e-ISSN:2320-0189
p-ISSN:2347-2308

Research & Reviews: Journal of Botanical Sciences

Purification and Characterization of β-1,3-glucanase from *Candida Oleophila* for the Biocontrol of *Penicillium expansum*

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Research Article

Received date: 24/12/2015
Accepted date: 15/03/2016
Published date: 17/03/2016

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Keywords: Cell wall degrading enzymes, Postharvest fungal diseases, Disease control.

ABSTRACT

Candida oleophila was originally isolated from apple fruit peel in the region of Cuauhtémoc, Chihuahua, Mexico, and utilizes exo-β-1,3-glucanase production as a mode of action against *Penicillium expansum* in postharvest apples. β-1,3-Glucanase activity generated by *C. oleophila* on a minimal-salt medium using glucose, laminarin or cell wall fragments of *P. expansum* as a sole carbon source was determined. Highest β-1,3-glucanase activity (249 U/L) was obtained after 48 hours of incubation using only glucose. β-1,3-Glucanase was purified from a growth medium using anion exchange chromatography on DEAE Sepharose, which increased the specific activity of β-1,3-glucanase 74-fold, compared to the crude extract after ultrafiltration. SDS-PAGE demonstrated a purified β-1,3-glucanase with an estimated molecular weight of 48.3 kDa. The purified enzyme hydrolyzed laminarin to glucose as a final product and was identified on native polyacrylamide gels as a monomeric protein. Optimum activity occurred at pH 5 and at 40°C, and the enzyme was stable at 30°C for 1 hour. The purified exo-β-1,3-glucanase, when challenged against *P. expansum* conidia, reduced conidial germination to 51.8% and mycelial growth to 31%. Results indicate that one mode of action of *C. oleophila* for controlling *P. expansum* is by producing β-1,3-glucanase and that the purified enzyme reduces conidial germination and mycelial growth of the same fungus.

INTRODUCTION

The development of biocontrol agents for use on postharvest fruits in recent years has been considered an alternative to the use of synthetic chemical products [1,2]. The extent of the economic loss due to postharvest pathogen damage to fruits depends on the country and can reach 50% in developing countries [3]. The most common way to control these postharvest pathogens is with chemical synthetic fungicides, which also represent a risk to both humans and the ecosystem. There are reports from around the world that microorganisms, such as yeast, bacteria and fungi, have the capability of controlling postharvest fungi on fruit [4-7]. In addition, commercial bio products based on *C. oleophila* have been developed, such as those by BioNext (Belgium) and Lesaffre International (France) and other products [8,9]. These microorganisms are reported as being capable of controlling postharvest fungus, such as *Penicillium expansum*, and their use, either alone or in combination, by way of several modes of action, such as

nutrient and space competition, resistance induction, antibiosis and lytic enzymes production, is able to degrade the cell walls of pathogenic fungi [1]. Enzymes such as β-1,3-glucanases are now considered an important component in the biocontrol of postharvest pathogenic fungi [1,7,10-12].

C. oleophila is an epiphytic yeast that was isolated from the "Golden Delicious" fruit peel from Cuauhtemoc, Chih., Mexico [13]. This yeast has shown several modes of action in controlling *P. expansum* and *B. cinerea* on postharvest apple fruit [5,14].

In this work, the isolation, characterization and purification of an exo-β-1,3-glucanase produced by *C. oleophila* L-06 strain was performed in order to evaluate the enzyme for use as a biocontrol agent against *Penicillium expansum* on postharvest apple fruit.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

C. oleophila isolates were obtained from the "Temperate Zone Microorganisms" collection from CIAD, A. C. Unidad Cuauhtémoc, Chih. Mexico. Isolates were stored at -80°C. The *C. oleophila* L-06 was selected for this work because of its higher level of β-1,3-glucanase production. The L-06 was grown for 72 h in NYDA (15 g/L Agar-Agar, 8 g/L nutrient broth, and 5 g/L yeast extract and 10 g/L dextrose). The *P. expansum* strain used in the experiment was obtained from the Oregon State University Mid-Columbia Agricultural Research Station, Hood River, Oregon, USA. Prior to use, *P. expansum* was grown in apple fruit and then maintained in PDA (Potato, dextrose, agar) at 4°C and grown at 25°C for one week in the same medium without agar (PD).

Extraction of *P. expansum* Cell Walls

For this procedure, the methodology of Bar-Shimon et al. was followed [15]. One ml of a *P. expansum* conidial suspension (2 × 106 cells/mL) was inoculated into one L of PD broth and grown for six days at 25°C at a constant shaking rate of 155 rpm. The mycelia produced by *P. expansum* were collected by filtration using Whatman No. 1 paper and then washed three times with sterile water, which included homogenization for two min (T 25 Basic ULTRA-TURRAX®, IKA®, Rioja, Spain). Cell wall samples were frozen at -20°C for 8 h and then thawed and homogenized again as previously described until the precipitate was completely dissolved. Approximately 20 mL of the homogenized mycelia was stored at 4°C for 10-15 min. These samples were centrifuged (Beckman GS-15R, Ramsey, MI USA) at 1,625 × g for two min, the supernatant was discarded, and the precipitate was suspended again in water and centrifuged six additional times until a transparent supernatant was obtained. The supernatant was lyophilized for 48 h and stored at -20°C until used.

Growth of *C. oleophila* L-06

A starter culture of the L-06 of *C. oleophila* was prepared in 50 mL of NYB (Nutrient broth, Yeast extract, broth) medium, which was then inoculated into 200 mL of the same medium and then incubated (Precision Scientific Model 31534, USA) for 100 h at 30°C with shaking at 165 rpm. The L-06 concentration was adjusted to 4 × 103 UFC/mL (the suspensions were prepared using a hemocytometer) and an initial Optical Density (OD) of 0.001 (A500) (spectrophotometer VARIAN UV-Vis Cary 1E, USA). One mL aliquots were aseptically drawn every hour during the 100 hours of incubation to determine the OD. Colony Forming Units (CFU) was determined every three hours during L-06 isolate growth phase using Petri dish counting on NYDA diluted solutions. The CFUs were Log10 transformed to identify the exponential growth phase.

β-1,3-Glucanase Activity on Different Substrates

To obtain a greater number of L-06 cells and a higher enzymatic activity, the yeast was grown in NYB to the end of the exponential phase. The yeast cells were centrifuged at 4000 × g and then transferred to a minimum salts medium (MSM, 2.5 g/L thiamin, 0.8 g/L urea, 2.06 g/L NH_4NO_3, 0.5 g/L MgSO4, 1 g/L KH2P04, 0.01 mg/L FeSO4, 8.7 mg/L ZnSO4, 3 mg/L $MnSO_4$ and 2 mg/mLcontaining either *P. expansum* cell wall fragments, laminarin or glucose as the sole carbon source) to obtain the maximum amount of enzymatic activity. Enzymatic activity was measured in 200 mL of MSM solution at 24, 48, 72, 96 and 120 h of incubation at 30°C and shaking at 165 rpm. β-1,3-glucanase production. To obtain the enzymatic extract, the methodology of Bar-Shimon et al. was followed [15]. Five mL MSM aliquots were drawn at 24, 48, 72, 96 and 120 h of incubation and were centrifuged at 6,136 × g to remove the yeast cells. The supernatant was filtered using 0.2 µm Millipore filters and was then used for the enzymatic assay. The precipitate was washed twice in distilled water and resuspended in 2 mL of distilled water to measure the biomass at 600 nm. The precipitates were dehydrated at 37°C to a constant weight to obtain the yeast dry weight during several days. β-1,3-glucanase assay. To determine the β-1,3-glucanase activity in the enzymatic extract, the procedure of Ippolito et al. 2000 was followed, and the activity was determined for each incubation time point. The assay solution contained 62.5 µL of enzymatic extract plus 62.5 µL of 0.5% laminarin (Laminarin was dissolved in a 50 mM, pH 5.0 acetate buffer), and the reaction was stopped at 20, 40, 80 and 100 min of incubation at 40°C. To stop the reaction at each time point, 125 µL of dinitrosalicylic acid (Sigma, D0550-100G, prepared by mixing 300 mL of 4.5% NaOH with 880 mL of a solution containing 8.8 g of 3,5-dinitrosalicylic acid and 255 g of double tartrate of sodium and potassium) was added to each solution. The solutions were incubated in a water bath at 100°C for five min. and absorbances were determined by spectrophotometry (Microplate reader Bio-Rad 550) at 490 nm. Absorbance readings for each time point of the enzymatic kinetic study were transformed to g/L of

glucose using the regression equation Y=0.322x + 0.009 (R^2=0.994). Glucose concentrations were transformed to units/L of growth medium by pooling the glucose concentrations at each of the different times of the enzymatic kinetic study, generating equations that represented the total glucose concentration, mg/mL/min, for each enzymatic kinetic time point, which were then transformed to units and then converted to micromoles/min*L = U/L.

Total Protein Determination

The Bradford, 1976 procedure was used to determine the total protein content of each sample. Twenty five µL of each sample was mixed with 200 µL of Bradford reagent (1X) and incubated for five min at room temperature. The absorbance was measured at 595 nm and was then transformed to µg/L using the regression equation Y= 0.0049x + 0.0057 (R^2=0.991) based on bovine serum albumin.

β-1,3-Glucanase Purification

Purification of β-1,3-Glucanase. Enzyme purification was performed by concentrating the sample by ultrafiltration (Amicon Filter Membrane 10 kDa, Sigma Z706345-8EA, USA) and centrifugation at 4,000 × g for 15 min followed by diethylaminoethyl Sepharose (DEAE) anionic exchange chromatography. The ultrafiltered enzymatic extract was applied to a DEAE Sepharose Fast Flow (Sigma) column, which was equilibrated with five column volumes, 230 mL, of 20 mM Tris HCl, pH 8.0. The column was eluted with a NaCl gradient (0.2, 0.3, 0.4 M) in 20 mM Tris HCl buffer, pH 8.0, at a flow rate of 500 µL/min. Three mL fractions were collected and total protein content and β-1,3-glucanase specific activity were determined. Enzyme purity was determined by SDS-PAGE under denaturing and reducing conditions. Polyacrylamide gels were prepared using a mixture of 10% polyacrylamide and 4% bis-acrylamide solutions. Electrophoresis was conducted at 100 millivolts (mV) for 1.5 h at room temperature. The migrated samples were stained with a $AgNO_3$ solution. Native PAGE procedures were performed and by using a discontinuous system at 20 mA for 1.5 h at room temperature, with 2 mg/mL laminarin in the separator gel. After electrophoresis, the gels were incubated for 1 h at 40°C in 200 mL of 50 mM sodium acetate buffer, pH 5.0. Gels were submerged in 0.005% aniline blue in 150 mM potassium phosphate, pH 8.6, for 15 min. Bands were observed under UV illumination (UV Trans illuminator High performance Kodak EDAS 290, USA).

Characterization of β-1,3-Glucanase

The optimal pH, temperature and thermal stability were determined as follows. A 50 mM sodium acetate buffer was used to determine the optimal pH for the enzyme. The pH range tested was from 5 to 8. A mixture containing 62.5 µL of purified β-1,3-Glucanase solution and 62.5 µL of 0.5% laminarin was incubated for 90 min at 40°C for each different pH value tested. The maximum enzymatic activity was considered 100% relative activity. Several temperatures were evaluated for β-1,3-glucanase. A 50 mM sodium acetate buffer was used to determine the optimal temperature and the optimal pH that as determined was used for these trials. A mixture containing 62.5 µL of purified β-1,3-Glucanase solution and 62.5 µL of 0.5% laminarin was incubated for 1.5 h at 0, 4, 10, 20, 30, 40 and 50°C. The maximum enzymatic activity observed was considered 100% relative activity. To determine the thermal stability of the enzyme, 62.5 µL of purified β-1,3-glucanase was previously incubated at 30, 40, 50, 60, 70, 80 and 90°C in 50 mM sodium acetate buffer for 60 min at the optimal pH that was obtained for the enzyme in previous trials. After this step, 62.5 µL 0.5% of laminarin was added to the previously incubated solutions before initiating a new incubation period at the optimal temperature. The maximum enzymatic activity was considered 100% relative activity.

Effect Of β-1,3-Glucanase on *P. expansum* Conidial Germination and Mycelial Growth

Varying amounts of purified β-1,3-glucanase, 1.9, 3.75 and 7.5 U/mg, were incubated with 300 *P. expansum* conidia in an ELISA (ELISA microplate reader) plate for 48 h at 27°C. In addition, a negative control was included that consisted of β-1,3-glucanase that was inactivated by heating at 100°C for five min, and a positive control, *A. niger* (Sigma 49101, USA) β-1,3-glucanase, was also included. After the incubation, *P. expansum* conidial germination was evaluated by counting those which have had germinated. The maximum conidial germination was considered 100% of relative activity. At the same time, *P. expansum* was grown for seven days at 27°C in PDA. Then, 0.5 mm^2 of agar containing mycelia was removed and inoculated in PDA again with 1.9, 3.75 and 7.5 U/mg of purified β-1,3-glucanase and incubated for 120 h at 27°C. Additionally, a negative control was included that consisted of β-1,3-glucanase inactivated by heating at 100°C for five min, and a positive control, *A. niger* β-1,3-glucanase, was also included. After the incubation, *P. expansum* mycelial growth was evaluated. The maximum mycelial growth was considered 100% of relative activity.

Statistical Analysis

Each experiment was repeated three times. When results were obtained as percentages, they were transformed to ArcSen and ANOVA analysis was applied. The statistical design was completely randomized using three replicates. Comparison of means was performed using the Tukey test (α=0.05). SAS (Statistical Analysis System, 8.0, Cary, NC, USA) for Windows.

RESULTS AND DISCUSSION

Growth of *C. oleophila* L-06

The growth curve for the *C. oleophila* L-06 is shown in **(Figure 1)** An optical density (OD) value of one is equivalent to 5 X 105

CFU/mL for 100 h incubation at 30°C. When the CFU/mL was Log10 transformed, there was a linear response, which indicates an exponential growth rate for the yeast cells. The results also demonstrate an exponential phase span up to 27 h. Bar-Shimon et al., reported a similar growth curve for *C. oleophila*. Therefore, maximum growth occurred (in NYB) at 27 h, and each life cycle (one generation) for the yeast lasted 2.6 h. Madigan et al., suggested that at the highest point of growth, the enzymatic machinery of the yeast cells is complete and thus able to produce a greater amount of enzyme. Based on these results, the *C. oleophila* L-06 was incubated for 27 h [16]. The yeast cells were then separated from the NYB medium by centrifugation and then transferred to a MSM in order to proceed with the following experiments.

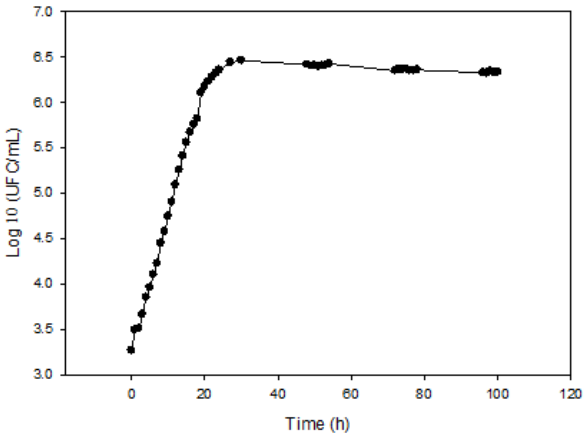

Figure 1. *C. oleophila* L-06 growth curve in NYB medium. (Shaking at 165 rpm, at 30°C, for 100 h). The arrow indicates the highest point of growth.

β-1,3-Glucanase Production In Different Substrates

The highest β-1,3-glucanase specific activity was obtained at 48 h of incubation and with 1.7 U/mg protein **(Figure 2A).** These results confirm that the highest level of enzyme activity of *C. oleophila* L-06 occurs during the period of greatest growth, as has been noted by Madigan et al. [16]. Following that period, the enzymatic activity was drastically reduced. The enzymatic specific activity diminished as much as 0.04 U/mg throughout the incubation period. The enzyme activity varied ($P<0.01$) when glucose, laminarin and *P. expansum* cell walls were used separately as the sole carbon source. **(Figure 2A)** shows the relationship between the enzymatic activity (U/L) and time (h), for which the carbon source also had a significant ($P<0.01$ effect. The greatest amount of enzymatic activity was observed after 48 h with glucose, at 249 U/L (Tukey $p<0.05$), followed by laminarin, at 233 U/L. The enzyme activity decreased after this period of time, but some enzyme activity remained until the end of the incubation. The lowest amount of enzyme activity, 173.9 U/L at 72 h, was produced using *P. expansum* cell walls. The response of the microorganisms to a carbon source is variable (Martin et al.). Some microorganisms, such as *Trichoderma harzianum* and *Trichoderma asperellum*, produced high levels of β-1,3-glucanase when the growth medium contained fungal cell walls Bara et al. [17]. Others, such as *Neurospora crassa* and *Trichoderma viride*, produced low or no activity when grown in a medium containing glucose. In this work, *C. oleophila* L-06 produced a higher level of β-1,3-glucanase with glucose than with PeCW, which is similar to Saccharomyces cerevisiae when the growth medium was supplemented with glucose. The results with both glucose and laminarin suggest that *C. oleophila* L-06 requires glucose to produce high levels of β-1,3-glucanase activity Bar-Shimon et al., however, at a certain time in the incubation period, the high glucose level inhibits β-1,3-glucanase activity by feedback inhibition Wisniewski et al. [15,18,19]. **(Figure 2B)** shows the relationship between dry weight and time and enzyme specific activity and time.

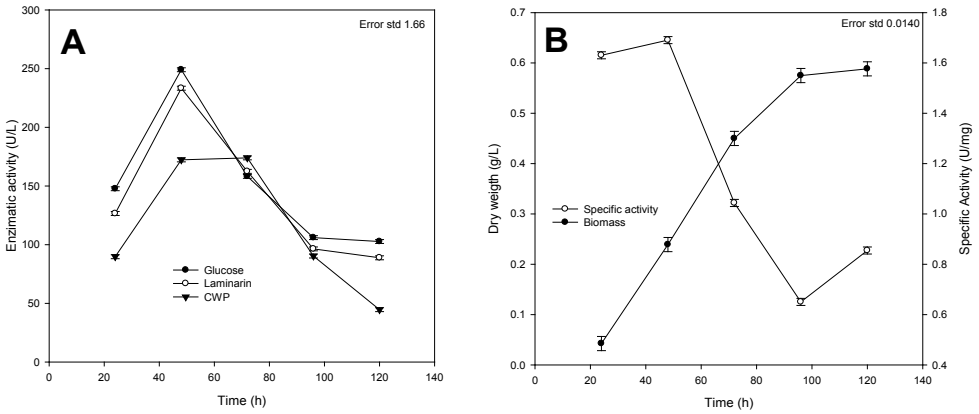

Figure 2. *C. oleophila* β-1,3-glucanase activity. (A) β-1,3-Glucanase activity using different carbon sources. (B) Relationship between biomass and β-1,3-glucanase specific activity using glucose as the sole carbon source.

β-1,3-Glucanase Purification

Non-gel attached eluted samples had a β-1,3-glucanase specific activity peak when hydrolyzing laminarin to form glucose

e-ISSN:2320-0189
p-ISSN:2347-2308

as the sole product. Gel-attached eluted samples had two specific activity peaks, peak b (a), corresponding to fraction 49, and peak c (b), corresponding to fraction 62 **(Figure 3).** The highest specific enzymatic activity corresponded to the fraction of peak c, which was ultrafiltered to obtain a greater enzyme concentration for later characterization and use as a biocontrol agent against *P. expansum*. Peak b had a specific activity similar to that of a glucosidase and may be due to the presence of a β-1,3-glucanase with a low specific activity Peng et al., [20]. At the end of the chromatography process, other proteins were eluted using a 0.4 M NaCl solution. These proteins did not show β-1,3-glucanase specific activity. There have been up to ten isoforms or isoenzymes of β-1,3-glucanase reported from *T. harzianum*, with seven of them being inhibited by 2% glucose [20]. Three Acremonium persicinum exo-β-1,3-glucanases are expressed from different genes, with two of them, GNI and GNII, having identical amino acid sequences; these enzymes have shown differences when compared to the third β-1,3-glucanase (GNIII). Further analysis concerning the regulation of these enzymes suggests that they may also have different functions. The β-1,3-glucanase isolated from the *C. oleophila* L-06 was purified 74 times compared to the total protein, with a specific enzymatic activity of 15.5 U/mg, indicating a protein purification of approximately ~74-fold. Enzyme recovery was greater than 20% compared to the crude extract **(Table 1).** Because the enzyme was recovered in small fractions, it was necessary to purify the extract ten additional times.

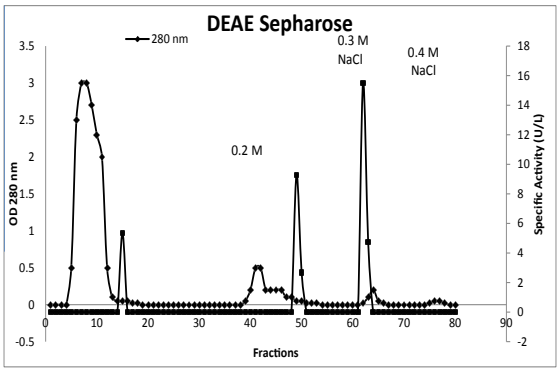

Figure 3. Anion exchange chromatography of an ultrafiltered extract of growth medium for *C. oleophila* using DEAE Sepharose Fast Flow. The column was eluted with a 0-0.4 M NaCl gradient. Each fraction was 3 mL.

Table 1. Purification process for β-1,3-glucanase from the L-06 *C. oleophila* isolate.

Purification process	Total volume (mL)	Total protein (mg/L)	Total activity (U)*	Specific activity (U/mg)	Purification degree (times of)	Yield (%)
Crude extract	1000	1380.4	288.7	0.21	1	100
Ultrafiltration	20	740.9	233.7	0.32	1.5	81
DEAE Sepharose	4	4.2	65.5	15.5	74	22.7

*Unit (U), amount of enzyme that catalyzes the conversion of 1 µMol of glucose/min.

The molecular weight and purity of purified β-1,3-glucanase were determined using fraction 62, due to its highest specific activity (DEAE Sepharose). **(Figure 4A)** Shows one protein band with a relative mass of 48.3 kDa, which corresponds to β-1,3-glucanase from the *C. oleophila* L-06. Similar β-1,3-glucanases were reported for yeast by Peng et al., (47.5 kDa). **(Figure 4B)** shows β-1,3-glucanase activity when analyzed by native PAGE electrophoresis for both ultrafiltered crude extract (well 1) and β-1,3-glucanase from DEAE Sepharose (well 3). Laminarin (2 mg/mL) was used as the substrate in the gel for this electrophoresis, allowing the identification of lytic zones that correspond to β-1,3-glucanase activity, here represented as dark bands. The observation that only one band is observed with the crude extract, DEAE Sepharose, and in both types of electrophoresis, denaturing/reducing and native conditions, indicates that β-1,3-glucanase obtained from the *C. oleophila* L-06 is monomeric, with a molecular weight of 48.3 kDa. These results are in agreement with those reported by Bar-Shimon et al. [15].

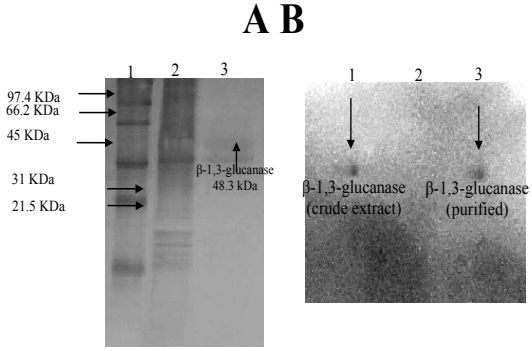

Figure 4. Electrophoretic analysis of β-1,3-glucanase from *C. oleophila*. (A) Denaturing and reducing PAGE (10% gel). The gel was stained with silver. Well 1, molecular weight markers: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and trypsin inhibitor (21.5 kDa). Well 2, crude extract C. oleophila growth medium (6 µg/mL). Well 3, eluted β-1,3-glucanase (2 µg/mL) with an estimated molecular weight of 48.3 kDa. (B) Native PAGE electrophoresis (10% gel). Well 1, C. oleophila (6 µg/mL) crude extract from growth medium. Well 2, eluted β-1,3-glucanase (0.4 U/mL), inactivated by boiling at 100°C for 5 min. Well 3, eluted β-1,3-glucanase (0.4 U/mL) (the enzyme activity in the gel is represented by a dark band).

e-ISSN:2320-0189
p-ISSN:2347-2308

Characterization of *C. Oleophila* L-06 Exo-β-1,3-Glucanase

pH: Enzymatic activity was present at all pH values evaluated, except for pH 3.0 **(Figure 5B).**The lack of activity at this low pH could be due to degradation or to modification of the active site due to the low pH [21]. The optimal pH was determined to be 5.0, and the enzyme had a specific activity of 13.2 U/mg (100% relative activity) and a relative activity higher than 80% between pH 4.0 and 6.0. A loss of greater than 50% of the relative activity was observed at pH 7.0 and 8.0. There were significant (α=0.05) differences among the pH values evaluated for β-1,3-glucanase production. The results obtained in this work are similar to those of most β-1,3-glucanases from different microorganisms [22,23].

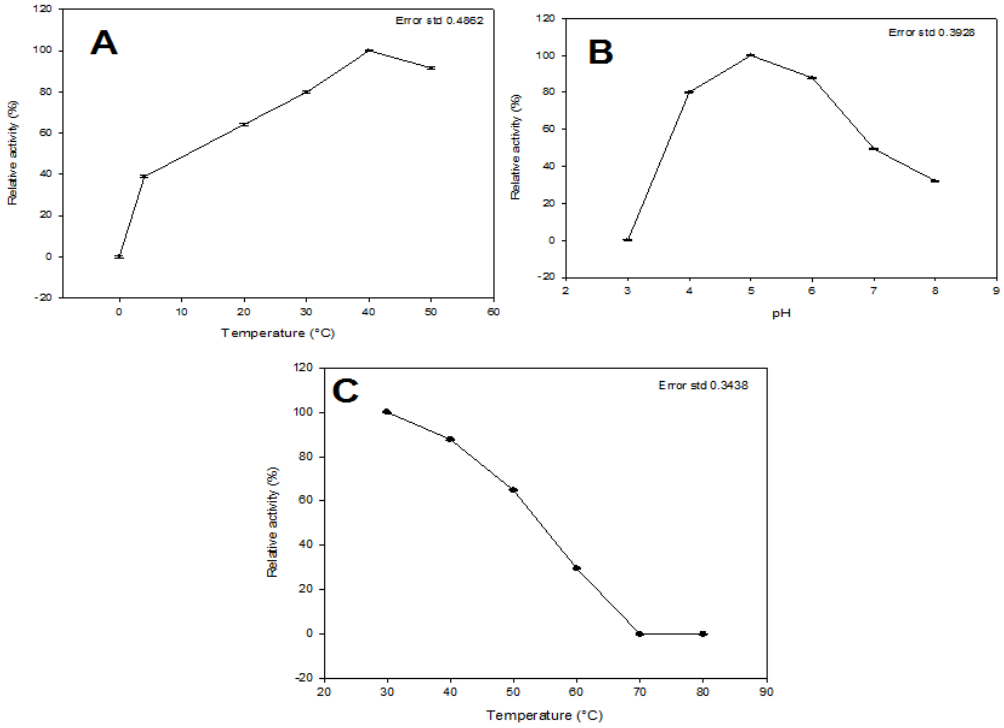

Figure 5. The effect of pH (A) and temperature (B) on β-1,3-glucanase specific activity. The highest specific activities, 13.2 U/mg and 11.2 U/mg, for pH and temperature optima, correspond to 100% relative activity, respectively. β-1,3-Glucanase thermal stability (C). The enzyme was incubated for one h at different temperatures, for determining specific activities. The highest specific activity, 8.9 U/mg, corresponds to 100% relative activity. Each experiment was performed three times.

Temperature: There was specific activity at all temperatures evaluated, except at 0°C **(Figure 5A).** The optimal temperature for the enzyme was 40°C, with a specific activity of 11.2 U/mg. There was also a specific activity of 90% up to 50°C. There were significant (α=0.05) differences among the treatments evaluated. Low temperatures, such as 4°C, lower the specific activity to 40%; at 0°C, there was no specific activity present.

Thermal stability: A temperature range from 30 to 60°C for 60 min was used to evaluate the relative enzymatic activity. A greater thermal stability was observed at 30°C, with a specific activity of 8.9 U/mg **(Figures 5B and 5C).** There was specific activity up to 60°C, with a loss higher than 70%. Significant (α=0.05) differences were detected among all treatments for enzyme activity. The enzyme isolated in this work can be considered a moderately thermo-stable enzyme because it exhibits specific activity from 50 to 60°C. The low or null activity for this enzyme at 4°C or lower may represent a disadvantage because apple fruit is stored cold at 0°C. The *C. oleophila* L-06 isolate has been reported to be able to grow at low temperatures [13]. It is also probable that L-06, when challenged against phytopathogenic fungi, may show β-1,3-glucanase activity with several temperate fruits at cold storage temperatures of 0 and 5°C [24].

Because β-1,3-glucanase has specific activity at high temperatures, such as 60°C, this enzyme may be considered effective as an industrial bio-catalyzer. Furthermore, it has a higher resistance to denaturation agents, such as co-solvents, chaotropic agents and detergents, and a long life span. These characteristics may allow the *C. oleophila* L-06 to be produced commercially. The enzyme that was isolated and purified in this work has characteristics similar to those reported by Peng et al. when working with Williopsis saturnus, which had an optimal pH of 4.0, an optimal temperature of 40°C and thermo stability at 70°C.

Effect of β-1,3-glucanase on P. expansum conidial germination and mycelial growth: *P. expansum* conidia were subjected to the following doses of purified enzyme: 1.9, 3.75 and 7.5 U/mg. Conidial germination was similar to that of the positive control, A. niger β-1,3-glucanase **(Figure 6A).** There were significant (α=0.05) differences among the treatment doses, including both *C. oleophila* and *A. niger* β-1,3-glucanases. Higher doses of enzyme produced greater inhibition of conidial germination. The *A. niger* enzyme produced greater inhibition of conidial germination (55.4%) than the *C. oleophila* (50%) enzyme, with significant (α=0.05) differences being observed among these and the other treatments.

e-ISSN:2320-0189
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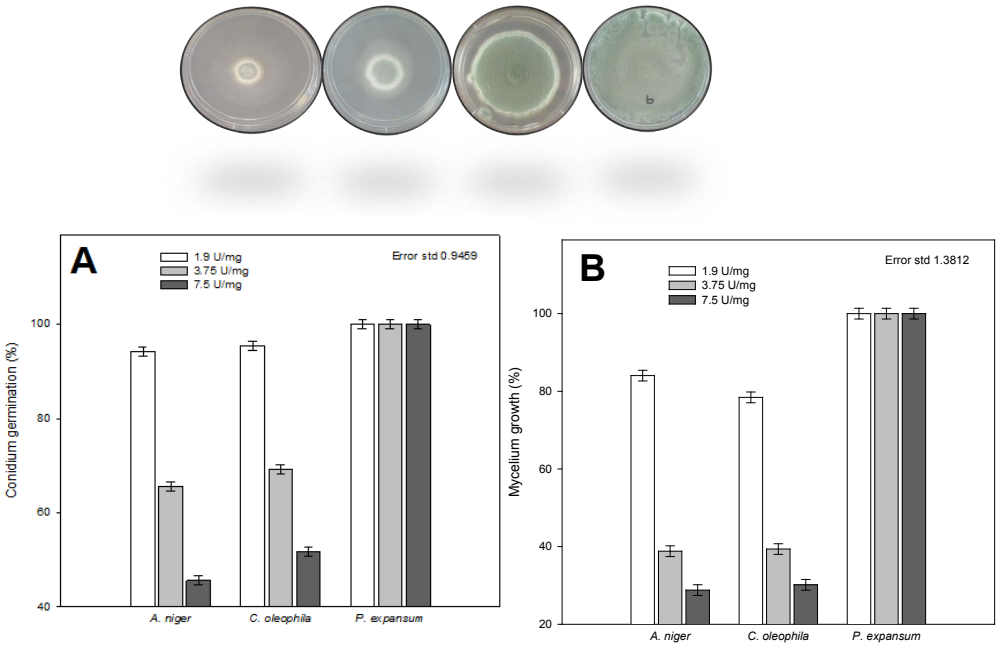

Figure 6. The effect of purified *C. oleophila* β-1,3-glucanase on *P. expansum*. (A) Conidial germination. (B) Mycelial growth. The *A. niger* β-1,3-glucanase was used as a positive control. Above graphs A and B are images of *P. expansum* mycelial growth inhibition. Left to right: 7.5 U/mg, 3.75 U/mg, 1.9 U/mg and a negative control. Each experiment was performed three times.

The percentage of *P. expansum* mycelial growth **(Figure 6B)** is shown after being subjected to the different β-1,3-glucanase treatments. There were significant (α=0.05) differences among the treatment doses, for both *C. oleophila* and *A. niger* β-1,3-glucanases. The larger the doses were, the greater the inhibition of *P. expansum* mycelial growth. *A. niger* had greater mycelial control than *C. oleophila*. The *C. oleophila* enzyme, at the lowest dose, produced a significant (α=0.05) difference compared to the other treatments. *C. oleophila* β-1,3-glucanase destroys *P. expansum* cell wall glucans [25,26]. *P. expansum* conidial germination and mycelial growth inhibition by the *C. oleophila* L-06 isolate β-1,3-glucanase may indicate that the production of lytic enzymes is a mode of action of the *C. oleophila* L-06 isolate. Guerrero-Prieto et al. reported that *C. oleophila* L-06, L-07 smooth and L-07 rugose exhibited had biocontrol in *P. expansum* and *B. cinerea* in postharvest apple fruit. The results reported in this work may indicate that it is preferable to use the yeast itself, instead of the enzyme alone, because *C. oleophila* may incorporate several modes of action, such as the production of lytic enzymes, competition for nutrients and space and the induction of resistance against *P. expansum* [5].

CONCLUSION

C. oleophila L-06 produces a β-1,3-glucanase with a molecular mass of 48.3 kDa, an optimal pH of 5.0, an optimal temperature of 40°C, an interval of thermal stability between 20 and 40°C, and enzymatic activity up to 60°C. β-1,3-Glucanase inhibited *P. expansum* conidial germination by 50% and mycelial growth by 70% when using a dose of 7.5 U/mg of purified enzyme. The results indicate that the production of β-1,3-glucanase by *C. oleophila* is one of the modes of action against *P. expansum*.

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