Amylase Activities of *Ascochyta Pisi* Causing Anthracnose of Peas (*Pisum sativum*).

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**ABSTRACT**

The anthracnose of pea caused by the phytopathogenic fungus *Ascochyta pisi* Lib. is one of the fungal diseases transmissible by the seeds causing necrotic spots on the different organs of pea plants (*Pisum sativum* L.), and making them improper for commercialization. Indeed, this parasite is carried out of very powerful enzymatic equipment which plays a role in its pathogenicity among which the amylase. The determination of the amylolytic activity in the solid medium (*Czapek* modified with different concentrations of starch), can confirm the presence of an enzyme complex necessary for the hydrolysis of starch present in the medium and shows that the optimum growth for the three isolates of *A. pisi* were obtained from a higher concentration of starch (*Czapek* + 2% of starch). The study of the amylolytic activity of the three isolates on medium *Czapek* + 2% of starch with different pH (4, 6, 8) and at various times from incubation, shows that the optimum for the amylolytic activity for all of our isolates occurs at pH values between 6 and 7 and away from either side of these limits the amylolytic activity declines.

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**INTRODUCTION**

Pea (*Pisum sativum*) is an annual leguminous plant grown worldwide and used in human and animal nutrition. World production in peas reached its optimum in 1990 with a production that is around 16.5 million tonnes [8]. From 2000, world production has stabilized at around 10 million tonnes. In Algeria the climate and soil conditions are very favorable for its cultivation which covers an area of 21200 ha with an annual production of 632900 quintals, representing a yield of 29.9 quintals / ha [7], producing wilaya are Mascara, Bouverdes, Biskra and Tlemcen.

From air-borne diseases, anthracnose caused by *Ascochyta pisi* is the most common and most damaging agent for pea culture in Algeria.

The interaction between a host and its pathogen bring into play various molecules which allow or not the disease development. Parasites and in particular fungi frequently act with enzymes or toxins.

Ameziane [1] noted that the progression of the fungus in the host tissue is accompanied by a chemical alteration of the main components of the wall of which pectins and cellulose, in addition to the destruction of the starch which is linked to production of enzymes by the pathogen.

The main objective of this study was to determine the amylolytic activity and assay of this activity for the three isolates of *A. pisi* by Bernfield method at different pH values and different incubation times.

**MATERIALS AND METHODS**

*Ascochyta pisi* used in this study were obtained from the fruit of pea (*Pisum sativum*), that show characteristic symptoms of the anthracnose. Samples of the isolated strains were collected during the month of March 2013 in north-west zone of Algeria represented by two departments: Mostaganem (Stidia) and Relizane (Yellel). The pathogenic isolation consists in taking the fragments of seeds and pods presenting the characteristic disease of the pea. After their disinfection with sodium hypochlorite at 2% for 5 min, they are
rinsed successively 3 times with distilled water, than they are plated on PDA (Potato Dextrose Agar), and incubated at 22°C with a 12 h alternating photoperiod. Each organ of the plant is treated separately [13]. Single spore isolation of *A. pisi* were germinated and grown on PDA medium at 20°C with a 12 h light-photoperiod.

**Study of mycelial growth and detection of amylolytic activity:**

The isolates of *Ascochyta pisi* are cultivated in medium Czapek (2 g of NaNO₃, 1 g of KH₂PO₄, 0.5 g of KCl, 0.5 g of MgSO₄, 0.01 g of FeSO₄, 16 g of agar, 1L of distill water) containing different concentration of starch (Czapek +2% of glucose (0% of starch), Czapek +1% of starch and Czapek+2% of starch). After 10 days of incubation at 22°C, the evaluation of the radial growth of the isolates is carried out by the measurement of two perpendicular diameters, the average diameter of each limps was then calculated by cutting off that from the disc of starting inoculums. Amylase activity was assessed by flooding the plates after incubation with 1% iodine in 2% potassium iodide (Lugol’s solution).

**Effect of pH and cultivation period on Amyloytic activity:**

The study of the mycelial growth on solid medium, allowed us to choose the concentration of starch for which our isolates had a good growth.

The Czapek liquid cultures supplied with 2% of starch was use for the amylase production, after autoclaving, the medium pH was adjusted from 4, 6 and 8 with using either sterile 0.1N NaOH or 0.1N HCl, medium (50 ml) in a 250 ml erlenmeyer flask was inoculated by three pieces 6 mm diameter of marginal fresh colony, and incubated at 22°C for 10 days on a rotary shaker at 100 rpm. Culture filtrate was collected on filter paper by filtration. It constitutes the crude enzymatic extract which will be analyzed for amylolytic activity. pH of each Culture filtrate was measured daily using a pH meter.

**Enzyme assay:**

Amylase activity was determined by employing a modified method of Bernfield [2]. One millilitre of fungus filtrate was added to 1 ml of a standard starch solution (containing 1% soluble starch in 0.02M Na₂HPO₄ and 0.006 M NaCl at pH 6.9) and incubated at 30°C for 1 h. The reducing sugars produced (maltose and glucose) were determined by adding 1 ml of dinitrosalicylic acid (DNSA) reagent [11], boiling for 15 min and cooling on ice to room temperature for 3 min, 9 ml of distilled water were added to this mixture. The absorbance of the resultant solution was determined at 540nm with the aid of an Spectrophotometer (OPTIZEN 2120UV). One millilitre of uninoculated medium similarly treated was used to set transmittance at zero. The amounts of sugars produced were read off from a standard curve obtained by recording absorbance of increasing concentrations of aqueous maltose solutions (1.2, 2.3, 3.5, 4.6 and 5.8 µmol/mL). The results are reported as units of amylase, one amylase unit being the amount of enzyme in 1 ml of filtrate which releases 1 µmol of reducing sugar from a 1% starch solution in one hour at 30°C at pH 6.9.

**Results:**

**Study of mycelial growth and detection of amylolytic activity:**

Mycelial Growth of *A. pisi* with different concentration of starch is presented in Table 01. Radial growth on medium czapek showed that the growth of *Ascochyta pisi* is optimal on the medium containing starch; but with a slight preference for the middle czapek+ 2% of starch, strain A2 recorded its optimum growth on Czapek medium + 2% of starch with a diameter of 6.35 cm after 10 days.

**Table 01:** Mycelial growth radia (cm) of three isolates of *A. pisi* on czapek medium with different concentration of starch after 10 days of incubation:

<table>
<thead>
<tr>
<th>Substrate Isolates</th>
<th>Cz+ 2% glucose</th>
<th>Cz+1% starch</th>
<th>Cz+2% starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>6±0.2</td>
<td>6.07±0.73</td>
<td>6.2±0.24</td>
</tr>
<tr>
<td>A2</td>
<td>5.9±0.45</td>
<td>6.25±0.25</td>
<td>6.35±0.35</td>
</tr>
<tr>
<td>A3</td>
<td>5.8±0.2</td>
<td>6±0.2</td>
<td>6.1±0.08</td>
</tr>
</tbody>
</table>

According to Figure 01, the three strains develop a clear zone formed around the fungal colony on both media (Czapek +1% of starch, Czapek + 2% of starch); This indicates that there's been a degradation of starch by the fungus, so it is able to produce Amylase.

The addition of Lugol’s solution to the cultures grown on Czapek + 1% of starch and Czapek + 2% of starch, showed the appearance of two zones; a pale yellow zone who indicating the hydrolysis of starch into maltose, and a blue-black zone indicating the presence of starch. These results are illustrated in Figure 02.
The evolution of the amylolytic activity according to the variation of the three initial pH:

In this study we tested the effect of different pH, in order to determine the optimum pH for amylase production by Ascochyta pisi.

When the initial pH 4 of the medium increases to pH 6, the amylase activity increased proportionally to this variation of pH for the three isolates, and attained at 7th day of incubation a maximum value of 1.33 U / mL and 1.66 U / mL for isolates A1 and A2 at pH 6.09 and 6.15 respectively, below these pH values the amylolytic activity decreased, whereas a maximum of activity (0.55 U / mL) was obtained by the isolate A3 after 10 days of incubation and at pH 6.03.

Figure 03 shows that the enzyme activity is proportional to the variation of pH 6. However, the maximum activity is obtained when the initial pH 6 reaches its maximum value for the three isolates. Maximum activity (1.23 and 1.95 U / mL) was recorded at 7th day at pH 6.35 and 6.30 with the isolate A1 and A2 respectively.

The tendency of initial pH 8 to neutral pH 7 is favorable for amylolytic Production. Maximum activity (1.93 U / mL) was recorded by the isolate A3 at 9th day of incubation and at pH 6.81, followed by the isolate A1 with an activity of 1.69 U / mL at pH 6.98 and at the 7th day of incubation. Above these pH values, this activity decreases, whereas the isolate A2 reaches its maximum value (1.20 U / mL) at pH 6.80 until 10th day of incubation.

So we concluded that the optimum pH for amylase production for the three isolates of A.pisi is found between pH 6-7. Enzyme activity decreased drastically at pH below 6.0 or above 7.0.
Fig. (a): Effect of pH and cultivation period on amylolytic activity by the isolate A1.
Fig. (b): Effect of pH and cultivation period on amylolytic activity by the isolate A2.

Fig. 03: Effect of pH and cultivation period on amylolytic activity by the isolate A3. Effect of pH and cultivation period on amylolytic activity.
B- Discussion:

The detection of the amylolytic activity on the solid medium (Czapek amended with different concentration of starch), shows that the optimum growth of the three isolates of A. pisi was obtained from a higher concentration of the starch, this possibility of development is associated with their enzymatic equipment and substrate to be transformed. As SHu and Blackwood [15] found for Aspergillus niger, the amount of extracellular amylase was increased greatly by the inclusion of starch in the medium. Maximum amylase was found in media containing the largest amount of starch.

In general, plant pathogens are able to produce enzymes which allow them to degrade the polysaccharides of the cell wall and reserves. The study carried out by Cochrane [5] on many of the lower fungi, allowed to observe that extracellular amylase production is widespread in microorganisms but not universal. Despite the extensive characterization of amylases secreted by industrially important fungi, very few works are devoted to the study of amylase produced by phytopathogenic fungi.

However, in our study we have tested the Czapek medium + 2% of starch with different pH (4, 6, 8); in order to determine the optimum pH for amylase production for Ascochyta pisi. The three strains produce varying quantities of amylase according to the variation of initial pH and the time of incubation. The results of the variations of the initial pH(4, 6, 8) are identical to those shown by Sani et al., [14] where the final pH of the cultures differs from the initial pH; the initial pH 3 of Aspergillus flavus and Aspergillus niger increases to 6.8 and 5.0 respectively while the initial pH 8 decreases to 7.3 for Aspergillus flavus and 6.8 for Aspergillus niger.

Further, the pH is known to affect the secretion of α-amylase and its stability [9], The optimum pH for amylase production for the three isolates of A. pisi was found between pH 6.0-7.0. Enzyme activity decreased drastically at pH below 6.0 or above 7.0; this decline was certainly due to the effect of pH on the ionization of groups of the side chains that maintain the structure of the enzyme on the one hand and are involved in the activity of the active site on the other hand, the variation of pH also affects membrane permeability so leads to a slowdown in the enzyme activity and mycelial growth [3]. Similarly, such findings were reported by Olama and Sabry [12], where the amylase activity was maximum at pH 7.0 in Aspergillus flavus. Aspergillus oryzae, A. ficuum and A. niger were found to give significant yields of α-amylase between pH 5.0–6.0 [4, 6, 10]

Conclusion:

The search for enzyme activity in the culture filtrate of the three isolates of A. pisi allowed us to demonstrate the presence of a group of protein metabolite responsible for the degradation of cellular host reserves: amylases. The quantitative determination of this activity, confirmed to us the production of these metabolites recognized responsible in the pathogenesis of other microorganisms. However this production is significantly influenced by a number of factors related as well to the pathogen (age of cultures) as environmental conditions (changes of the initial pH of the medium). In the study of the search for amylolytic activity, initial pH (4, 6, 8) of Czapek medium supplemented at a concentration of 2% starch tended to pH plus or minus neutral, the optimum of the amylolytic activity for all of our isolates occurs at pH between 6 and 7.

REFERENCES