.3 on a PDA plate. A spore-suspension was made with a sterile glass rod to collect the spores and was diluted serially before the concentration was counted using a haemocytometer.

2.3 Submerged fermentation in different growth media

The spore suspension was inoculated into 500 mL Erlenmeyer flasks containing 250 mL sterilized medium. There are five types of medium were used in this study which are Richard medium, Czapek dox medium, Malt extract broth, Sabouraud dextrose medium and Potato dextrose broth. The cultures were grown at 30^{o}C, 120 rpm in rotary incubator shaker (Lab companion/S-971R) for 12 days. The samples were withdrawn at regular intervals of one day (destroy samples). The biomass was separated by the filtration through Whatman No 1 filter paper and the cell-free culture broth was extracted using organic solvent for further used in disc diffusion test.

2.4 Dry cell weight of mycelia

The samples of fermentation for each medium were harvested every day until the twelve day of the fermentation. The weight of Whatman filter paper was recorded before the filtration process. Then, the fungal mycelia were filtrated using the vacuum filtration. The filter papers containing the cells were dried at 60^{o}C overnight. Then the weight of the dried filter paper containing the cells was recorded.

2.5 Biolog FF microplate analysis

Biolog identification and carbon utilization test was done according to the method described by Tosiah, 2013. FF Microplate with 95 wells prefilled with different carbon sources and a single well prefilled with water as control were used to identify the carbon utilization of strain T3.3. A volume of 100 µL of spore suspension with inocula turbidity of 75%±2% at A590 nm was dispersed into each the microplate well and incubated at 26^{o}C. The mycelia growth based on turbidity and the change of optical density were measured at dual wavelength 490nm and 750nm using microlog 3 software. After 72 h of incubation, the mycelia growth and metabolic reaction of *P. oxalicum* T3.3 were measured at 490 nm and 750 nm respectively.

2.6 Cultivation with different carbon sources

A volume of 250 µL of *P. oxalicum* spores suspension (10^4 spores mL^{-1}) was inoculated into fresh medium containing: KNO_3 0.25 g, KH_2PO_4 0.125 g, MgSO4 0.0625 g, $FeCl_3$ $2.5x10^{-4}$ g and carbon source 7.5 g. The fermentation was carried out in

500 mL Erlenmeyer flasks containing 250 mL medium on rotary shaker at 120 rpm, 30°C for 10 days, and the culture filtrates were used for the extraction process. The carbon sources used; glucose, maltose, sucrose, xylitol, starch and *U. pinnatifida*, edible seaweed, were substituted into fermentation media.

2.7 Extraction of metabolites

After 10 days of fermentation, the fermented broths were filtrated to separate the cells by using 90 mm Whatman filter paper. The supernatant was then centrifuged at 10000 x g for 15 min before extracting with organic solvent, ethyl acetate. An equal volume of ethyl acetate was used for liquid-liquid extraction of the chemical compounds from *P. oxalicum* T3.3 supernatant. The suspension was vigorously mixed by using magnetic stirrer. After 1 h, the suspension was poured into a separatory funnel with an equal volume of ethyl acetate as the solvent and the organic layer was collected. The organic layer was dried and concentrated using a rotary evaporator (Rotavapor R-3, BüCHI, Switzerland). The extraction process was repeated twice to maximize the extraction of the organic compounds. The pooled fractions were dried, weighed and stored before subjected to antagonist test and NMR analysis.

2.8 In-vitro susceptibility test against *C. gloeosporioides*

2.8.1 Disc diffusion test

Ten mg of the dried samples from previous extraction was dissolved with 1 mL ethyl acetate. A sterile Whatman filter paper with diameter 1 cm was dipped into the sample solution and was dried in sterile condition for 1 hour. Then, seven days old 5 mm disc of pathogen will be cut near the periphery of the colony using sterile cork borer and place on one side of the PDA plate. Similarly, the filter paper impregnated with the sample solution be place on other side of PDA plate and was kept at a distance of 1 cm at an angle of 180°. Disc impregnated with ethyl acetate was act as a negative control while disc impregnated with cyclohexamide (commercial antibiotics) was act as a positive control. All PDA plates were incubated at 30 ± 1°C for seven days and the inhibition zone around the disc was observed and measured. The percentage of inhibition was measured according to this formula:

Percent inhibition of radial growth (PIRG): (R1-R2)/R1

Where, R1 = radial growth of pathogen in control plate

R2 = radial growth of pathogen in opposed plate

2.8.2 Spores suspension preparation

Inoculum suspensions of filamentous fungi were prepared by the method of NCCLS M38-A (NCCLS, 2002). Briefly, *C. gloeosporioides* were grown on a PDA at 30°C for 5 days. The five-day-old colonies were covered with approximately 10 mL of sterile 0.85% saline, and suspensions were made by gently probing the colonies with the tip of Pasteur pipette. The resulting mixture of conidia and hypha fragments was withdrawn and transferred to a sterile tube. After heavy particles were allowed to settle for 3 to 5 min, the upper homogenous suspensions were collected and mixed with a vortex mixer for 30 s. The concentration of the conidia was count using the counting chamber, haemocytometer.

2.8.3 Minimum Inhibition Concentration (MIC)

Individual MICs was determined following the broth microdilution method recommended by NCCLS, approved standard M38-A (NCCLS, 2002), as modified by Espinel-Ingroff *et al.* (2002) and Santos *et al.* (2006). The broth microdilution tests were performed using sterile and disposable 96-well flat bottomed microtitration plates. Each microdilution well containing 100 µL of the two-fold antifungal concentration was inoculated with 100 µL of medium of the diluted of inoculum suspension. For each test plate, two antifungal free controls were included, one with medium alone and the other with 100 µL of medium plus 100 µL of inoculum suspension. Each type of inoculums suspension was treated with cyclohexamide (Sigma, USA) and T3.3 fungal extracts. The concentration assayed ranged from 0.039 to 10 mg mL^{-1}. The plates were incubated at 30°C and the endpoints were read visually after 5 days incubation. Assays were always run in duplicate (Rukayadi and Hwang, 2007).

2.9 Nuclear magnetic resonance analysis

Ten milligrams of each dried fraction was dissolved with 0.6 mL of deuterated acetone containing 0.05% Tetramethylsilane (Merck, Germany) as the reference peak. The samples were sonicated and centrifuged for 3 minutes and 10 minutes, respectively before directly transferred into 5 mm NMR tubes and subjected to ^{1}H-NMR analysis. ^{1}H-NMR spectra were

acquired at 25^0C on a Varian Unity INOVA 500 MHz spectrometer (Varian Inc, CA). All spectra were manually phased and baseline corrected. For each sample, 64 scans were recorded and the spectral width was adjusted to a range between 0.00 and 10.00 ppm.

2.10 Data processing and analysis

The ^{1}H-NMR spectra were automatically reduced to ASCII format file using Chenomx software (v. 5.1, Alberta, Canada). Spectral intensities were scaled in reference to TMS and binned into regions of 0.04 ppm width for the spectral region of 0 to 10 ppm. The signals between δ2.04-2.1 were excluded from the analysis since this due to residual signal from the deuterated acetone. The binned ^{1}H-NMR data were then subjected to PCA and PLS, performed using SIMCA-P+ version 12.0.1.0 (Umetrics AB, Umeå, Sweden) in unit Pareto scaling was applied in all analyses.

III. RESULTS AND DISCUSSION

3.1 Dry cell weight and antifungal activity of *P. oxalicum* T3.3

Based on Fig. 1, the highest dry cell weight was achieved in Richard medium on day 10 of fermentation compared to other media. Meanwhile, Malt extract broth and Sabouraud medium recorded lowest dry cell weight until the end of fermentation. The antifungal activities of the crude extracts of *P. oxalicum* T3.3 grown in different media were not detected during the early fermentation day. It is interestingly to note that the antifungal activity through disc diffusion test was only detected for Richard and Czapek dox medium on 10^{th} day of fermentation and decreased on subsequent day. Solvent fractions from both media showed the highest antifungal activity with 20% PIRG value during tenth and eleventh day of fermentation period. In contrast, Santamarina *et al.* (2002) reported that extracts from isolates of *P. oxalicum* Corrie and Thom cultured in PDB was more suitable for the production of antibiotic compounds. It can be deduced that the antifungal production was directly proportional to the biomass produced as the antifungal activity showed highest activity when the biomass was at the highest weight. Since the minimum antifungal activity of this strain was detected during 10^{th} day of the fermentation using Richard medium, this medium was hence selected in maximizing the production of antifungal compound in different carbon sources.

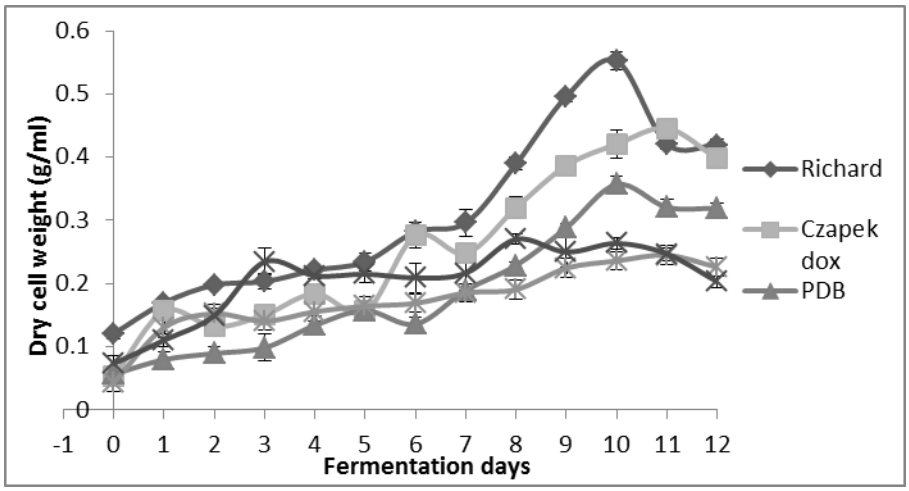


FIG. 1: DRY CELL WEIGHT OF *P. OXALICUM* T3.3 GROWN IN DIFFERENT FERMENTATION MEDIA AT 30^oC AND PH 5.5. ERROR BARS REPRESENT STANDARD DEVIATION FROM THREE REPLICATES

3.2 Biolog FF Microplate Analysis

Based on Biolog FF Microplate analysis, the growth of *P. oxalicum* T3.3 at 72 hour on 95 carbon sources and water as a control had shown variation. In this study, glucose, maltose, sucrose and xylitol were selected from the Biolog FF Microplate analysis and were used as the carbon sources in the fermentation medium (Table 1). This finding proved that *P. oxalicum* T3.3 is a versatile filamentous fungus as it able to utilize wide range of carbon sources including monosaccharides, disaccharides, polysaccharides and sugar alcohols. However, there are some of the carbon sources which are not suitable for the growth of this strain. According to Singh (2009), the growth of the cultures used has shown strong correlation between substrate utilization, antifungal activity and presence of the responsible secondary metabolite produced. However, some of the substrates tested did appear to support growth but not the production of the antimicrobial metabolite. Papaspyridi *et al.* (2011) supported that substrate utilization fingerprint obtained from Biolog FF Microplate analysis was useful in the

optimization of media components for maximum biomass as well as secondary metabolite production for the various cultures (Singh, 2009).

TABLE 1
CARBON-BASED SOURCES CONSUMPTION BY STRAIN T3.3 IN BIOLOG FF MICROPLATE ANALYSIS AFTER 72H

Well	Carbon	Turbidity (+)*
C11	Maltose	+++
E7	Sucrose	+++
E11	Xylitol	+++
C12	Maltotriose	+++
B12	α-D-glucose	++
A12	D-cellobiose	++
B5	D-fructose	++
E9	D-trehalose	++
A3	N-acetyl-D-galactosamine	-
E5	L-sorbose	-

'+++' represent the turbidity of Strain T3.3 is high, '++' means that turbidity is moderate, and '-' means no turbidity can be observed. Turbidity is referring to the biomass concentration of Strain T3.3.

3.3 In-vitro antagonist tests against *C. gloeosporioides*

3.3.1 Disc diffusion test

Disc diffusion methods are widely used to investigate the antimicrobial activity against certain fungi or bacteria using the Kirby-Bauer method (Bauer *et al*. 1966) with a minor modification. These assays are based on the use of discs as reservoirs containing solutions of substances to be examined (Bartner *et al.* 1994). A filter paper disc impregnated with sample fraction was placed on the agar in which the chemical constituents in it would be diffused from the disc into the agar around the disc. The solubility of the components and their molecular sizes determined the circumference of the area around the disc. Once *C. gloeosporioides* was placed on the agar, after the incubation period, it would not grow in the area around the disc when it was susceptible to the components. This area of no growth around the disc is known as a "zone of inhibition". The findings from this study revealed that, crude extracts of *P. oxalicum* T3.3 in all carbon sources shows a significant reduction in colony radial growth against *C. gloeosporioides* as showed in Fig. 2. Glucose crude extract of *P. oxalicum* T3.3 exhibited the highest antifungal activity with 75% inhibition against *C. gloeosporioides.* This was followed by sucrose crude extract and maltose crude extract which exhibited 40% to 30% inhibition, respectively. Xylitol and *U. pinnatifida* crude extracts, less than 25% antifungal activity were recorded (25 to 20% inihibition), respectively. Starch crude extract of *P. oxalicum* T3.3 shows the lowest reduction in colony radial growth with only 10% inhibition against *C. gloeosporioides.*

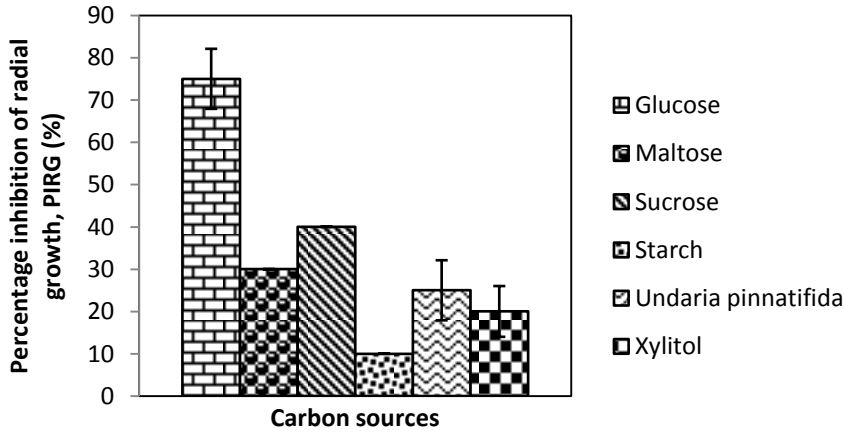


FIG. 2 PERCENTAGE INHIBITION OF RADIAL GROWTH, PIRG (%) FOR DIFFERENT CARBON SOURCES USED

Glucose extract of *P. oxalicum* T3.3 was found to be the most effective against *C. gloeosporioides* with more than 70% inhibition of radial growth (7.5 mm) compared to other carbon sources. Carbon sources played an important role in the onset and intensity of secondary metabolites produced (da Silva *et al.* 2012). Specificity in the production of antifungal compound towards *C. gloeosporioides* related to the carbon sources might be suggested as the reason why *P. oxalicum* T3.3 could not

give a high antifungal activity in all carbon sources. The finding in this study is in total agreement with Gebreel *et al.* (2008), wherein reported a higher production of antifungal activity was observed to be at the highest when glucose was used as the carbon sources. As reported by Lucas *et al.* (2007), the isolation of bioactive compounds such as pencolide, sclerotiorin and isochromophilone VI from the cultivation of *Penicillium sclerotiorum* in a liquid medium rich in glucose has shown a diameter of inhibition zone ranges from 10-16.75 mm against various bacteria species.

The utilization of glucose as a sole carbon source is preferable compared to others because glucose is the simplest sugar (monosaccharide) where it can be easily consumed by the cells as a source of energy and metabolic intermediate without any breakdown process. The inhibition zone produced by the crude extracts of *P. oxalicum* T3.3 was considered higher since the metabolites are in a mixture form where the crude did not undergo any isolation or fractionation of the bioactive compounds. This proved that the inhibitory action of the crude extracts showed a clear indication that the crude extracts contained active components that have antifungal properties. However, the efficiency of substituting starch as carbon source was not efficient enough since the starch extract of *P. oxalicum* T3.3 shows the lowest inhibition zone against *C. gloeosporioides.* This result was in total agreement with Mathan *et al.* (2013), where starch was the least utilized carbon compound by *Aspergillus terreus* KC 582297and produce lowest inhibition zone (6mm) whereas starch crude extract of *P. oxalicum* T3.3 producing 1 mm inhibition zone.

3.3.2 Minimum Inhibition Concentration (MIC)

Minimum inhibition concentration (MIC) of extracts of glucose, sucrose, maltose, xylitol, starch and *U. pinnatifida* were examined and recorded (Table 2). Minimum inhibitory concentration of glucose crude extracts the most active with the lowest MIC value with 78 µg mL^{-1}, followed by sucrose crude extracts with 156 µg mL^{-1}. Both maltose and *U. pinnatifida* crude extracts resulting on a MIC value of 313 µg mL^{-1}. In contrast, both crude extracts of xylitol and starch were much less active for all the tested strains with the highest MIC value, 1250 µg mL^{-1}.

TABLE 2
MIC VALUES FOR *P. OXALICUM* T3.3 EXTRACTS AGAINST *C. GLOEOSPORIOIDES*

Carbon sources	MIC (µg mL^{-1})
Glucose	78
Maltose	313
Sucrose	156
Starch	1250
Xylitol	1250
U. pinnatifida (seaweed)	313

The MIC of the glucose extract in this study is 78 µg mL^{-1} which is comparable with the MIC recorded for the metabolites produced by *P. sclerotiorum* isolated from Brazilian's cerrado soil samples (Lucas *et al.* 2009). Both Pencolide (I) and Isochromophilone VI (III) showed MIC values 64 µg mL^{-1} against *C. albicans*. Meanwhile, the sucrose extract with 156 µg mL^{-1} against *C. gloeosporioides* obtained from this study was considered higher when compared with Sclerotiorin (II) against *S. aureus* with MIC 128 µg mL^{-1}, metabolite produced from the same fungus (Lucas *et al.* 2007). According to Petit *et al.* (2009), there are three new naphthalenoids has successfully characterized from a *Penicillium* sp. isolated from Brazillian cerrado soil which consumed glucose as their carbon source in fermentation media. The corresponding minimum inhibition concentrations were determined and natural secondary metabolite methyl 6-acetyl-4-methoxy-5,7,8-trihydroxynaphthalene-2-carboxylate showed to be the most active compound against *Candida albicans* with MIC 32 µg mL^{-1} meanwhile against *Listeria monocytogenes* and *Bacillus cereus* with MIC value 64 µg mL^{-1} for both. Since there were no any isolation and purification steps for the crude extracts, the compounds responsible for the susceptibility test cannot be determined. However, from this test we can conclude that the metabolites produced by *P. oxalicum* T3.3 were able to suppress the growth of the pathogen, *C. gloeosporioides.* There are many secondary metabolites and chemical constituents produced by *P. oxalicum* extracts that may contribute to the observed positive antifungal effects. The MIC value obtained in this study explains that *P. oxalicum* T3.3 extracts act as antifungal agent against *C. gloeosporioides.*

3.4 Metabolomics of different carbon sources used using PCA and PLS data analysis

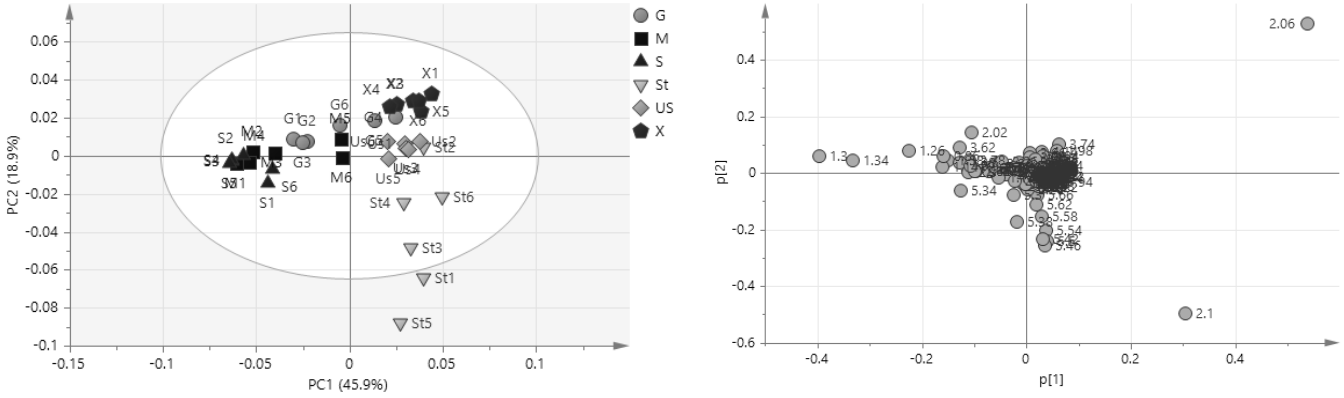
3.4.1 Principal component analysis (PCA)

Visual inspection of the ^{1}H NMR spectra of the six different carbon sources used showed the presence of different classes of metabolites which included fatty acids, amino acids, organic acids, alcohols, and sugars. Principal component analysis (PCA)

was used for the clustering of the six samples of different carbon sources and the metabolites contributing to the variability. The application of PCA in multivariate analysis by disclosing the samples to different principal components (PCs) is to identify the pattern and cluster of the samples depending on their variance (Mediani *et al.* 2012). All assignments of the ^{1}H NMR signals were accomplished by comparison with ^{1}H NMR spectra of reference compounds from Chenomx database and Yeast Metabolome Database (YMDB, http://www.ymdb.ca/) as shown in Table 3.

TABLE 3
LIST OF ^{1}H-NMR CHEMICAL SHIFTS AND VIP VALUES OF THE MAJOR COMPOUNDS CONTRIBUTING TO THE CLASSIFICATION IN THE PLS MODEL

^{1}H NMR chemical shift (δ)	Compound	VIP value	References
2.06	Acetic acid	6.56	Kim *et al.* (2011)
2.1	Methionine	4.97	Chenomx database
1.3	Threonine	4.77	Seung *et al.* (2012)
1.34	Lactate	3.62	Chenomx database
1.26	Isopropanol	3.02	Chenomx database
1.14	2,3-Butanediol	2.2	Georgiev *et al.* (2011)
3.74	Glycerol	2.06	YMDB
3.66	Leucine	1.98	Chenomx database
0.86	Butyrate	1.78	Chenomx database
5.42	Sucrose	1.72	Seung *et al.* (2012); Georgiev *et al.* (2011)
2.3	Valine	1.67	Chenomx database
2.46	*N*-carbamoylaspartate	1.62	Chenomx database
1.58	Butyrate	1.61	Chenomx database
3.94	Serine	1.25	Chenomx database
3.98	Serine	1.24	Chenomx database
0.9	Valine	1.23	YMDB
3.58	Threonine, fructose	1.17	Chenomx database
2.78	5-aminolevulinate	1.16	Chenomx database
6.58	Cinnamate	1.15	Chenomx database
4.18	*o*-Phosphoserine	1.13	Chenomx database
4.14	Uridine	1.07	Chenomx database
3.7	Leucine,glucitol	1.02	Chenomx database
7.46	Cytosine	1.01	Chenomx database



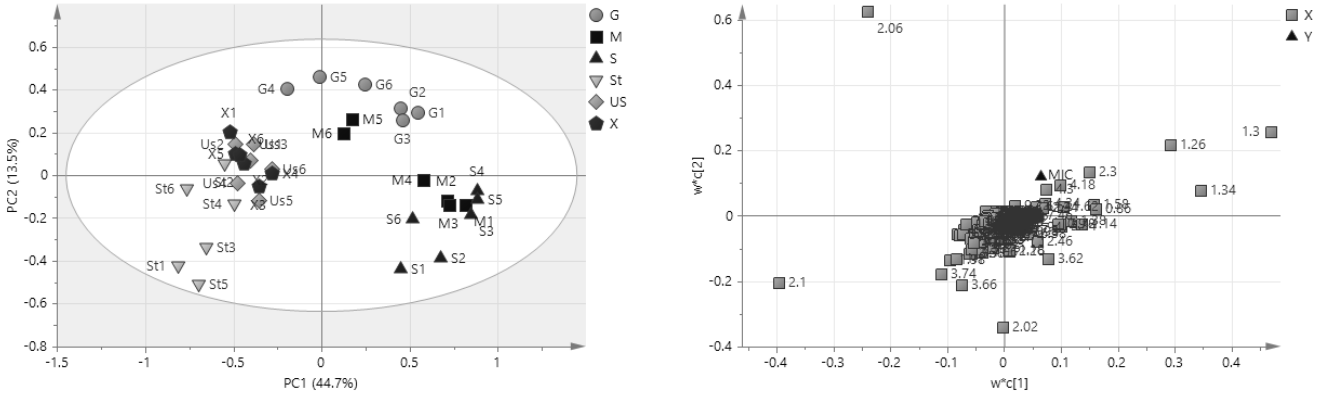
(A) **(B)**

FIG. 3: SCORE SCATTER PLOT (A) AND LOADING SCATTER PLOT (B) OF PCA SEPARATED BY PC1 AND PC2 OF THE STRAIN T3.3 EXTRACTS. SCORE PLOT (A) SHOWS THE DISCRIMINATION OF SIX CARBON SOURCES USED. LOADING PLOT (B) INDICATE THE 1H-NMR SIGNALS OF COMPOUNDS THAT ARE RESPONSIBLE FOR THE SEPARATION OF THE SIX CARBON SOURCES. (A) (●): glucose, (■): maltose, (▲): sucrose, (▼): starch, (♦): *U. pinnatifida*, (): xylitol

In this study, initial PCA which is an unsupervised analysis method was performed on the NMR data of the *P. oxalicum* T3.3 grown in different carbon sources to determine the primary observation between the clustering samples and to evaluate the variance in their metabolite content and concentration. The score plot was implemented to evaluate the variation within the processed samples with regard to the six carbon sources used, whereas the loading plot indicated the metabolite signals that may contribute to cluster differentiation as shown in Fig. 3(A) and (B). From the PCA score scatter plot (Fig. 3A), six samples were separated into two clusters between the carbon sources along PC1 with two notable outliers (St1 and St2). PC1 showed the most sample variation, followed by PC2. An eigen value of about 64.8% was described by the first two PCs wherein the first component PC1 contributed 45.9% of the variance, meanwhile by PC2 with 18.9%. The loading plot of the carbon sources (Fig. 3A) indicated the important chemical shifts responsible for discrimination in the score plots. The accumulation of the signals at the centre of the PC1 and PC2 axes indicating the consumption of these carbon sources might produce similar compounds. However, the intensity and concentration of each compound produced may differ and can be determined from the NMR spectra. The loadings illustrated the correlations between the original variables (chemical shifts) and the new variables which are the PCs (PC1, PC2, etc.). The signals in ppm on the loadings plot that fall within the same quadrant represent specific NMR spectral regions and the location of clusters in a score plot situated in the same quadrant/dimension in the loading plot is remarkably influenced by these variables (Khatib *et al.* 2011).

3.4.2 Partial least square (PLS)

The previous MIC results showed *P. oxalicum* T3.3as having the ability in producing metabolites that could inhibit the growth of *C. gloeosporioides*. Thus, in order to have further investigation between the correlation of MIC and the impact of the six carbon sources used, a supervised analysis which is known as Partial least square (PLS) was applied using a validation model with a degree of over fit between the variables and the responses (Maisuthisakul *et al.* 2008). PLS expands a regression of PCA and uses class data to maximize the separation between groups of observations. This frequently used classification approach is categorical which pronounces the class membership of the statistical units (Eriksson *et al.* 2006; Barker and Rayens, 2003). Furthermore, PLS was performed to acquire a more precise view on the correlation between the MIC activity and the carbon sources used. PLS has been reported as a better statistical model in linking DPPH with phytochemicals and also serves as a model for prediction (Kim *et al.* 2011). According to Maisuthisakul *et al.* (2008), the PLS model could illustrate the correlation between chemical and DPPH data, which used in predicting variations among dried samples. The determination of a correlation between anti-radical activity ($1/IC_{50}$) and phytochemical composition of extracts from different drying effects samples also has been done by using PLS evaluation (Mediani *et al.* 2012).



(A) **(B)**

FIG. 4: PLS-DERIVED SCORE PLOT (A) AND PLS-DERIVED LOADING PLOT (B). (A) (●): glucose, (■): maltose, (▲): sucrose, (▼): starch, (♦): *U. pinnatifida*, ⬟): xylitol. (B) (■):^{1}H-NMR signals of compounds that are responsible for the separation of the six carbon sources, (▲): MIC value as the Y-variable

In this study, PLS was performed using the ^{1}H NMR data of the extracts/fractions and the data were scaled using Pareto (par) by the SIMCA-P 12.0 software. In the PLS analysis, samples were well separated and showing a significantly obvious discrimination between the carbon sources along PC1 (Fig. 4A). Meanwhile, the monosaccharide and disaccharide sugar samples were strongly correlated with MIC rather than complex sugar samples as shown in Fig. 4B.The metabolites produced by the extracts from monosaccharide and disaccharide group of sugars, glucose, maltose and sucrose were well separated along PC1 from those extracts of the polysaccharide and complex sugar of starch, xylitol and *U. pinnatifida*. The

spectral data of the glucose and sucrose samples were relatively close with those of maltose and xylitol, while *U. pinnatifida* and starch samples were found to partially overlapped with each other.

The monosaccharide and disaccharide sugar samples were strongly correlated with MIC. Meanwhile the complex sugars were projected in the negative side of the plot far away from MIC. The compounds contributed to this separation by PC1 were identified as threonine (δ1.3), 2-heptanone (δ1.26), lactate (δ1.34), valine (δ2.3), butyrate (δ1.58 and δ0.86), and *o*-phosphoserine (δ4.18). All of these compounds were located closer to monosaccharide, disaccharide samples and MIC activity rather than complex sugar samples as shown in Figure 4B. However, the complex sugar samples were separated from the others by their high content of acetic acid (δ2.06), methionine (δ2.1) and leucine (δ3.66). This finding confirmed that the MIC, which showed that the extracts from glucose and sucrose had the highest positive impact on the in-vitro susceptibility test against *C. gloeosporioides.*

PLS model validation was carry out by checking the Q^2 and R^2 accumulative means after cross validation and permutation test (Mediani *et al.* 2012). The goodness of fit of the PLS model was performed and represented by R^2Y, where R^2 determines the model fitness, while the predictive ability was indicated by Q^2Y in which, Q^2 provides the predictive quality of the model (Barker and Rayens, 2003). Generally, R^2Y varies between 0-1 and 1 indicates a model with a perfect fit. Meanwhile models with Q^2Y values >0.5 and >0.9 are considered to have excellent and good predictability. As shown in Table 4, R^2Y and Q^2Y values for PLS model in this study are 0.830 and 0.687, respectively which are a good fitness and predictive abilities. Furthermore, permutation test was performed with 20 permutations to validate the PLS model. According to Eriksson *et al.* (2006), permutation test provides the statistical significance of the estimated predictive power of models by comparing R^2Y and Q^2Y values from the original model with the values for a reordered model, which was created newly whenever Y-data was permutated at random. It was noted that R^2Y-intercept should not exceed 0.3-0.4 and that Q^2Y-intercept should not exceed 0.05. The R^2Y-intercept and Q^2Y-intercept values for the PLS model in the present study were 0.449 and -0.408 respectively as shown in Table 4. Even though the R^2Y-intercept value is slightly higher than the validation value, with proper Q^2Y-intercept value that was under recommended cut-off, it is suggested that the PLS model in this study was successfully validated by permutation testing.

TABLE 4
PLS MODEL PARAMETERS DERIVED FROM THE DATA SAMPLES

PLS parameters	Values	Validation values
R^2Y	0.830	0-1
Q^2Y	0.687	0.5-0.9 and 0.9-1.0
R^2Y-intercept	0.449	0.3-0.4
Q^2Y-intercept	-0.408	<0.05

IV. CONCLUSION

In conclusion, among 6 carbon sources used, glucose crude extract showed the highest inhibition zone with 75%. PIRG and exhibited the lowest MIC and MFC values with 78 and 2500 µg/mL, respectively. Meanwhile, from PLS analysis, it was observed that the sugar crude extracts which are glucose, maltose and sucrose extracts contained more threonine, 2-heptanone, lactate, valine, butyrate, *o*-phosphoserine whereas the complex sugar extracts which are starch, *U. pinnatifida* and xylitol have higher acetic acid, methionine and leucine. The results obtained in this study gave a representative impression of the metabolites present in *P. oxalicum* T3.3 with different carbon sources.

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