CHAPTER IV

CHARACTERIZATION OF ANTIFUNGAL ACTIVITY OF LAB AND CELL FREE SUPERNATANT AGAINST PHYTOPATHOGENS *Fusarium* SPECIES

4.1 INTRODUCTION

The antifungal activity of LAB isolates could be more sensitive because of consistent of protieneous compound which could be useful for removal of pathogenic compound for example, myco-toxinogenic moulds such as *Fusarium, Aspergillus,* and *Penicillium* play a major role in the deterioration of the marketable quality and safety of food and feeds by synthesizing highly toxic metabolites known as mycotoxins (Desjardins, 2006). *Fusarium* species are phyto-pathogens that cause serious plant diseases on many economically important plants worldwide (Darnetty et al., 2008; Nur et al., 2008). This phyto-pathogen attacks especially roots and shoots of plants, causing damping off or root-rot (Thomasho, 1996). The food and Agriculture Organization (FAO) of the United Nations approximated more than 25% of food crops globally are lost yearly because of mycotoxin contamination with the *Fusarium* species (Chelkowski et al., 1998).

Fungal contamination is usually removed mostly by heat treatment and chemical treatment. However, there have been issues related to the use of chemical treatments which are basically soil pollution and associated health problems. Therefore it is essential

to replace chemical pesticides and fungicides with alternative antifungal agents produced by microorganisms which can act as a biocontrol agent against pathogenic fungi spoilage organisms or plant pathogens (Stiles, 1996; Mataragas et al., 2003; Magnusson et al., 2003; Yaqub & Shahzad 2005; Abdel-Fattah et al. 2011; Alwathnani & Perveen 2012). Different type of compounds are produced by LAB such as organic acids, hydrogen peroxide, cyclic dipeptides, phenolic and proteinaceous compounds that could be responsible for the antifungal activity. Recently, Ogunbanwo et al. (2014) reported that lactic acid, diacetyl and hydrogen peroxide compounds produced by *Lb. brevis* I and II, *Lb. plantarum* I and II have the ability to reduce the biomass germination of phyot-pathogenic fungi *Fusarium* species chiefly *F. fujikuroi*, *F. solani* and *F. acuminatum*. The inhibitory activity of compounds in LAB supernatants are affected by temperature, pH and enzymes.

Indeed, Samuel et al. (2014) observed that heating the LAB-CFS isolated from fresh and spoilage tomato samples had different effects against *A. fumigatus* and *Fusarium* species. The heating of the CFS of *Lb. brevis*at 60°C was effective against *A. furmigatus* and *F. solani, F. acuminatum, F. fujikuroi*. However, heating the CFS of *Lb. brevis* at 80 and 100°C destroyed the antifungal activity. In contrast, the antifungal activity of CFS in strains of *Lb. plantarum* could be maintained at higher temperatures between 80 and 121°C (Rouse et al., 2008; Niku- Paavola et al., 1999; Gerez et al., 2009). Previously, Miescher et al. (2005) also reported that the heating of the supernatant of *Lb. paracasei* sub sp.*paracasei* SM20, SM29, and SM63 for 10 min at 100°C did not change the inhibitory activity. Recently, Marwa et al. (2015) was to demonstrate the stability of

the bacteriocin extracted from *Lactobacillus acidophilus* treated at different pH values, heat treatments and proteinase K enzyme noticed that inhibitory effectiveness of bacteriocins was higher on Gram-positive bacteria than Gram-negative bacteria. Because of, the largest inhibition zone was obtained by *Lb. acidophilus* bacteriocin against *Bacillus subtilis* while the smallest one was against *E.coli.* The extracted bacteriocin exhibited broad spectrum of inhibition at concentration 6400AU/ml against *Staph aureus*, *Bacillus subtilis* and *E.coli.* The antimicrobial activity of crude supernatant fluid was stable after heating at 100°C for 30 min and declined thereafter. Stability of antimicrobial activity was observed at pH ranged from 2.0 to 8.0. Its active principle was proteinaceous in nature since the bacteriocin was inactivated by proteinase K enzyme.

The value of pH adjustment of the supernatant has variable effect on the inhibitory activity of LAB-CFS. The antifungal activity of *Lb. plantarum* LB52, *Lb. plantarum* LB20, *Lb. plantarum* LB51, *Lb. farciminis* LB53 and *Lb. plantarum* LB54, isolated from silage, camel milk and carrots showed antifungal activity at pH 2.0. However, increasing the pH of the CFS to 3.0, 4.0, and 5.0 decreased the inhibitory activity of all LAB isolates against spoilage fungi *Aspergillus* species (Laref & Bettache, 2013). Furthermore, changing the pH of CFS of *Lb. plantarum* LB20; *Lb. plantarum* LB52 to pH 6.0 and 7.0 did not diminish the antifungal activity and thus concluded that the antifungal activity was not only due to un dissociated organic acids but also by dissociated organic acids. In contrast, De Muynck et al. (2004) reported that the neutralization of the supernatant of *Lb. amylovorus* DSM 20532, *Lb. brevis* LMG 6906, *Lb. acidophilus* LMG 9433 and *Lb.*

coryniformis sub sp. *coryniformis* LMG 9196 isolates destroyed the inhibitory activity at pH 5.0, 5.5 and 6.0.

Proteolytic enzyme treatments seem to have different effect on the inhibitory activity of CFS either decreasing or increasing. Ndagano et al. (2011) reported that the treatment of supernatant by protelotytic enzymes (pronase, pepsin, Proteinase K and α chymotrypsin) maintained the inhibitory activity. In contrast, the antifungal activity against *Aspergillus parasiticus* CBS97197, *Aspergillus versicolor* CBS117286, and *Penicillium bialowiezense* CBS110102 was lost after treating the LAB-CFS with trypsin enzyme (Coda et al., 2011). Similarly, Rouse et al. (2008) reported that addition of proteinase K to supernatant of *Lb. plantarum* removed their antifungal activity. Mauch et al. (2010) and Guo et al. (2011) reported that the proteolytic treatment reduced the inhibitory activity of *Lb. reuteri* R2 and *Lb. brevis*. Therefore, the objectives of this study was to determine the effect of temperature, pH, and proteolytic enzyme on antifungal activity of CFS of selected LAB isolated from Malaysian soil sample, fermented chilli fruit and ATCC culture against *Fusarium* species. This study also determined the amount of LAB-CFS and the proteolytic activity of the LAB isolates.

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4.2 MATERIALS AND METHODS

4.2.1 Detection of proteolytic activity on skim milk agar

Proteolytic Activity five selected LAB was determined on Skim Milk Agar and dtails of procedure as following:

4.2.2 Lactic acid bacteria strains and culture conditions

Five selected LAB strains were used to evaluate the proteolytic activity of extracellular cell-bound proteinase. These LAB strains were *Lb. plantarum*1, *P. pentosaceus*1 and *Lb. plantarum*1 was isolated from fermented chilli fruits (LAB-FF11) and *Lb. acidophillus* ATTCC314, *Lb. plantarum* ATCC8014 obtained from the Faculty of Science & Technology, Universiti Sains Islam Malaysia. The pure cultures were grown on MRS agar plate and were incubated at 37°C for 24 h. The freshly grown cultures were used to detect the proteolytic activity of extracellular cell-bound proteinase.

4.2.3 Preparation of skim milk agar and cultures

Non fatty dry milk (Spray Process Pasteurized Skim Milk, Grade A, Farmers Cooperative Creamery, McMinnville or USA) were used to evaluate the proteolytic activity of extracellular cell-bound proteinase using the method described by Pailin et al. (2001) with modification. Twenty five grams of non-fat dry skim milk was reconstituted with 250 mL

of distilled water. The mixture was stirred thoroughly and autoclaved at 121°C for 15 min. Simultaneously, 500 mL of 2.5% technical agar was prepared for plating, skim milk and agar solutions were held in a water bath at 50°C and was then poured into the agar bottle and mixed thoroughly. The skim milk agar was poured quickly into plates. Twenty four hours old LAB colony spotted with sterile wire loop on skim milk agar and incubated at 37°C for 48 h in an anaerobic chamber. After 48 h inoculated plates were incubated at 4°C for 3 d cooling in a refrigerator to detect extracellular cell-bound proteinase. The diameter of clear haloes zone surrounding the individual LAB culture was measured. The experiment was proceeded triplicates.

4.3 Preparation of cells free supernatants of lactic acid bacteria

The isolates were inoculated into MRS broth and incubated for 24 h at 37°C. The LAB-CFS was prepared by centrifuging the broth at 11500 × *g* for 10 min at 4°C. The bacteria were grown and measured at OD of 600nm by Nanophotomerter (P330 BRAND IMPLEN) as (39×10^8 LAB 1), (64×10^8 LAB 5), (608×10^7 LAB 6), (419×10^7 LAB 7), and (605×10^7 LAB 11) then the supernatant of each isolates were filtrated using sterile filtered 0.45µm pore size filter, Millipore (Schleicher and Schuell, Dassel, Germany).

4.4 Titrimetric assay of lactic acid production by LAB isolates

Lactic acid production was achieved using the methods described by Bamidele et al. (2011) with slight modification. The LAB isolates were grown in MRS broth at 37°C for

24 h and the broth culture was centrifuged at 4°C 11500 × *g* for 10 min. Lactic acid was determined by pipetting 2 mL of the LAB-CFS to 50 ml test tubes and 2 drops of phenolphthalein were added as indicator. The supernatant was titrated with 0.1 M NaOH until a pink color appeared and percentage of lactic acid was calculated using the following equation. The titration was determined in triplicate and percent lactic acid was reported as means of three readings.

$$TLA\ (\%) = ML \times N \times 90 / V \times 1000 \times 100$$

Where,

TLA =Titrimetric (%) of lactic acid

ML= 0.1 NaOH

N= Normality of 0.1 NaOH

V= Volume (ml) supernatants sample used

90= Molecular weight (Mw) of lactic acid

4.5 Micro titer plate assay

4.5.1 Effect of LAB-CFS on growth of *Fusarium* spp. by micro titer plate assay

MEB in 96 wells micro titer plates was inoculated with 10^5/ml of seven days fungal spores at ratio of 1:1 v/v (LAB-CFS: spore suspension) incubated at 30°C for 72 h.

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24 h and the broth culture was centrifuged at 4°C 11500 × *g* for 10 min. Lactic acid was determined by pipetting 2 mL of the LAB-CFS to 50 ml test tubes and 2 drops of phenolphthalein were added as indicator. The supernatant was titrated with 0.1 M NaOH until a pink color appeared and percentage of lactic acid was calculated using the following equation. The titration was determined in triplicate and percent lactic acid was reported as means of three readings.

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Fungal growth in micro titer plates was observed at 630 nm using Microtitr plate Autoreader reader (EL 309, Biotek Instruments, Winooski, Vt.) following the method described by Schilinger & Lucke (1989) with modification. The percentage growth inhibition of fungi was calculated using the equation as mentioned below.

$$[GI\ (\%)] = [TC\text{-}TT/TC] \times 100$$

Where,

GI (%) = growth inhibition,

TC = Total fungal growth in control sample),

TT= Total fungal growth in treatment sample),

4.5.2 Effect of heating treatments on antifungal activity of LAB-CFS

The LAB-CFS were heat treated at 80°C, 90°C in water bath and 121°C in the autoclave for 30 min and immediately cooled in iced water following the method described by Muhialdin et al. (2011) with modification. The heat treated supernatants were then tested against fungi CL, CS, FC and LR in microtiterplate assay. The plates were incubated at 30ºC for 72 h. Fungal growth was measured by optical density at 630nm (Microtitr plate autoreader EL 309, Biotek Instruments, Winooski, Vt.). The percentage growth of fungi was measured using same equation mentioned in section 4.5.1 and tests were done in duplicate.

4.5.3 Effect of pH treatments on antifungal activity of LAB-CFS

The initial pH of the *Lb. plantarum*1, *P. pentosaceus*1, *Lb. acidophillus* ATTCC314 and *Lb. plantarum* ATCC8014 were determined with pH metre (Mettler Toledo Greifensee, Switzerland) and adjusted to different pH values 2,4, 6, and 8 using 0.1 N HCl or 0.1 N NaOH, respectively. The adjusted pH supernatant was then tested against phytopathogenic fungi CL, CS, FC, and LR in microtiterplate assay. Need 100 µL ME broth containing 10^5 conidia/ml were placed in the 96 wells plate and 100 µL pH adjusted supernatant were added into the well. The plates were incubated at 30 °C for 72 h. Fungal growth was measured by optical density at 630 nm (Microplate Autoreader EL 309, Biotek Instruments, Winooski, Vt.) and visually. The percentage growth of fungi was also measured using the equation as mentioned in section 4.5.1.

4.5.4 Effect of enzymes treatments on antifungal activity of LAB-CFS

The five LAB-CFS was assessed to determine their sensitivity to three different proteolytic enzymes at different concentrations using the method described by Thornton (1996) with modification. A mixture 1:50 ratio consisting of enzyme and LAB-CFS (60 µL enzyme + 2940 µL LAB-CFS) were prepared and the mixtures were adjusted to pH value of pepsin (pH 1.5), papain (pH 6.5) and proteinase K (pH 7.5) as required for enzyme reaction before mixing. The LAB-CFS samples were treated separately with pepsin (pH 1.5), papain (pH 6.5) and proteinase K (pH 7.5) (Sigma) at a concentration of 0.1 milligram enzyme/mL. The mixtures of pepsin and proteinase K were incubated at

37°C for 4 h in incubator shaker (SASTEC) at 150 rpm. The mixture containing papain and LAB-CFS was incubated in water bath (MEMMERT) at 65°C for 4 h and manually gently shaken at 30 min interval. The enzymes reaction was stopped with changing pH of LAB-CFS as same as crude supernatants. Enzyme treated LAB-CFS 100µL was added to100 µL of freshly prepared fungal spore suspension (1×10^5 spore/ml) in micro titer plates and incubated at 30°C for 72 h. Growth of fungal mass in the micro titer plates was measured at OD 630 nm using microtiter plate assay (Micro plate Auto reader EL 309, Biotek Instruments, Winooski, Vt.). The percentage growth inhibition was measured by using the equationas mentioned in section 4.5.1.

4.5.5 Inhibitory activty of combined LAB-CFS isolates

An equal volume of LAB-CFS (ratio 1:1) were combined and vortexed properly to mix well and labled in group A to J as shown in Table 16. From mixture solution 100 µL LAB-CFS mixture and 100 µL freshly prepared fungal spore suspension was obtained (1×10^5 cells/ml) using serial dilution and were used for treatments. The inhibitory activity of LAB-CFS mixtures were tested against four fungi *Fusarium* species and incubated at 30^0C for 72h. The inhibitory activity of LAB-CFS mixtures were measured OD 630 nm by micro titer plate assay (Micro plate Auto reader EL 309, Biotek Instruments, Winooski, Vt.). The percentage inhibitory activity of LAB-CFS mixtures were measured using the equation as mentioned in section 4.5.1. All tests were done in duplicate.

Table 16: Designation of CFS of LAB was combined in different group

Groups	Combination of LAB-CFS	Volumes (ml)
A	*Lb. plantarum*1-MSS1 + *P. pentosaceus*1-MSS5	1:1
B	*Lb. plantarum*1-MSS1 + *Lb. acidophilus* ATTCC314	1:1
C	*Lb. plantarum*1- MSS1 + *Lb. plantarum*ATCC8014	1:1
D	*Lb. plantarum*1-MSS1 + *L. plantarum*1- FF11	1:1
E	*P. pentosaceus*1-MSS5 + *Lb. acidophilus* ATTCC314	1:1
F	*P. pentosaceus*1-MSS5 + *Lb. plantarum*ATCC8014	1:1
G	*P. pentosaceus*1-MSS5 + *Lb. Plantarum*1-FF11	1:1
H	*Lb. acidophilus* ATTCC314 + *Lb. plantarum*ATCC8014	1:1
I	*Lb. Acidophilus* ATTCC314 + *Lb. plantarum*1- FF11	1:1
J	*Lb. plantarum ATCC8014* + *L. plantarum*1-FF11	1:1

4.6 Data analysis

Mean (±) and standard deviation obtained from each analysis was analyzed using One and/or Two-way analysis dependent of variance (ANOVA and the significant was done by the Tukey test at ($P<0.05$). The statistical analyses were performed using Minitab 16 software.

4.7 RESULTS

4.7.1 Extracellular proteinase activity

The extracellular proteinase with LAB cells on skim milk agar is shown in Figure 11. The colony of LAB-MSS5 which was isolated from soil sample showed clear zones of 7.0 mm diameter. The proteolytic activity of LAB did not showed significantly different

(P>0.05) in LAB strains in Table 17, whereas LAB ID6 and LAB7 showed clear zones of 5.3 mm. The LABMSS1 and LAB FF11 which were isolated from soil and fermented chilli fruit samples also showed proteolyic activity with clear zones surrounding colony of 6.0 mm and 6.2 mm, respectively. The proteolytic activity clear zone was showed with LAB-MSS5 around the colony 7.0 mm wider than other isolates (Figure 13). It was clear that the antifungal actvity of LAB agaist *Fusarium* species because of proteinous compouds consist in LAB strains which were resposible to inhibited the fungal growth of *Fusarium* species.

TABLE 17: Extracellular proteolytic activity of LAB isolates on skim milk agar

LAB Isolates	Diameter of clear zone (mm)
LAB-MSS1	6.06±0.64[a]
LAB-MSS5	7.06±0.64[a]
IDLAB6	5.33±0.05[a]
IDLAB7	5.30±1.34[a]
LAB-FF11	6.23±0.15[a]

Notes:- Means with the same letter in the same column are not significant different (P>0.05); LAB-MSS1 = *Lb. plantarum* from Malaysian soil sample, LAB-MSS5 = *P. pentoceous* from Malaysian soil sample, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum* from fermented chilli fruits

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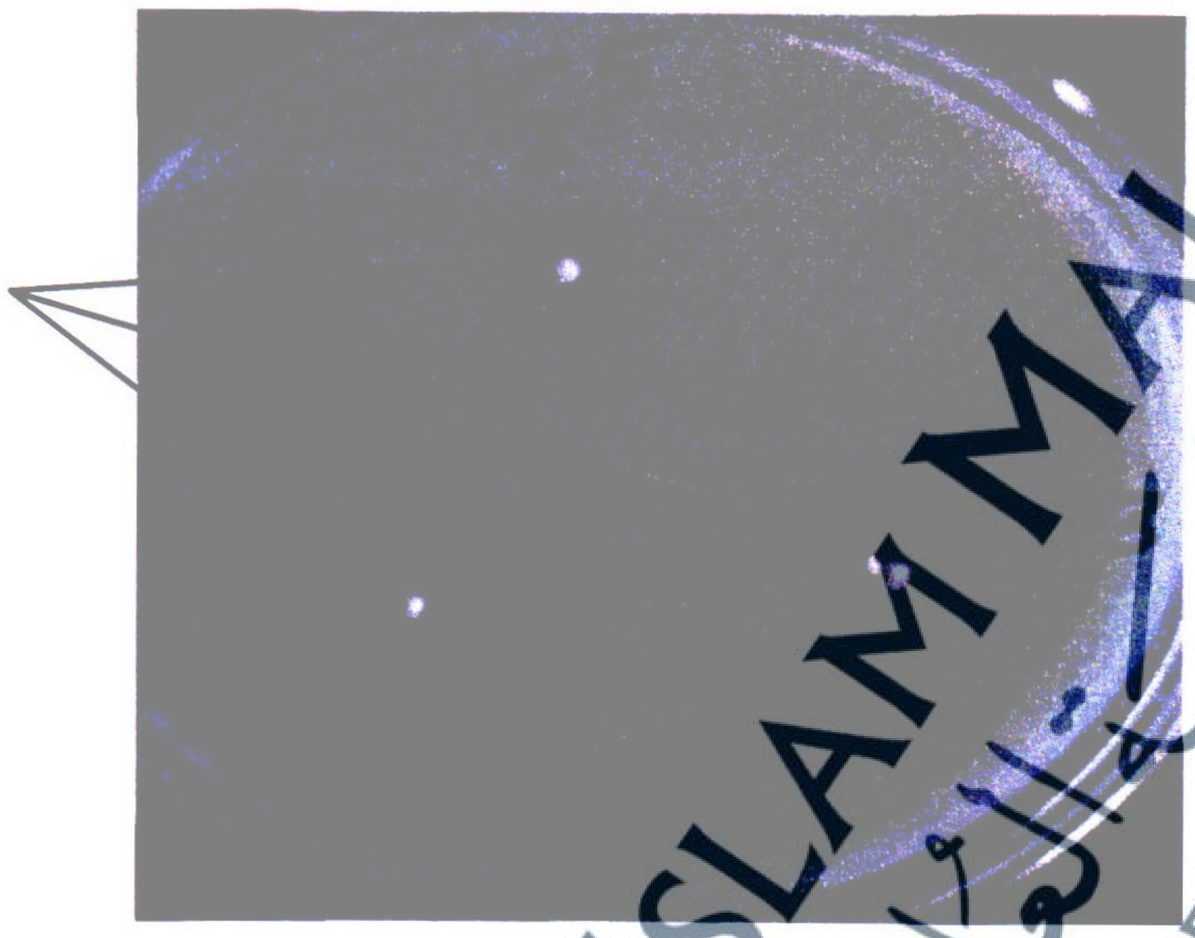
FIGURE 13: Clear zones around LAB-MSS5 colonies grown on skim milk agar showing proteolyic activity



Proteolyic activity of LAB-MSS5 = *P. pentosaceus*1

4.7.2 Lactic acid in LAB-CFS by titrimetric assay

Titrimetric assay is a technique that is very useful to know that the concentration of unkown solution in liquid form by using titration method. For instance, the percentage of lactic acid produced by LAB-CFS variable and showed and significantly different ($P<0.05$) in LAB strains. It was determined the percentage of lactic acid produced by LAB-MSS1 and LAB-FF11 were 2.02% and 2.30%, respectively. However, the percentage of lactic acid in IDLAB6, IDLAB7 and MSS5 CFS were 2.02%, 1.94% and 1.55%, respectively as shown in Figure 14 and appendix-B-Figure 47).

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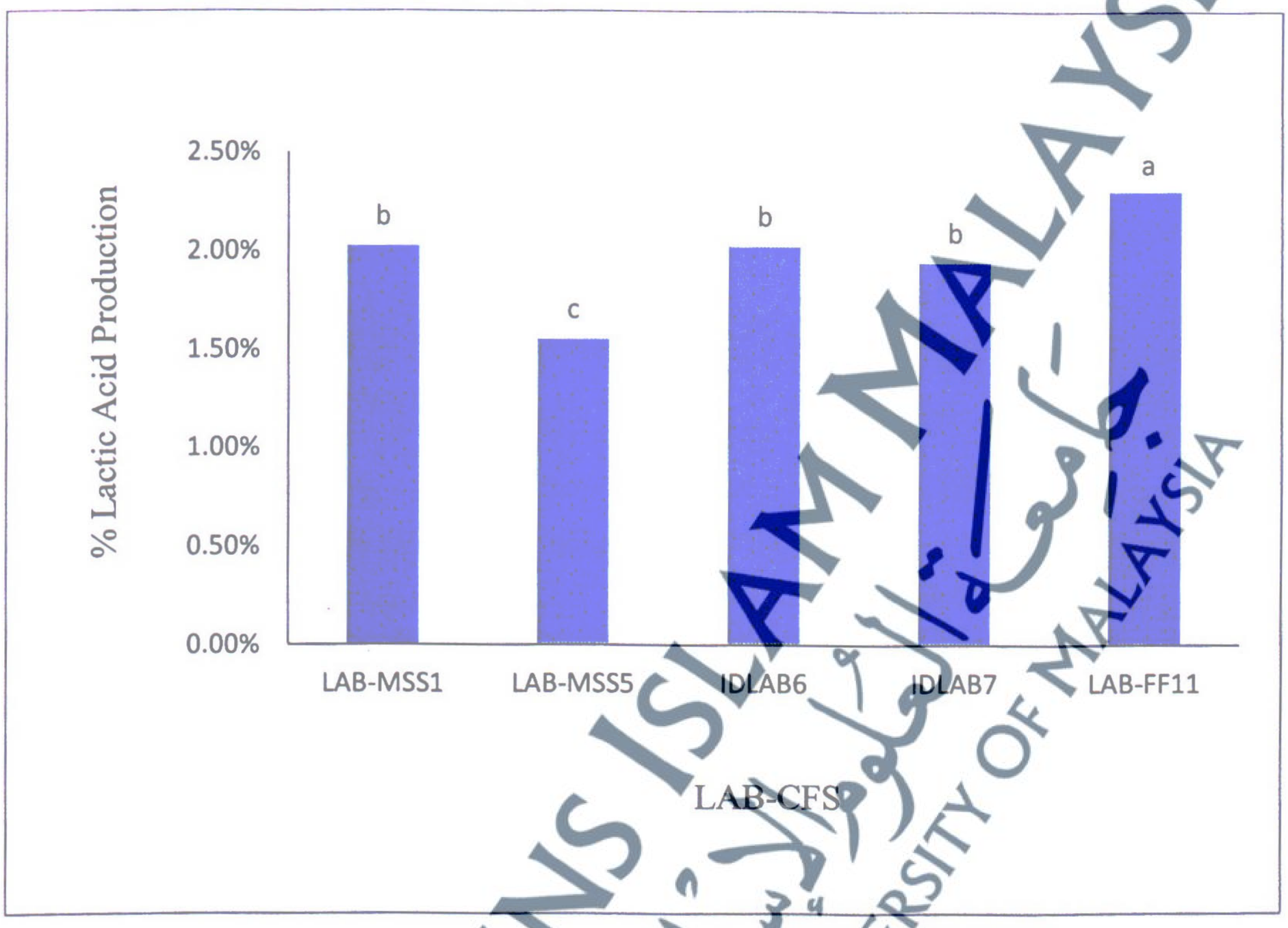
FIGURE 14: Percentage of lactic acid in LAB-CFS



Notes:- The Means with the same letters are not significant different (P>0.05) and Means with the different letters are significantly different (P<0.05); LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb.* plantarum ATCC8014, and LAB-FF11 = *Lb. plantarum*

4.8 Different factors effect on LAB-CFS against *Fusarium* species

4.8.1 Effect of crude LAB-CFS on growth of *Fusarium* species

The effect of LAB-CFS did not inhibit significantly (P>0.05) against *Fusarium* spp. However, all the supernatants from the LAB isolates showed good inhibitory activity greater than 90% against the four species of *Fusarium* spp. evaluated after 72 h incubation at 30°C (Table 18).

TABLE 18: Percentage of growth inhibition of fungi *Fusarium* species by LAB-CFS after 72 h incubation at 28°C

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	93.0^{Aa}	96.6^{Aa}	93.8^{Aa}	93.7^{Aa}
LAB-MSS5	94.2^{Aa}	94.3^{Aa}	90.0^{Aa}	94.6^{Aa}
IDLAB6	94.0^{Aa}	94.0^{Aa}	94.6^{Aa}	94.5^{Aa}
IDLAB7	93.4^{Aa}	93.7^{Aa}	94.2^{Aa}	94.0^{Aa}
LAB-FF11	93.3^{Aa}	93.3^{Aa}	94.1^{Aa}	94.0^{Aa}

Notes:- Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum*; fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

The CFS of *Lb. plantarum*1 MSS isolated from soil inhibited fungal spore germination of *F. solani*-CS (96.6%) and *F. oxysporum* f. sp. *lycopersici*-CL (93.0%). The CFS of IDLAB6 (*Lb. acidophilus* ATCC314) and LAB-MSS5 (*P. pentosaceus*1 isolated from soil) showed good inhibitory activity against *F. acuminatum*-FC and *F. proliferatum*-LR with percentage growth inhibition of 94.6% and 94.7%, respectively. In comparison to other *Fusarium* spp., it was observed that *F. oxysporum* f. sp. *lycopersici*-CL showed slightly less inhibition by other LAB-CFS to CFS of *Lb. plantarum*1MSS.

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4.8.2 Effect of heating treatment on antifungal activity of LAB-CFS

Heating the CFS-LAB showed variable effects on the inhibitory activity on spore germination of *Fusarium* spp. Heating at 80°C significantly difference ($P<0.05$) change the inhibitory activity of LAB-CFS compared to the crude LAB-CFS to all *Fusarium* species. However, significant difference ($P<0.05$) in the inhibitory activity among the CFS-LBS on *Fusarium* spp. was observed when the LAB-CFS-MSS1 showed 95.6% growth inhibition against *F. solani*-CS while LAB-CFS-MSS5 showed 92.0% growth inhibition against *F. oxysporum* f. sp. *Lycopersici*-CL (Table 19).

TABLE 19: Percentage mass growth inhibition of *Fusarium* species by LAB-CFS heated at 80°C

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	92.0^{Aa}	95.6^{Aa}	93.8^{Aa}	93.7^{Aa}
LAB-MSS5	92.0^{Ba}	94.0^{Ba}	90.0^{Ba}	94.6^{Ba}
IDLAB6	94.0^{Ba}	94.0^{Ba}	94.2^{Ba}	94.5^{Ba}
IDLAB7	93.4^{Aa}	93.3^{Aa}	94.6^{Aa}	94.0^{Aa}
LAB-FF11	93.3^{Aa}	93.7^{Aa}	94.1^{Aa}	94.0^{Aa}

Notes:- Means with the same small letters in the same column are not significant different ($P>0.05$) and Means with the different capital letters in the same same are not significantly different ($P>0.05$); LAB-MSS1= *Lb. plantarum*, LAB-MSS5 = *P. pentoceous* from, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11= *Lb. plantarum*; fungi CL= *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

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Heating all the LAB-CFS evaluated at 90°C maintained the antifungal activity against growth of all *Fusarium* spp. The mass growth of *F. acuminatum*-FC was greatly reduced (93.8%) by heating CFS of LAB-FF11 (Table 20). Furthermore, no significant difference (P>0.05) was observed in the antifungal activity of heated LAB-CFS-MSS1 and IDLAB7 against *Fusarium* spp., the growth of *F. solani*-CS was greatly inhibited by CFS- LAB-MSS1 (92.5%) and growth of *F. proliferatum*-LR was inhibited by LAB-CFS -IDLAB7 (92.7%).

TABLE 20: Percentage mass growth inhibition against *Fusarium* species by LAB-CFS heated at 90°C

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	92.0^{Aa}	91.5^{Aa}	92.3^{Aa}	92.5^{Aa}
LAB-MSS5	93.0^{Ca}	92.6^{Ca}	93.1^{Ca}	93.3^{Ca}
IDLAB6	92.8^{BCa}	92.1^{BCa}	93.0^{BCa}	93.1^{BCa}
IDLAB7	92.0^{Aa}	91.5^{Aa}	92.6^{Aa}	92.7^{Aa}
LAB-FF11	92.5^{ABa}	91.7^{ABa}	93.8^{ABa}	92.8^{ABa}

Notes:- Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum*; fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

Similarly, heating the LAB-CFS at 121°C for 30 min resulted in significant reduction (P<0.05) in the inhibitory activity ranging between 80.2% and 88.9% among LAB-CFS and fungi evaluated (Table 21). The LAB-CFS-IDLAB7 showed the highest antifungal activity 88.67% against *F. oxysporum* f. sp. *lycopersici*-CL which was isolated

from infected chilli leaves. High temperature treatment also reduced the antifungal activity of LAB-CFS-LAB-FF11 against *F. proliferatum*-LR and *F. oxysporum* f. sp. *lycopersici*-CL by 80.2% to 84.3%, respectively. It was clear that from results that the antifungal activity LAB-CFS was reduced by high heat treatment at 121°C compared to heating at 80 and 90°C.

TABLE 21: Percentage mass growth inhibition against *Fusarium* species using LAB-CFS heated at 121°C

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	87.9^{Ba}	87.4^{Ba}	87.6^{Ba}	86.6^{Ba}
LAB-MSS5	84.3^{Ba}	84.3^{Ba}	84.8^{Ba}	83.3^{Ba}
IDLAB6	88.9^{Ca}	88.7^{Ca}	88.3^{Ca}	86.8^{Ca}
IDLAB7	88.6^{Ca}	87.8^{Ca}	87.6^{Ca}	85.8^{Ca}
LAB-FF11	84.3^{Ba}	82.8^{Ba}	82.5^{Ba}	80.2^{Ba}

Notes:- Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum*; fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

4.8.3 Effect of pH treatment on antifungal activity of LAB-CFS

It was observed that adjusting pH of LAB-CFS siginifcantly (P<0.05) affected the fungal growth inhibition with variable results. The antifungal activity was maintained at pH 2 and pH 4 but with slight reduction compared to unadjusted LAB-CFS. The percentages of fungal growth inhibiton were between 47.5 to 91.3% at pH 2 and, 60.93 to 89.6% at pH

4 against all the *Fusarium* spp. (Table 22 & 23). However, the inhibitory activity of all LAB-CFS lost when the LAB-CFS of isolates were adjusted to pH 6 against fungi *F. oxysporum* f. sp. *lycopersici*-CL and *F. solani*-CS (Table 24). The antifungal activities of LAB-MSS1 and IDLAB6 at pH 6 were low as the values were 8.5% and 6.8% respectively against *F. proliferatum*-LR. Also at pH6, the LAB-CFS lost its antifungal activity against fungi *F. oxysporum* f. sp. *Lycopersici*-CL and *F. solani*-CS. However, the percentage inhibitory activity against fungi *F. acuminatum*-FC by all LAB-CFS ranged between 0.9% and 10.7% after 72 h incubation. Likewise, LAB-MSS5 IDLAB7and LAB-FF11 also lost their antifungal activity against fungi *F. proliferatum*-LR. However, LAB-MSS1and IDLAB6 showed little inhibition and the inhibitory activity of both LAB-CFS were 8.5% and 6.8% against fungi *F. proliferatum*-LR respectively.

TABLE 22: Percentage mass growth inhibition against fungi *Fusarium* species by LAB-CFS adjusted at pH 2

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	69.8^{ABa}	88.1^{ABb}	91.3^{ABab}	91.3^{ABab}
LAB-MSS5	47.5^{Aa}	80.2^{Ab}	87.0^{Aab}	87.0^{Aab}
IDLAB6	55.2^{Ba}	82.3^{Bb}	87.3^{Bab}	87.3^{Bab}
IDLAB7	53.9^{Aa}	81.4^{Ab}	86.6^{Aab}	86.6^{Aab}
LAB-FF11	54.3^{Aa}	81.9^{Ab}	86.8^{Aab}	86.8^{Aab}

Notes:- Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum*; fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

TABLE 23: Percentage mass growth inhibition against fungi *Fusarium* species by LAB-CFS adjusted at pH 4

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	79.5^{Aab}	87.4^{Abc}	89.6^{Aa}	89.1^{Ac}
LAB-MSS5	60.9^{Aab}	74.3^{Abc}	80.1^{Aa}	76.2^{Ac}
IDLAB6	71.3^{Aab}	85.2^{Abc}	84.6^{Aa}	87.2^{Ac}
IDLAB7	66.3^{Aab}	81.8^{Abc}	84.3^{Aa}	84.4^{Ac}
LAB-FF11	71.4^{Aab}	85.4^{Abc}	87.1^{Aa}	87.0^{Ac}

Notes:- Significant Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATTCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum*; fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

TABLE 24: Percentage mass growth inhibition against fungi *Fusarium* species by LAB-CFS adjusted at pH 6

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	g^{*Ba}	g^{*Ba}	7.8^{Ba}	8.5^{Ba}
LAB-MSS5	g^{*Aa}	g^{*Aa}	5.8^{Aa}	g^{*Aa}
IDLAB6	g^{*Ca}	g^{*Ca}	14.3^{Ca}	6.8^{Ca}
IDLAB7	g^{*ABa}	g^{*ABa}	0.9^{ABa}	g^{*ABa}
LAB-FF11	g^{*Ba}	g^{*Ba}	10.7^{Ba}	g^{*Ba}

Notes:- Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); g* = growth of fungi was seen; LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATTCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum*; fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

4.8.4 Effect of enzymes treatments on antifungal activity of LAB-CFS

The CFS-LABs treated with different proteolytic enzymes (pepsin, papain and proteinase K) resulted in variable in significantly difference ($P<0.05$ of antifungal activity against *Fusarium* species. Treating CFS of LAB-MSS1, IDLAB6, and LAB-FF11 with pepsin destroyed the inhibitory activity against *Fusarium* CL. However, the antifungal activities against other *Fusarium* species were reduced when pepsin treated with CFS-IDLAB7 inhibited the growth of both *F. acuminatum* and *F. proliferatum*-LR with more than 86.8% (Table 25). In contrast, pepsin treated CFS of LAB-MSS5 and CFS of IDLAB7 suppressed the growth of *F. oxysporum* f. sp. *lycopersici*-CL by 30.2% and 57.0%, respectively. Similarly, growth of *F. proliferatum*-LR was inhibited more than 85.0% when the spores were inoculated in broth containing papain treated CFS of both LAB-MSS1 and IDLAB7. However, it was observed that CFS of both LAB-MSS5 and IDLAB6 treated with papain showed inhibitory activity less than 11.0% against *F. oxysporum f.* sp. *lycopersici*-CL (Table 26). The inhibitory activity of CFS-LAB-FF11 against *F. solani*-CS reduced to 22.0% inhibition and antifungal activity of CFS-LAB-FF11 and lost after papain treatment against *F. oxysporum* f. sp. *lycopersici*-CL. In contrast, growth of *F. acuminatum*-FC was inhibited (80%) by papain treated LAB-CFS-MSS1. It was observed that growth of *F. oxysporum* f. sp. *lycopersici*-CL was not inhibited by proteinase K treated with CFS-LAB-MSS5 (Table 27). However, growth of *F. proliferatum*-LR and *F. acuminatum*-FC were inhibited 87% and 80% with proteinase K treated CFS-IDLAB7, respectively. However, lower antifungal activity was observed

against *F. solani*-CS for proteinase K treated LAB-CFS of both LAB-FF11 and LAB-MSS1 with 10.0% and 25.0% percentages of inhibition respectively.

TABLE 25: Percentage growth inhibition of *Fusarium* species by LAB-CFS treated with pepsin

LAB-CFS	Inhibition activity (%)			
	CL	**CS**	**FC**	**LR**
LAB-MSS1	g*^{Ca}	59.40^{Ca}	71.87^{Ca}	70.10^{Ca}
LAB-MSS5	30.27^{CDa}	52.67^{Da}	74.59^{CDa}	80.88^{CDa}
IDLAB6	g*^{Ba}	12.60^{Ba}	59.53^{Ba}	63.85^{Ba}
IDLAB7	56.90^{Da}	79.43^{Da}	86.83^{Da}	86.70^{Da}
LAB-FF11	g*^{Aa}	11.00^{Aa}	29.98^{Aa}	54.15^{Aa}

Notes:- Significant Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous* from Malaysian soil sample, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum;* fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

TABLE 26: Percentage of growth inhibition of *Fusarium* species by LAB-CFS treated with papain

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	34.76^{Da}	70.25^{Da}	81.84^{Da}	83.40^{Da}
LAB-MSS5	13.33^{BCa}	60.64^{BCa}	73.44^{BCa}	78.36^{BCa}
IDLAB6	10.56^{Ba}	58.81^{Ba}	71.82^{Ba}	75.71^{Ba}
IDLAB7	29.02^{CDa}	67.27^{CDa}	75.22^{CDa}	82.11^{CDa}
LAB-FF11	g*^{Aa}	22.54^{Aa}	56.39^{Aa}	55.37^{Aa}

Notes:- Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1= *Lb. plantarum*, LAB-MSS5= *P. pentoceous* from Malaysian soil sample, IDLAB6= *Lb. acidophilus* ATTCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum;* fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

TABLE 27: Percentage growth inhibition of *Fusarium* species by LAB-CFS treated with proteinase K

LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
LAB-MSS1	7.44^{Ba}	61.09^{Ba}	71.06^{Ba}	83.60^{Ba}
LAB-MSS5	g^{*Aa}	15.08^{Aa}	53.76^{Aa}	58.88^{Aa}
IDLAB6	46.64^{Ba}	68.31^{Ba}	77.72^{Ba}	74.97^{Ba}
IDLAB7	54.29^{Ca}	80.42^{Ca}	85.34^{Ca}	87.21^{Ca}
LAB-FF1	26.31^{BCa}	70.88^{BCa}	79.06^{BCa}	84.11^{BCa}

Notes:- Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1= *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATTCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum*; fungi CL = *F. oxysporum* f. sp. *lycopersici*, CS = *F. solani*, FC = *F. acuminatum* and LR = *F. proliferatum*

4.8.5 Effect of combined LAB-CFS antifungal activity against *Fusarium* species

Combining the LAB-CFS did not improve the antifungal activty against the *Fusarium* spp. as compared to the LAB-CFS when evalauted singly. There was significantly difference (P<0.05) inhibition of biomass reduction against *F. solani*-CS, *F. acuminatum* FC, and *F. proliferatum*-LR in Table 28. The CFS of mixture (A) isolated from soil inhibited fungal spore germination of *F. oxysporum* f. sp. *lycopersici*-CL (96.6%) and *F. oxysporum* f. sp. *lycopersici*-CL (93.0%). However, combination of (H) showed 73.9% inhibition against fungi *F. solani*-CS. The inhibitory activity of combination of (H) was

showed against *F. proliferatum*-LR (84.8 %). Whereas, LAB-CFS mixture (A) inhibited fungal spore germination of *F. acuminatum*-FC by 83.2% after 72 h incubation.

TABLE 28: Percentage mass growth inhibition of *Fusarium* species by combined LAB-CFS evaluated at OD630nm in micro titer plate

Combined of LAB-CFS	Inhibition activity (%)			
	CL	CS	FC	LR
A =LAB-MSS1+LAB-MSS5	90.8^{BCb}	81.1^{BCa}	83.2^{BCa}	83.7^{BCa}
B =LAB-MSS1+ IDLAB6	89.9^{Cb}	83.3^{Ca}	82.8^{Ca}	83.4^{Ca}
C =LAB-MSS1+ IDLAB7	90.6^{Cb}	82.5^{Ca}	83.1^{Ca}	84.8^{Ca}
D =LAB- MSS1+ LAB-FF11	90.3^{BCb}	83.8^{BCa}	82.7^{BCa}	81.5^{BCa}
E =LAB-MSS5+ IDLAB6	86.6^{Ab}	76.9^{ABa}	77.3^{ABa}	76.8^{ABa}
F =LAB-MSS5+ IDLAB7	86.7^{ABCb}	76.0^{ABCa}	79.0^{ABCa}	79.1^{ABCa}
G =IDLAB7+ LAB-FF11	87.8^{ABCb}	77.9^{ABCa}	78.3^{ABCa}	81.1^{ABCa}
H = LAB-MSS5+LAB-FF11	86.3^{BCb}	73.9^{BCa}	77.1^{BCa}	78.6^{BCa}
I =IDLAB6+ IDLAB7	85.9^{ABCb}	78.3^{ABCa}	77.7^{ABCa}	79.8^{ABCa}
J =IDLAB6+ LAB-FF11	87.5^{ABCb}	78.9^{ABCa}	79.4^{ABCa}	80.6^{ABCa}

Notes:- Means with the same small letters in the same column are not significant different (P>0.05) and Means with the different capital letters in the same same are not significantly different (P>0.05); LAB-MSS1 = *Lb. plantarum*, LAB-MSS5 = *P. pentoceous*, IDLAB6 = *Lb. acidophilus* ATCC314, IDLAB7 = *Lb. plantarum* ATCC8014, and LAB-FF11 = *Lb. plantarum;* fungi CL = *F. oxysporum* f. sp. *lycopersici,* CS = *F. solani,* FC = *F. acuminatum* and LR = *F. proliferatum*

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4.9 DISCUSSIONS

Strains of LAB are recognized to produce extracellular cell-bound proteinases (Nippunpre et al., 2013). In this study the extracellular cell-bound proteinases of LAB cells MSS5 showed strong extracellular cell-bound proteinases with clear zones 7.0 mm compared to other LAB (Table 17 & Figures 13). The clear zone on skim agar of MSS5 was bigger than the result reported from different spp. of *Lb. bulgaricus* (Pailin et al., 2001). Lactic acid is one of the organic acids produced by LAB. Indeed, LAB-FF11 which was isolated from fermented chilli fruit produced about 2.30% lactic acid that was higher compared to isolates LAB-MSS1, LAB-MSS5 from soil and from ATCC culture IDLAB6, ATCC LAB IDLAB7 (Figure 14). LAB isolates selected for this study have the ability to produce lactic acid which was also proteolytic. Similarly, Merih et al. (2011) observed that the LAB isolated from different boza samples namely *Leuconostoc citreum, Lb. brevis, Lb. plantarum, Lb. graminis, Enterococcus faecium, Lactococcus lactis* and *Lb. paraplantarum* showed the ability to produce lactic acid and proteolytic activity. The LAB has ability to produce a variety of antimicrobial compounds such as lactic acid, acetic acid, propionic acids, antibiotics, bacteriocins as well as hydrogen peroxide and carbon dioxide (Roy et al., 1996; Ouwehand, 1998; Samuel et al., 2014).

Many *in vitro* studies indicated that LAB has potential application as bio control against phytopathogenic fungi (Stephane et al., 2005; Rosalia et al., 2008; Ashgar & Mohammad, 2010; Wang et al., 2011; El-Mabrouk et al., 2012). Therefore, the use of LAB is not only limited to the production of fermented foods but can also be used as

biocontrol agents in plants. Magnusson et al. (2003) suggested that inhibitory activity of LAB used against phytopathogenic fungi and spoilage fungi namely *F. sporotrichioides*, *A. fumigates, A. nidulans, Pencillium commune* and *Pencillium requefoti*. All selected LAB-CFS strains showed inhibitory activity when crude supernatants were tested against spore germination of four phytopathogenic *Fusarium* species *F. solani*-CS was strongly inhibited by LAB-CFS -MSS1 about 96.6%. However, other LAB-CFS also showed good inhibition against fungi *Fusarium* spp. with more than 90.0% (Table 18). In micro titer plate at OD630nm, the results is in agreement with that of Samuel et al. (2014) reported that CFS of *Lb. plantarum* II showed biomass inhibition against *A. fumigates, F. solani* and *F. fujikuroi*. However, LAB-CFS of *Lb. plantarum*1 showed inhibition of biomass of *F. solani* and *F. funjikuroi.*

Heating the LAB-CFS could affect the inhibitory activity. Heating the supernatants of LAB at 80°C did not change the inhibitory activity of LAB-CFS and similar inhibitory activity obtained with the unheated crude of LAB-CFS. The strong inhibitory activity was recorded with LAB-CFS-MSS1 soil isolated 95.6% against *F. solani*-CS (Table 19). However, the antifungal activity were also maintained by all LAB-CFS when heated at 90°C and tested against bio-mass growth of four *Fusarium* species. The inhibitory activity of 92% was recorded for LAB-FF11 against *F. acuminatum*-FC, *F. solani*-CS, and *F. proliferatum*-LR (Table 20). Thus, the LAB-CFS isolated from different sources were considered to produce heat stable antifungal compounds because all selected LAB-CFS heated at 80°C, 90°C did not change the inhibitory activity of the four *Fusarium* species after 72 h incubation at 30°C compared to non-heated LAB-CFS

isolates. This result was similar to study by Francesca et al. (2009) who observed that heating the LAB-CFS of *Lb. mensenteroides, Lb. citreum, Lb. plantarum* and *Lb. rossiae* at 100°C did not change their inhibitory activity against *Penicillium roqueforti, Aspergillus niger,* and *Endomyces sfibliger.* The antifungal peptides was heat stable, with a size of approximately 3kDa and had maximum activity at pH 3.0 to 4.5 of *Lactobacillus coryniformis* was against mould *A. fumigates* (Magnusson, 2003).

There was no loss of inhibitory activity observed after heating LAB-CFS of *Lb. plantarum* for up to 15 min at 120°C (Niku- Paavola et al., 1999; Rouse et al., 2008). In contrast, LAB-CFS heated at 121°C and tested against biomass reduction four *Fusarium* spp. The biomass reduction were noticed that slightly reduced after 72 h incubation at 30°C even thought still showed inhibitory activity compare to control (Table 22). Report from Miescher et al. (2005) heat treatment (100°C for 10 min) of the supernatant of *Lb. plantarum* was maintained the antifungal activity and (Gerez et al., 2009) when heating the supernatants of *Lb. plantarum* at 80°C for 1h and 121°C for 15 min showed to reduce the antifungal activity. Laref & Bettache (2013) suggested that the antifungal activity of *Lb. plantarum* LB52, *Lb. plantarum* LB20, *Lb. plantarum* LB51, *Lb. farciminis* LB53 and *Lb. plantarum* LB54, isolated from silage, camel milk and carrot showed activity at pH 2.0. However, adjusting the pH of the LAB-CFS to 3.0, 4.0 and 5.0 decreased the inhibitory activity of all LAB isolates against spoilage fungi *Aspergillus* species. In this study the fungal growth inhibition diminished when the supernatants were adjusted to pH 6 but was maintained at pH 2 and 4. The percentage of inhibiton against *F. oxysporum* f. sp. *lycopersici*-CL was 47.5% with LAB-CFS-MSS5 and 91.3% against *F. acuminatum-*

FC with LAB-CFS-MSS1 at pH 2 (Table 23). Thus, changed of the pH 2 growth of *F. acuminatum*-FC that was isolated with severely infected plant was inhibited by LAB-CFS of LAB-MSS1 isolated from soil samples. However, report from Vina & Carol (2005) culture supernatant from *L. acidophilus* maintained inhibitory against growth of *A. niger* and *A. pullulans* at pH 5.5 and 6.0, whereas *Lb. casei*s supernatant did not change the inhibitory activity against *P. chrysogenum* or *T. viride* at pH 4.5. However, *Lb. acidophilus* lost its antifungal activity against *P. chrysogenum* or *T. viride*at at pH 5.5 and 6. The inhibitory activity of all LAB-CFS was showed in this study against *F. oxysporum* f. sp. *lycopersici*-CL and *F.solani*-CS lost when the CFS was adjusted to pH 6 (Table 24). The antifungal activity LAB-CFS of LAB-MSS1 and IDLAB6 at pH 6 was still noticeable against *F. proliferatum*-LR, but the inhibition percentages were very poor because the growth of fungi *Fusarium* species were reported to be very fastidious in alkaline conditions. However, this fungi is also very difficult to inhibit with LAB-CFS when maintained at pH 6. This is because LAB-CFS losses its inhibitory activity at this pH.

The inhibitory activity of crude LAB-CFS was observed that more than 92% against fungi *F. proliferatum*-LR. Similarly, De Muynck et al. (2004) suggested that the neutralization of the CFS of *Lb. amylovorus* DSM 20532, *Lb. acidophilus* LMG 9433, *Lb. brevis* LMG 6906 and *Lb. coryniformis* sub sp. *coryniformis* LMG 9196 isolates to pH at 5.0, 5.5 and 6.0 destroyed the inhibitory activity. All LAB-CFS lost their inhibitory activity or showed poor antifungal activity at pH 7, 8, and 9 against all *Fusarium* species after 72 h incubation. Treatment of proteolytic enzymes to LAB-CFS would indicate if

proteins or peptides are involved in antifungal activity. The inhibitory activity against *F. oxysporum* f. *sp. lycopersici*-CL was destroyed when LAB-CFS of LAB-MSS1, IDLAB6, and LAB-FF11 were treated with pepsin indicating the presence of antifungal peptides in the CFS (Table 25). However, percentage of growth of *F. solani, F. acuminatum*-FC and *F. proliferatum* reduced when these fungi were treated with pepsin digested LAB-CFS - FF11 and the IDLAB7. Antifungal activity of LAB-CFS-FF11 against all fungi decreased after treatment with papain which further supports the presence of protein-like compounds in LAB-CFS of LAB-FF11 (Table 26). The percentage of inhibitory activity with LAB-CFS -MSS1 against growth of spore germination on *F. proliferatum*-LR recorded a value of 83.4% after incubation by proteinase K treated LAB-CFS, however, the growth to reduced against *Fusarium* spp. LAB-CFS-MSS5 lost its antifungal activity against *F. oxysporum f. sp. lycopersici*-CL (Table 27). This results were in line with Wang et al. (2011) in that *Lb. plantarum* IMAU 10014 LAB-CFS treated with enzyme proteinase K lost the inhibitory activity against *F. oxysporum* which indicated that there are proteinaceous compound responsible for antifungal activity. The inhibitory activity reduction effect of proteolytic enzymes on LAB-CFS is considered to feature the presence of proteinaceous compounds (Gourama, 1997). It was reported that *Lb. pentoceou* produced bacteriocins like peptide namely pentocin which showed fungi static effect on *Candida albicans*.

Currently, Marwa et al. (2015) was demonstrated the stability of the bacteriocin extracted from *Lb. acidophilus* treated at different pH values, heat treatments and proteinase K enzyme noticed that inhibitory effectiveness of bacteriocins was higher on

Gram-positive bacteria than Gram-negative bacteria such as, *Lb. acidophilus* bacteriocin against *Bacillus subtilis* while the smallest one was against *E.coli.* The antimicrobial activity of crude supernatant fluid was stable after heating at 100°C for 30 min and declined thereafter. Stability of antimicrobial activity was observed at pH ranged from 2.0 to 8.0. Its active principle was proteinaceous in nature since the bacteriocin was inactivated by proteinase K enzyme. Because, the the proteinaceous nature of bacteriocins can be degraded by proteolytic enzymes (Perez et al., 2014) and trypsin, alpha-chymotrypsin and pepsin, which are active to degraded proteinaceous nature of LAB-CFS of LAB strains (Dallas et al., 2012).

Combining the LAB-CFS did not improve the antifungal activty compared to when they were used singly against the *Fusarium* species. *F. solani, F. acuminatum*-FC and *F. proliferatum*-LR. The percentage of spore germination was evaluated with mixture A 90.81% against *F. oxysporum* f. sp. *lycopersici*-CL in Table 28. Similarly, mixture of LAB-CFS showed that mixture A inhibited the growth of *A. niger* and *A. oryzae*. Mixture B inhibited the growth of both fungi with good activity (Muhialdin et al., 2011).

4.10 CONCLUSION

This study showed that LAB isolated from soil sample was found to have antifungal activity against *Fusarium* species. It produced heat stable LAB-CFS, active at pH 6-9) and their antifungal activity completely changed after treatment with proteolytic enzymes. Because of, proteolytic enzymes such as pepsin, papain and proteinase K were interacted

different actions and function to degrade the protieneous nature of the LAB-CFS isolates. Furthermore, combining the crude LAB-CFS did not improve the antifungal activty when tested against isolated *Fusarium* species. These antifungal compounds have potential to be used as plants protection as well as food biopreservation to inhibit conidia germination and mycelia growth of spoilage fungi depending on food type, and pH of food especially in heat, and cold processed foods. Thus sensitivity of LAB-CFS treatments with different factors such as heat, pH, and enzymes were showed to can be protieneous nature. These protieneous nature could be has ability to enhance the seeds germination of Solanaceous plant seeds. Therefore, the objectives were selected chilli seeds belonging to Solanaceous plants seeds namely, chilli seeds in next chapter.

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