Enhancement of Novel Isolate

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Research Article
Enhancement of a Novel Isolate of *Serratia plymuthica* as Potential Candidate for an Antianthracnose

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Abstract

Background and Objective: A new rhizobacteria isolate of *Serratia plymuthica* (strain UBCR_12) exhibited a promising potential as a biocontrol agent for anthracnose causing agent *Colletotrichum gloeosporioides*. The aim of this study was to characterize its antagonistic activity and explore the factors contributing to a higher inhibition activity. **Materials and Methods:** The antifungal effect of UBCR_12 against *C. gloeosporioides* was assayed under various pH values and nutritional sources. Culture supernatant obtained from UBCR_12 and *C. gloeosporioides* co-culture was also tested for its inhibitory activity. In addition, the antagonistic range of this isolate was examined against *Sclerotium rolfsii* and *Fusarium oxysporum*. Statistical analysis was done using one way analysis of variance and further processed using Fisher's Least Significant Difference (LSD) test with a $p<0.05$. **Results:** The UBCR_12 induced inhibition was shown to be stable over time at pH 7, while peptone addition led to a faster induction (2 days after treatment) and glucose treatment to a higher activity. Of all these modifications, preliminary co-culture experiments with fungal cells resulted in the best antagonistic activity of UBCR_12 culture supernatant of about 30.66%. This isolate also showed a wide range of antagonistic activity due to its high suppression against *S. rolfsii* and *F. oxysporum* from soybean. **Conclusion:** Both environmental and biotic manipulations contributed an elevated inhibition rate of UBCR_12 against *C. gloeosporioides*. A proportional combination of the factors stimulating antagonistic activity of this strain is recommended to be utilized for the development of this strain as an antianthracnose. The enhanced antifungal effects of UBCR_12 resulted under each type of modification were varied indicating the difference of cell responses. It suggests that certain antifungal mechanism could be generated by modifying the environmental factor required for its induction. In addition, the application of cell-free culture supernatant provides an alternative solution in the utilization of biocontrol agents. For large scale application, it could minimize the risk of population outbreaks and harmful effects due to the living cells application.

Key words: *Serratia plymuthica*, rhizobacteria, antianthracnose, *Colletotrichum gloeosporioides*, pH, nutritions, co-culture, culture supernatant

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

As a biocontrol agent, *Serratia plymuthica* has been widely utilized to protect plants against infection of many phytopathogens, such as *Phytophthora capsici*, *Fusarium oxysporum*, *Ralstonia solanacearum*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Dickeya* sp. and *Rhizoctonia solani*[1-5]. Many factors and molecular mechanisms contribute to a broad spectrum of inhibition, including enzymatic activity (i.e., chitinolytic and proteolytic), production of certain antibiotics, formation of siderophore and induced systemic resistance[2,4,6-8]. In Gram negative bacteria like *Serratia*, these mechanisms are mediated by acyl-homoserine lactone (AHL) based quorum sensing system[9,10]. Taken together, *S. plymuthica* is a promosing candidate to be used as a biocontrol agent in plant diseases management.

Novel isolate of *S. plymuthica* strain UBCR_12 (GenBank Accession No. KU299959) showed potent antagonistic activity against the anthracnose causing agent *Colletotrichum gloeosporioides*. Infection causes fruit rot with serious yield loss in a wide range of hosts. Hence, this disease is considered as one of the most devastating plant diseases, especially in tropical region[11]. This seed-borne pathogen is known to be unmanageable at the early stage due to its slow symptom development, although penetration occurs before fruit formation[12].

However, the application of *S. plymuthica* to control *C. gloeoporioides* has not been reported yet. Previous study revealed that UBCR_12 inhibited the growth of *C. gloeosporiodes in vitro* when using either colony or culture supernatant[13]. To develop its potential as an antiantrachnose, optimal conditions to stimulate the activity of the strain were further identified.

MATERIALS AND METHODS

Preparation and cultivation of fungi and bacterial isolates: *Colletotrichum gloeosporioides* (isolated from chili pepper) and *S. plymuthica* strain UBCR_12 were obtained from internal collection of the Biotechnology Laboratoryof Andalas University. Other fungus isolates, such as *C. gloeosporioides* (isolated from dragon fruit), *Fusarium oxysporum* (isolated from soybean and banana) pand *Sclerotium rolfsii* (isolated from groundnut) were obtained from Phytopathology Laboratory of Andalas University. Fungal mycelium was grown on Potato Dextrose Agar (PDA) medium for 7 days at room temperature in darkness. Bacteria were grown on Nutrient Agar (NA) medium for 18 h at 30°C in darkness as well.

Modification of pH level and nutritional factors: To test for enhanced antifungal activity of UBCR_12 against *Colletotrichum gloeosporioides*, several antagonistic assays with different pH levels and nutritional compounds were performed on PDA medium. The pH levels were modified from 5-9. Nutritional factors were modified by adding 1% (w/v) of various carbon (glucose, sucrose and glycerol) or nitrogen (peptone, yeast extract and ammonium sulfate) sources to the antagonistic assays at pH 7.0.

Antagonistic assays under these modified conditions were performed using the agar spot method. An aliquot (5 µL) of UBCR_12 cell suspension (OD_{600nm} = 1.0) were applied to a plate which was subsequently grown with the fungus. The results of the assay were determined using this following equation:

$$\text{Inhibition } (\%) = \frac{DC - DT}{DC} \times 100$$

where, DC is the diameter of the untreated fungi and DT is the diameter of the treated fungi sample[14]. Each treatment was performed in five replicates.

Co-culture of UBCR_12 and *Colletotrichum gloeosporioides*: To further analyse the antagonistic activity, a co-culture system based on the method described by Zhang *et al.*[15] was adapted with some modifications. *Colletotrichum gloeosporioides* was cultured in a disk (5×5 mm) with 100 mL Potato Dextrose Broth (PDB) for 72 h at room temperature under shaking condition at 100 rpm. Bacterial UBCR_12 cells were cultured in Luria Broth (LB) medium until the Optical Density (OD_{600nm}) had reached a level 1.0. Cells were harvested by centrifugation (20.000×g for 15 min at 4°C) and the pellet were washed twice with dH_2O. Subsequently, 5 mL suspension of UBCR_12 cells (3×10^{8} CFU mL^{-1}) was added to the culture and grown for 24 h under shaking condition at 100 rpm and 30°C.

Fungal mycelia were removed using Whatmann paper No. 1. Culture supernatant was collected by centrifugation (20.000×g for 15 min at 4°C) and then filtered through membrane with a pore size of 0.22 µm. The resulting cell-free filtrate (50 µL) was then tested for its antifungal activity using agar well plate method on PDA medium. As a control, culture supernatant obtained from single culture of UBCR_12 grown at the same conditions as the co-culture was also tested.

Total protein extraction and SDS-PAGE: Co-culture of UBCR_12 and *C. gloeosporioides* was also subjected to total protein extraction, examining cellular and secreted proteins

to observe the proteome profile of UBCR_12 during its interaction with fungi. Cellular proteomes were extracted using a method described by Jangpromma *et al.*[16]. Bacterial pellets harvested from co-culture were lysed in 500 µL lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS) and homogenized by 5 min vortexing at room temperature. Supernatant was then collected after another centrifugation step at 10.000×g for 30 min at 4°C.

The extraction of extracellular proteins was performed using the supernatant from co-culture as described by Nouwens *et al.*[17] with slight modification. The cell free culture supernatant from co-culture was precipitated overnight in pre cooled 20% TCA-acetone and then centrifuged at 20.000×g and 4°C for 30 min. Collected pellets were washed 3 times using cold acetone, freeze-dried and resuspended with 2 mL sample buffer (7 M urea, 2 M thiourea, 4% CHAPS).

About 190 ng cellular and extracellular proteins were loaded on a 17% polyacrylamide gel with a 5% stacking gel. The gel was run for 15 min at 40 mA then continued at 100 mA for 50 min in SDS running buffer (0.025 M tris, 0.192 M glycine, 0.1% SDS). As size marker, 5 µL PageRuler™ prestained protein ladder (Thermo scientific) was used. For the visualization, gel was stained with Coomassie Brilliant Blue (CBB) G-250 and then destained with destaining solution (40% methanol and 10% acetic acid). Images of stained gels were documented using Canon CanoScan LiDE 110 scanner.

Antagonistic assay against other phytopathogenic fungus: In addition to *C. gloeosporioides* from chili pepper, the antifungal activity of UBCR_12 was also assayed against other pathogenic fungi to determine its inhibition spectrum. An assay was carried out using four isolates of phytopathogenic fungus, including *C. gloeosporioides* isolated from dragon fruit, *Fusarium oxysporum* isolated from banana and soybean and *Sclerotium rolfsii* isolated from groundnut. The assay was performed using agar spot method through by application of 20 µL UBCR_12 culture (OD_{600} = 1.0) on PDA medium containing a fungal colony.

Statistical analysis: Data were processed by one-way analysis of variance (ANOVA) using SPSS 16.0 and presented with mean and standard error. Significant differences between each treatment group were analysed using Fisher's Least Significant Difference (LSD) test with a $p<0.05$.

RESULTS

Antifungal activity of UBCR_12 at different levels of pH: Antagonistic interaction between the bacterial strain UBCR_12 and the pathogenic fungus *C. gloeosporioides* was significantly affected by pH value in the antagonistic assay medium. The inhibition level varied between different pH values even though activity patterns were rather similar over time, except for pH 8. According to its dynamic activity (Fig. 1), the highest level of inhibition was found at pH 8 (22.22%) one day after treatment. However, inhibition decreased at pH 8 over time, while the most stable inhibition was observed at pH 7. Acidic (pH 5 and 6) and highly basic pH values (pH 9) seemed to suppress the antifungal activity of UBCR_12 against *C. gloeosporioides*.

Effect of nutritional modification towards the antagonistic activity of UBCR_12: Nutritional modifications by using different carbon and nitrogen sources had strong effect on the antifungal activity of UBCR_12 against *C. gloeosporioides* (Fig. 2a, b). In the carbon source experiments, the antifungal

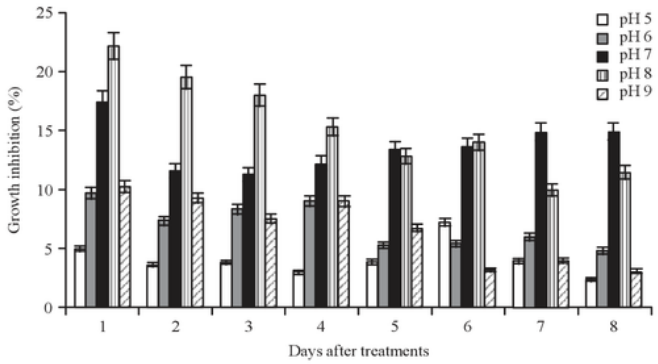

Fig. 1: Antifungal activity of UBCR_12 at different level of pH. The UBCR_12 inhibition activity against *Colletotrichum gloeosporioides* under different pH levels ov 8 days of incubation. An aliquot (5 µL) of UBCR_12 culture was spotted 3 cm away from the center of fungal growth. Values are Mean±SD (n = 5)

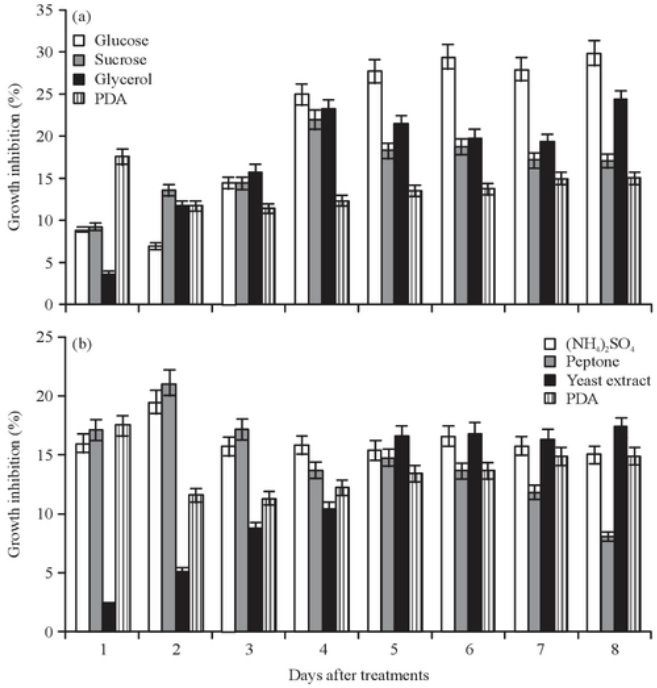

Fig. 2(a-b): Effect of nutritional modification towards the antagonistic activity of UBCR_12. Dynamics of UBCR_12 antifungal activity against *Colletotrichum gloeosporioides* under modified medium (pH 7.0) containing (a) Carbon and (b) Nitrogen sources. An aliquot (5 µL) of UBCR_12 culture was spotted 3 cm away from the center of fungal growth. Values are Mean±SD (n = 5)

activity increased consistently and reached its maximum inhibition at 8 days after treatment with the addition of glucose (29.79%) and glycerol (24.14%) (Fig. 2a). Addition of sucrose showed the highest inhibition at 4 days after treatment (Fig. 2a). Glucose supplemented medium displayed the best inhibitory activity.

Optimization using various nitrogen sources resulted in a rapid induction of UBCR_12 antagonistic activity, where the best inhibition was obtained from peptone addition at 2 days after treatment (21.18%). The UBCR_12 also showed striking inhibition against *C. gloeosporioides* on medium containing $(NH_4)_2SO_4$ about 19.53% at 2 days after treatment (Fig. 2b). However, these high suppression levels induced by peptone and $(NH_4)_2SO_4$ were quite unstable and slowly decreased until 8 days after treatments.

Presence of fungal cells stimulated higher antagonistic activity of UBCR_12: The interaction which occured between UBCR_12 and *C. gloeosporioides* during co-culture, significantly contributed to an elevation of UBCR_12 antifungal activity. Culture supernatant of UBCR_12 produced from this co-culture displayed the highest suppression about 30.66% at 8 days after treatments (Fig. 3).

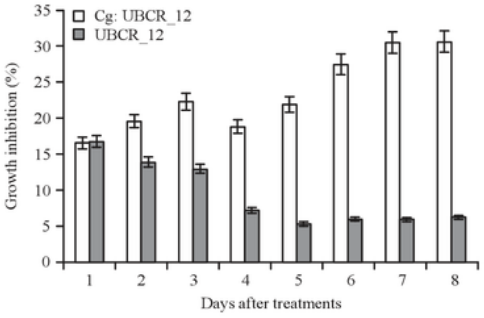

Fig. 3: Presence of fungal cells stimulated higher antagonistic activity of UBCR_12. Inhibitory effect of cell free culture supernatant extracted from single bacterial culture and co-culture of UBCR_12 *Colletotrichum gloeosporioides* over 8 days of incubation. An aliquot (50 µL) of culture supernatants was applied 3 cm away from the center of fungal growth. Values are Mean±SD (n = 5)

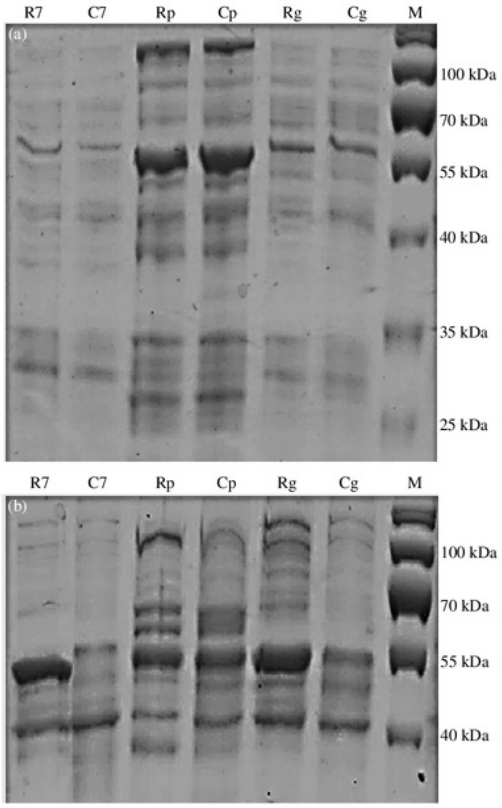

Fig. 4(a-b): SDS-PAGE of UBCR_12 after single and co-culture with *Colletotrichum gloeosporioides* under modified conditions. Visualization of UBCR_12 (a) Cellular and (b) Extracellular proteomes after 24 h co-culture with *Colletotrichum gloeosporioides* under modified conditions showing the best antagonistic activity (pH 7, peptone or glucose treatments). M: Protein ladder, R: Single culture of UBCR_12, C: Co-culture of UBCR_12 *Colletotrichum gloeosporioides*

This was significantly higher than the inhibition levels induced by the cell-free supernatant of single-cultured UBCR_12 (16.76%, Fig. 3). The co-culture of *C. gloeosporioides* and UBCR_12 may therefore, induce the production of antifungal compounds.

SDS-PAGE of UBCR_12 after single and co-culture with *Colletotrichum gloeosporioides* under modified conditions: Antagonistic interaction between UBCR_12 and *C. gloeosporioides* was further analysed by SDS-PAGE. From several modified conditions, the best inhibition was found at pH 7, with 1% peptone and 1% glucose. This experiment was performed u sing both cellular and extracellular compartments of UBCR_12 proteome, obtained after 24 h co-culture. Figure 4a shows, UBCR_12 displayed a similar expression pattern of cellular proteome at all modified conditions which only could be distinguished by the thickness of protein bands. Several bands were stronger under peptone addition but showed weaker signals at pH 7 and with glucose addition. This result suggests that peptone content in the medium elevated the expression of some cellular proteins of UBCR_12, which might be associated with its antagonistic activity. Unlike pH 7 and glucose treatments, the presence of fungal cells in the peptone containing medium showed no significant difference in the cellular profile of UBCR_12 supporting the assumption that peptone could be the enhancer (inducer) of its antagonistic activity.

Visualization of the extracellular proteome of UBCR_12 displayed a more differentiated pattern compared to the cellular one (Fig. 4b). Nutritional modifications seemed to trigger the expression of more bands rather than pH modification, while UBCR_12 interaction with *C. gloeosporioides* affected some of those bands under each condition. Even though the cellular profile exhibited a similar

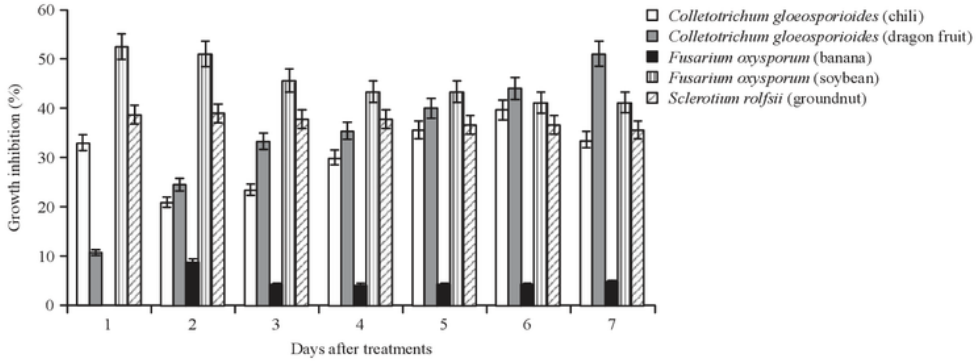

Fig. 5: Antagonistic spectrum of UBCR_12 against several phytopathogenic fungus. Antifungal activity of UBCR_12 against several phytopathogenic fungus over 7 days of incubation. An aliquot (20 µL) of UBCR_12 culture were spotted 3 cm away from the center of fungal growth. Values are Mean±SD (n = 5)

pattern, the expression of UBCR_12 extracellular proteins was quite different compared to standard growth medium. In this study, some high molecular weight bands (>55 kDa) were less detectable with pH modification but more visible with glucose and peptone additions (Fig. 4b). The effect of fungi-bacteria co-culture was rather supposed to affect the expression of several protein bands resulting in different profile pattern.

Antagonistic spectrum of UBCR_12 against several phytopathogenic fungus: The bacterial strain UBCR_12 not only inhibited growth of *C. gloeosporioides* from chili pepper, but also showed high and stable suppression against *F. oxysporum* in soybean and *S. rolfsii* from groundnut (Fig. 5). However, its antagonistic activity displayed weak influenceon *F. oxysporum* isolated from banana. However, its highest inhibition remained to be found in the interaction with *C. gloeosporioides*, especially that one isolated from dragon fruit about 51% at 7 days after treatment (Fig. 5).

DISCUSSION

The production of antimicrobial substances is greatly influenced by physical (such as pH, temperature, humidity) and nutritional factors[18-21]. The contribution of these factors is considered as one of crucial aspects and a main requirement to trigger antimicrobial activity of bacteria. In this study, effect of nutritional and pH level modifications were successfully used to elevate the inhibition activity of *S. plymuthica* strain UBCR_12 against *C. gloeosporioides*.

Several previous studies have reported that some *Serratia* species require nearly neutral pH level (6-7) for its optimum production of antifungal compounds. The optimum chitinase activity of *S. plymuthica* strain HRO-C48 was obtained[22] at pH 6.6, while some strains of *S. marcescens* had their highest chitinase activity at a pH ranging[23-25] from 6-7. In line with the previous studies, the antagonistic activity of UBCR_12 had the most stable activity over time at pH 7, which can be considered as the most optimal condition for its inhibition activity, even though the highest inhibition rate was shown under pH 8 at 1 day after treatment (Fig. 1a).

Nutritional factors, such as carbon and nitrogen sources have vital functions for bacterial growth and production of secondary metabolites. The presence of these factors is regarded as an environmental manipulation stimulating the production of certain antibiotics[26]. The production of some other antibiotics is triggered by nutrient starvation and growth rate decrease[27], which frequently occurs in the late stationary growth phase of bacteria[26]. In this present study, glucose addition resulted in the highest fungal inhibition at 8 days after treatment with a relatively slow induction compared to peptone addition (Fig. 2). One explanation for this phenomenon might be that the glucose content in the medium was mostly used to support UBCR_12 growth at the beginning, thus inhibited its antagonistic activity. When its availability becomes limited, it starts to trigger the production of UBCR_12 antifungal compounds. Previous study has shown that glucose is a vital carbon source which greatly influences bacterial growth and metabolism, for example by repressing the biosynthesis of several secondary metabolites[28].

Biosynthesis of natural products in Gram negative bacteria, such as *Serratia* is known for its sensitivity to carbon deficiency[26]. Due to this sensitivity, not all kinds of carbon sources promote the production of these metabolites. In addition, the application of easily metabolized carbon seems to suppress the production of bacterial antibiotics[29,30]. For instance, production of prodigiosin by *S. marcescens* attained its optimum activity in medium containing ethanol, but is strongly reduced in medium with low concentration of monosaccharides or disaccharides[31]. In other study, the use of colloidal chitin as sole carbon sources contributed to higher chitinase production of *S. marcescens* strain CBC-5 compared to some other simple sugar compounds[30]. Other Gram negative bacteria, *Pseudomonas aeruginosa* strain MAI2, exhibited optimum production of antimicrobial compounds by utilizing glycerol and starch[32].

In this study, unlike the carbon sources the presence of peptone contributed to a rapid stimulation of the the antifungal activity of UBCR_12 (Fig. 2b). This finding is in agreement with Wang *et al.*[33] who reported that the highest production of chitinase by *S. marcescens* strain JPP1 is achieved with the presence of peptone in the medium. Another strain of *S. marcescens* MO-1, also displayed optimal production of prodigiosin in ram horn peptone containing medium after 48 h of incubation[34]. Nitrogen contributes vital functions in the biosynthesis regulation of primary and secondary metabolites[35]. However, the necessity of this nitrogen source is specifically depending on the biosynthesis pathway of the compounds[36] and the cellular processes.

Shapiro[37] noted that types of nitrogen sources, whether organic or inorganic determine the optimum production of a metabolite. In bacteria, for example a compound produced by the same bacteria species probably requires different types of nitrogen sources depending on the strains. The optimum production of chitinase by some strains of *S. marcescens* is stimulated by different nitrogen sources, such as yeast extract, malt extract, peptone and ammonium sulfate[30,33,38,39]. Since the highest antagonistic activity of UBCR_12 under nitrogen modification was observed when using peptone (Fig. 2b), it could be concluded that this strain preferentially favors an organic nitrogen source to stimulate its activity. The addition of peptone has such a strong impact on the metabolism of UBCR_12, that within this study it could be easily show by simple SDS-PAGE an effect of the cellular and extracellular proteome (Fig. 4a, b). However, the connection between expression shifts occurs within both compartments of the proteome was still unclear and required to be further elucidated.

The co-cultivation method has been reported as a good tool to initiate a communicative response in antagonistic assay since it precisely mimics the actual condition occuring during antagonist-pathogen interaction[40,41]. Effect of *Lactobacillus plantarum* and *Aspergillus nidulans* co-culture successfully diminished biomass of *A. nidulans* to 36% compared to the control through higher production of *L. plantarum* inhibitory metabolites[42]. Other study reported that some secondary metabolites demanded the presence of pathogen cells to stimulate or enhance its production[43]. Similar to those previous studies, the presence of *C. gloeosporioides* cells in the UBCR_12 culture significantly enhanced the production of antifungal compounds released into the medium. Culture supernatant obtained from co-culture of UBCR_12 and *C. gloeosporioides* exhibited a remarkable higher suppression of fungal growth compared to the single-cultured one (Fig. 3). This co-culture also affected the expression of some cellular and extracellular protein bands of UBCR_12 under pH 7 and glucose addition. However, effect of peptone addition seemed to cover the effect of fungal cells presence resulting in similar expression pattern either in single culture or co-culture bacteria.

The broad antifungal activity of *S. plymuthica* against several phytopathogenic fungus has been widely reported[2,8,44-46]. Antifungal activity of this plant associated Gram negative bacteria results from various mechanisms, including production of diverse secondary metabolites, degradation of fungal cell wall and formation of siderophore[2,4,6,7,47,48]. In this study, UBCR_12 also showed high antifungal effect against *S. rolfsii* and *F. oxysporum* from soybean. These results suggest that this isolate is promising to be used as a biocontrol agent to overcome various infections from phytopathogenic fungi.

CONCLUSION

Elevated antifungal activity resulted either from environmental or biotic modifications, suggests that the activity of this isolate is environmentally dependent. The compatibility of these external factors would be a critical aspect to be considered for its further development as an antianthracnose. However, since all modifications assayed contributes to higher inhibition, it remains questionable how far the modifications affect the cell and subsequently result in this higher activity. Therefore, further investigation is required to find out the molecular mechanism which possibly involved in the regulation of this activity. This information is highly required as the main consideration in the production of more effective antifungal effect of this strain.

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