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Using Clover Husk for Avicelase Production from *Aspergillus flavus* Via Solid-State Fermetnation

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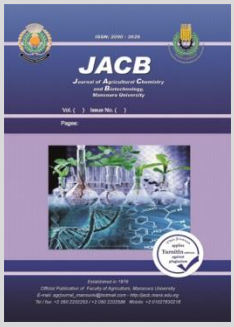

ABSTRACT

Avicelase is considered as an important component in the cellulases system, which has great importance in the saccharification of cellulosic materials for use in biotechnological industries. Recently, there is a trend to produce cellulolytic enzymes from microorganisms especially fungi through solid-state fermentation. In this study, a high avicelase producing local fungus was isolated and identified as *Aspergillus flavus* isolate L-2007/2012, then used for high avicelase production. Among some cellulosic wastes, clover husk was chosen as the best basal fermentation substrate. Results of avicelase production showed that the highest production of avicelase 799.23 U/g was obtained after 7 days of incubation by adjusting moisture ratio at 1.5:1.0 (v/w) and supplementing fermentation medium with 1% glucose as an additional carbon source and 1% ammonium sulfate as a nitrogen source. Furthermore, the highest production of avicelase occurs with incubating at 30ºC with adjusting initial pH at 6.0 and 2 ml of inoculum (10^6 spores/ml). By studying some factors affecting the enzyme activity, it revealed that the produced avicelase gave the highest activity at 50ºC and pH 6.0, furthermore the enzyme has good pH stability in acidic conditions and high thermal stability at 50ºC.

Keywords: *Aspergillus flavus*- avicelase - solid-state fermentation

INTRODUCTION

Cellulosic materials considered one of the most common plant wastes which are used in a wide range as raw materials for producing a lot of substances and involved in many industrial applications such as; food processing, animal feed production, laundry, pulp & paper industry, textile, wine and bioenergy industry (Skomarovsky *et al*., 2005 and Bharti *et al*., 2018). The most important step in these applications is the pretreatment of cellulolytic materials, which could be chemical, biological or biochemical. The enzymatic method is the most efficient for cellulose hydrolysis, which breakdown cellulosic materials to sugars by an enzymatic group called cellulases (Idris *et al*., 2017). On the other hand, cellulases including avicelase play an important role in the saccharification of cellulose to release glucose, which is more suitable for fermentation processes (Silva *et al*., 2019). Cellulases complex system includes the major types of enzymes; endo-1,4-β-glucanase (carboxymethyl-cellulase, or Cx-cellulase), cellobiohydrolase (exoglucanases, CBH, Avicelase, C1-cellulase), and β-glucosidase (cellobiase) (Ray, 2015), which act synergistically for efficient cellulose hydrolysis (Idris *et al*., 2017).

Avicelases (1,4-β-D-glucancellobio-hydrolase, EC 3.2.1.91) also called exoglucanases are classified into type I and type II, avicelase type I work from the reducing end of the cellulose chain, whereas avicelase type II work from the non-reducing end, cleaving two to four units from the end of the chain producing the disaccharide cellobiose. Avicelases also act on the terminal of the oligosaccharide chain releasing glucose or cellobiose (Ray *et al*., 2015).

Considering the huge role of cellulases including avicelase in various biotechnological applications, microbial production of avicelase took on its importance due to its high production, as microorganisms are excellent enzymes producers by fermentation processes (Marques *et al*., 2018). Avicelases are secreted by various types of microorganisms such as bacteria and fungi. Fungi are known for their high capacity to produce extracellular cellulases (Marques *et al*., 2018). Many fungi secrete avicelases when grown on cellulosic materials or microcrystalline cellulose via fermentation processes. *Rhizopus oryzae*, *Neurospora*, *pleurotus*, *Trichoderma* and *Aspergillus* are well known for high avicelases fungi producers (Yoo & Chang, 1999; Seki *et al*., 2011; Deswal, 2012; Lim *et al*., 2013; Mahmood *et al*., 2013)

Both submerged fermentation (SmF) and solid-state fermentation (SSF) processes could be used for fungal production of avicelase. *Aspergillus flavus* was found to produce avicelase in both submerged and solid-state fermentations (Hussain *et al*., 1999). Solid-state fermentation widely used as easy to operate, less capital intensive and the ability to use cheap cellulolytic material as raw material and cheap carbon source for avicelase production (Pandey, 2003).

The aim of this work is to use a plant cellulosic waste as a fermentation substrate via a solid-state fermentation process to optimize avicelase production from a high avicelase producing fungus isolate by providing optimum conditions for avicelase production.

MATERIALS AND METHODS

Isolation of fungi: To obtain the avicelase producing fungal isolates, soil samples were collected from the rhizosphere of some field crops from Mansoura, Dakahlia governorate, for the isolation of some avicelase producing fungi. Isolation process was applied on potato dextrose agar with addition of

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streptomycin to prevent bacterial growth. Isolated fungi were tested for avicelase production via SSF process.

Preparation of cellulosic wastes: Five plant cellulosic wastes including sawdust, rice husk, wheat husk, clover husk and Rice brane were used in a SSF process to determine the best basal fermentation medium for avicelase production. Cellulosic wastes were collected from local markets, then cut into small pieces and ground. After that, the cellulosic wastes were dried in the oven at 70ºC then stored in polythene bags at room temperature for further use.

Identification of the selected avicelase producing fungus: The morphological and molecular identification were carried out on a 7 days old culture of the isolated fungus grown on glucose peptone agar. The morphological examination includes; colonial and microscopic appearance to identify the species of the fungus according to Campell and Johnson (2013). To ensure the fungal strain, molecular identification was carried out by Sigma Scientific Services Co., Cairo, Egypt, using the partial sequence of the 5.8S rRNA.

Inoculum preparation: To each 5 days PDA fungus slant, 5 ml of distilled water with 0.1% tween 80 were added and scratched gently, then added to 0.8% NaCl solution to make a fungus spore suspension. The spore suspension was adjusted to make spore density up to 10^6 spore/ml.

Testing the ability of isolated fungi for Avicelase production via SSF: In the application of SSF for avicelases production, 5 grams of rice husk were added to 250 Erlenmeyer flasks and moistured with 5 ml of 1% ammonium nitrate solution, medium pH were adjusted at 5.0 then autoclaved at 121ºC for 20 min. and cooled. Flasks were inoculated with 1 ml of spore suspension having 10^6 spores/ml and incubated at 30ºC for 7 days.

Extraction of crude enzyme: Fifty ml of distilled water were added to each flask after SSF process ended, and mixed well, then shaked at 150 rpm for 30 min. to ensure homogeneity, then filtered through double-layer gauze. The filtrate was then centrifuged at 6000 rpm for 20 min. at 25ºC, then the clear supernatant was collected to use as a crude enzyme.

Enzyme assay: Avicelase activity was assayed by using DNS method according to Miller (1959). The enzymatic reaction applied by adding 0.3 ml of crude enzyme to 0.7 ml of 1% avicel solution in 0.1 mM acetate buffer (pH 5.5) and incubated at 40ºC for 30 min, the reaction mixture was shaked to prevent the precipitation of microcrystalline cellulose (avicel). The reaction was stopped by adding 1 ml of 3.5-Dinitrosalycilic acid (DNS) reagent, then the mixture was boiled for 5 min. then cooled for 5 min. in ice water. Reduced sugars were measured colorimetrically at 570 nm using glucose as standard. One unit of avicelase was defined as the amount of crude enzyme which released 1 µg of glucose per minute.

Factors affecting avicelase production:

Incubation period and cellulosic material wastes: SSF process were applied using five cellulosic wastes including sawdust, rice husk, wheat husk, clover husk and Rice brane. Fermentation media contain 5g of the used waste, moisture with 5 ml of ammonium nitrate solution (1%) and pH was adjusted at 5.0. SSF flasks were incubated at 30ºC for 5, 7 and 9 days to determine the best incubation period and the best cellulosic waste for avicelase production.

Initial moisture ratio: SSF medium were adjusted by different level of moisture ranging from 0.5-4.0 : 1.0 (ammoinium nitrate solution : substrate, v/w), at interval 0.5 to determine the best moisture ratio for avicelase production. Fermentation medium was adjusted at pH 6.0 and flasks were incubated at 30ºC.

Amendment with carbon source: SSF medium were supplemented with 1% (w/w) of different carbon sources; glucose, fructose, lactose, maltose, cellulose, sucrose, galactose and starch to study the effect of additional carbon source on avicelase production with control flask, which has no additional carbon source. All flasks were adjusted at the optimum moisture level, pH was adjusted at 6.0 and incubated at 30ºC for at 30ºC.

Amendment with nitrogen source: To determine the best nitrogen source for avicelase production, ammonium nitrate was replaced with 1% (w/w) of different organic and inorganic nitrogen sources in the SSF medium. Used nitrogen sources including; sodium nitrate, calcium nitrate, ammonium sulfate, ammonium chloride, yeast extract, peptone and beef extract, while control was amended with ammonium nitrate. All flasks were adjusted at the optimum moisture ratio, the best carbon source, pH 6.0 and incubated at 30ºC.

Incubation temperature: Flasks with best fermentation medium adjusted with the best moisture ratio, amended with the best carbon and nitrogen sources and adjusted at pH 5 were incubated at different temperatures; 20, 25, 30, 35, 40 and 45ºC to determine the optimum temperature for avicelase production.

Initial pH: Initial pH of fermentation medium was adjusted in 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 to determine the best pH value for avicelase production. SSF were adjusted at the optimum moisture ratio and amended with the best carbon as well nitrogen sources and incubated at the optimum temperature.

Inoculum size: Fermentation medium was inoculated with different sizes of inoculum to determine the best inoculum size for avicelase production. Inoculum size ranging from 1-5 ml (10^6 spores / ml).

Factors affecting enzyme activity

Optimum pH of enzyme activity: The reaction mixture was adjusted at pH; 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, where the enzymatic reaction was performed under these pH conditions. The relative activity of the enzyme was determined comparing with the highest enzyme activity to determine the optimum pH for the enzyme activity. The enzymatic reaction was carried out at 40ºC for 30 min.

Optimum temperature of enzyme activity: the enzymatic reaction was carried out at various temperatures at 30, 40, 50, 60, 70 and 80ºC. Relative activity of the enzyme was determined comparing with the highest enzyme activity to determine the optimum temperature for the enzyme activity, the reaction mixture was adjusted at the optimum pH and carried out for 30 min.

pH stability of the enzyme: the crude enzyme was stored at different conditions of pH; 4.0, 5.0, 6.0 and 7.0, then the enzyme activity was assayed every 1 hour from zero time to 7 hours. The relative activity of the enzyme was assayed to explore the enzyme stability on different pH conditions. The enzymatic reaction was assayed on optimum pH and temperature for enzyme activation.

Thermal stability of the enzyme: The crude enzyme was incubated at 50, 60 and 70ºC for different periods. The relative activity of the enzyme was assayed to explore the thermal stability of the crude enzyme. The enzymatic reaction was assayed on optimum pH and temperature for enzyme activation.

RESULTS AND DISCUSSION

Isolation and efficiency test of avicelase producing fungi under SSF process

Seven different fungal isolates were isolated from collected soil samples on PDA plates amended with avicel. To choose the most avicelase producing fungal isolate, the isolated fungi were grown on rice husk medium as an avicel source through a solid-state fermentation process for 7 days to determine their efficiency at avicelase production. Data in Fig. (1) Show the amount of avicelase produced by isolated fungi. In this connection the isolate no. 3 is the highest avicelase producer among the seven isolates, as it produced 252.28 U/g of the enzyme, so this isolate was chosen to be identified and to complete the subsequent experiments for the optimization of avicelase production under solid-state fermentation.

SSF has an advantage over SmF in fungi cellulases production, as SSF is a simple technology, low cost and high enzyme production process (Holker *et al*., 2004). As their natural adaptation for growing on solid surfaces, most of the filamentous fungi was reported to perform better in SSF production processes (Johansson, 1966). Dutt and Kumar (2014) reported that cellulases production increases in range of 40.08-45.86% by SSF compared with SmF processes by *A. flavus* and *A. niger*.

Identification of *Aspergillus flavus* isolate L-2007/2012

The colonial examination of the isolate No. 3 grown on glucose peptone agar showed that the isolated fungus has yellow to green flat colonies with creamy reverse and granular texture, Fig. (2a) show the fungus growth on peptone agar medium after 7 days. The microscopic examination showed a numerous large visculate conidiophores with roughened stalks, vesicles globose with radiate or columnar spore production. In some heads, phialides rises directly from the entire surface of the vesicle and in others it produced on metulae. Conidia are smooth and round with diameter ranging between 3.5-4.8 mm as shown in Fig. (2b). From these morphological characteristics and according to Campell and Johnson (2013), the fungal isolate could be identified as *Aspergillus flavus*. To confirm the identification of the fungus strain, the partial sequence of the 5.8S rRNA and phylogenetic tree was carried out by Sigma Scientific Services Co., Cairo, Egypt, and has aclose identification to the fungal strain, and the fungal strain identified as *Aspergillus flavus* isolate L-2007/2012. Figure (2c) show the phylogenetic tree of *Aspergillus flavus* isolate L-2007/2012.

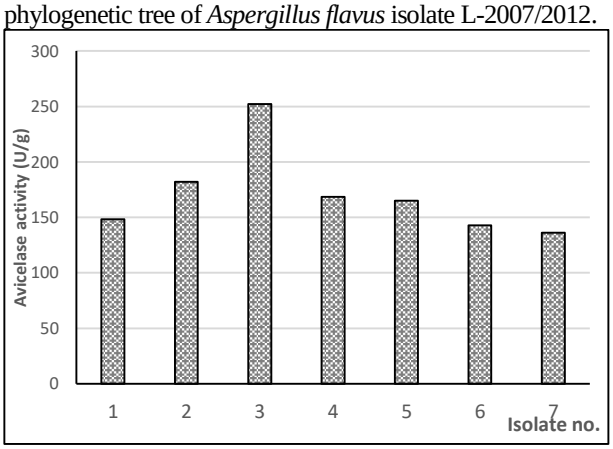

Fig. 1. Avicelase production by isolated fungi after 7 days of incubation under SSF process on rice husk medium. Flasks were adjusted at moisture ratio 01: 01, pH 5 and incubated at 30ºC. The enzymatic reaction was carried out at 40ºC and pH 5.5 for 30 min.

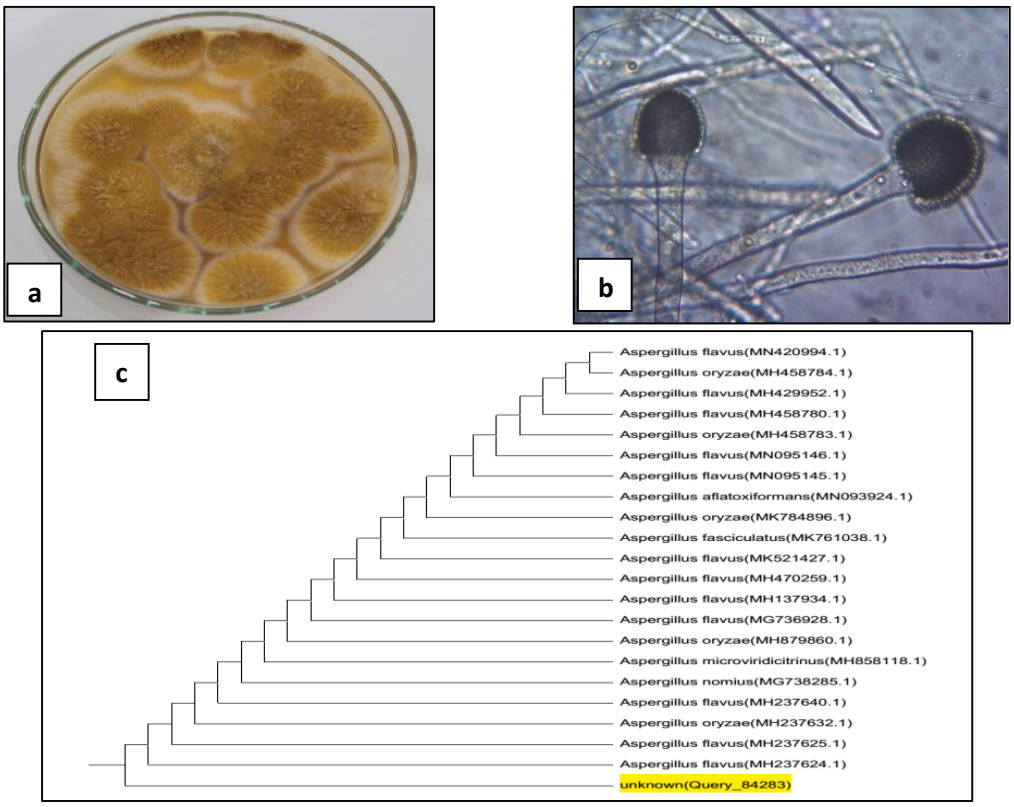

Fig. 2. a. Photograph of *A. flavus* colony on peptone agar medium after 7 days; b. Microscopic photograph showing the roughened conidiophore and rounded conidia of *A. flavus* with 40X magnification; c. Phylogenetic tree of *Aspergillus flavus* isolate L-2007/2012

The genus *Aspergillus* is distinguished by its ability to produce high amounts of extracellular cellulases including avicelases (Abo-state *et al*., 2010). *A. niger*, *A. fumigatus* and *A. flavus* are among the most productive fungal species for extracellular cellulases by solid-state fermentation on plant cellulosic wastes (Hussain *et al.,* 1999; Kumar, 2013; Mahmood *et al.*, 2013). Youssef *et al.,* (2014) reported a higher cellulases production by *A. flavus* compared with *A. niger*, *A. fumigatus* and *Penicillium* sp.

Optimization of avicelase production by *A. flavus* L-2007/2012

Effect of cellulosic wastes and incubation time

One of the main factors affecting cellulases production in SSF process is the composition of the fermentation medium which influences the supply of nutrients and metabolism of cells (Kumar, 2013). Plant cellulosic materials have been used on a large scale for the microbial production of cellulases under SSF, due to their availability and low-cost (Bharti *et al*., 2018). Data in Fig. (3) Show the effect of cellulosic material wastes and incubation time on avicelase production by *A. flavus* isolate L-2007/2012. Data show that the production of avicelase varies depending on the type of used cellulosic wastes. Gao *et al*., 2008 and Gautam *et al.*, 2015 reported that cellulases production varies from substrate to another depending on the chemical composition, substrate accessibility, substrate degradability and the presence of nutrients. Data reveal that the avicelase production raised from the 5th day to the 7th day then decreased on the 9th day of incubation with all used cellulosic wastes, which means that the highest avicelase production achieved on the 7th day of incubation. On the other hand, data show that clover husk gives the highest avicelase production, which reached 274.2, 327.96 and 297.03 U/g after 5, 7 and 9 days of incubation respectively. Clover husk medium was used in subsequent experiments as a basal substrate for avicelase production by *A. flavus* L-2007/2012 via SSF process.

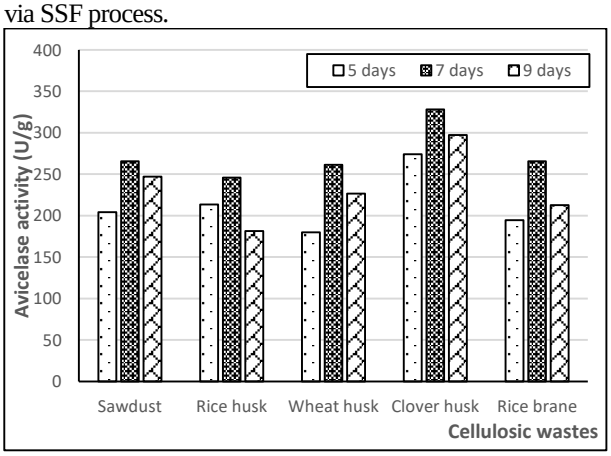

Fig. 3. Effect of cellulosic material wastes and incubation time on avicelase production by *A. flavus* L-2007/2012 after 5, 7 and 9 days of incubation under SSF process. Flasks were adjusted at moisture ratio 1.0: 1.0, pH 5 and incubated at 30ºC. The enzymatic reaction was carried out at 40ºC and pH 5.5 for 30 min.

Effect of moisture ratio

There is always an urgent need to adjust the moisture in SSF processes by using plant harvesting wastes because of its low moisture content. Data in Fig. (4) show that avicelase production increased with raising moisture content until the ratio of 1.5:1, which recorded the highest avicelase production (384.91 U/g) after 7 days of incubation. Avicelase production slightly decreased at the ratio of 2:1, then decreased greatly with increasing moisture content. Subsequent experiments were adjusted at the best moisture ratio.

Moisture content plays a vital role in microbial growth and products biosynthesis in SSF processes (Dutt and Kumar, 2014). Moisture enhances the substrate utilization by the microorganisms, beside the mass transfer in the solid phase particles under SSF growth depends on the appropriate moisture and the substrate. Lower moisture than the optimal reduces the swelling of the substrate, resulting in low nutrients transfer (Bharti *et al*., 2018), while higher moisture level than the optimal could inhibit the growth of fungi and encourage bacterial contamination due to the decrease of porosity and oxygen diffusion in the substrate (Raimbault and Alazard, 1980). The optimum moisture content for SS-fermentation differs depending on the type of the fermentation substrate (Chugh *et al*., 2016). Dutt and Kumar, (2014) reported that 3:1 was the best moisture ration for cellulases production by *A. flavus* and *A. niger* on rice straw.

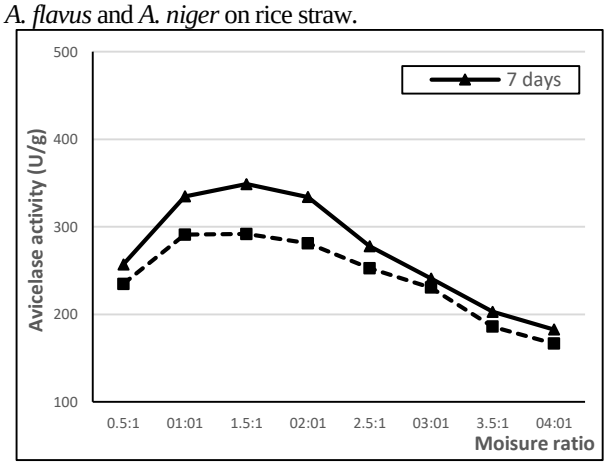

Fig. 4. Effect of moisture ratio on avicelase production by *A. flavus* L-2007/2012 under SSF process. Flasks were adjusted at pH 5 and incubated at 30ºC. The enzymatic reaction was carried out at 40ºC and pH 5.5 for 30 min.

Amendment with carbon sources

Supplementing the fermentation medium with carbon sources may affect the fungal growth as well as avicelase production (Fang *et al*., 2008). From data in Fig. (5), it appears that there are big differences between the effects of used sugars additives to SSF medium. Data reveal that only glucose caused an increase in avicelase production, while other sugars reduced the production by *A. flavus* L-2007/2012. The highest avicelase production was recorded on the 7th days of incubation with the addition of glucose, which recorded 411.65 U/g. In contrast with our results Fang *et al*., (2008) reported that lactose is more effective in enhancing cellulases production from *Acremonium cellulolyticum*, while Mahmood *et al*., (2013) found that fructose is better than glucose in avicelase production by *A. fumigatus*.

Effect of nitrogen source

Data in Fig. (6) show the effect of different nitrogen sources on avicelase production by *Aspergillus flavus* L-2007/2012 under SSF process. Data reveal that some

nitrogen sources such as ammonium sulfate and peptone increased avicelase production, while other nitrogen sources decreased avicelase production, compared with control (ammonium nitrate). Using ammonium sulfate as a nitrogen source achieved the highest avicelase production by *Aspergillus flavus* L-2007/2012, which reached 415.75 and 535.41 U/g at 5th and 7th days of incubation respectively.

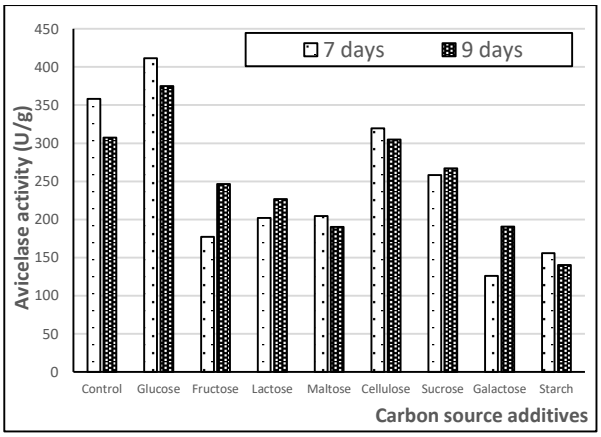

Fig. 5. Effect of carbon sources additives on avicelase production by *A. flavus* L-2007/2012 under SSF process. Flasks were adjusted at moisture ratio 1.5: 1.0, pH 5 and incubated at 30ºC. The enzymatic reaction was carried out at 40ºC and pH 5.5 for 30 min.

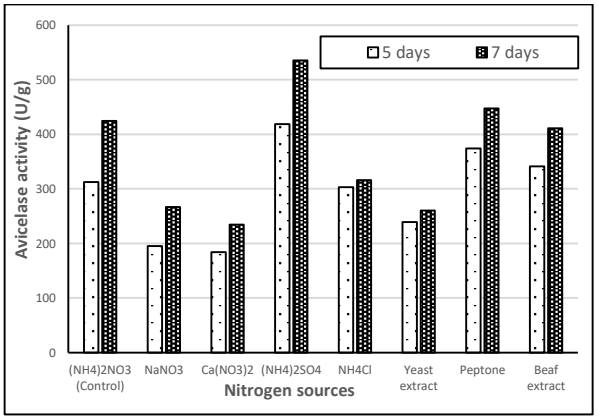

Fig. 6. Effect of nitrogen sources on avicelase production by *A. flavus* L-2007/2012 under SSF process. Flasks were amended with 1% glucose, adjusted at moisture ratio 1.5: 1.0, pH 5 and incubated at 30ºC. The enzymatic reaction was carried out at 40ºC and pH 5.5 for 30 min.

Nitrogen is considered the main constituent element for protoplasm, which is involved in protein formation (Kachlishvili, *et al.*, 2006). Cellulases biosynthesis is affected by the nitrogen sources; organic or inorganic. Organic nitrogen sources increase cellulases production, where peptone is considered one of the highest nitrogen source for cellulases production (Bansal *et al*., 2014). The effect of nitrogen supplement on the production of lignocellulolytic enzymes via SSF varies depending on the nature of the basal fermentation substrate (Kachlishvili *et al*., 2006). In line with our results, Kachlishvili *et al*., (2006) reported that among several sources of inorganic nitrogen, $(NH_4)_2SO_4$ was the highest lignocellulytic enzymes inducer with different fungal species.

Effect of incubation temperature

Incubation temperature affects the growth and metabolic activities of growing fungi. Avicelase production by *A. flavus* L-2007/2012 was affected by incubation temperature as shown in Fig. (7). Results show that avicelase production increased with raising incubation temperature from 20ºC through 25ºC to reach maximum avicelase production at 30ºC. On the other hand, raising temperature above 30ºC through 35ºC and 40ºC resulted in a great decrease in avicelase production. From data, the optimum temperature for avicelase production by *A. flavus* is 30ºC, in which enzyme production reached 526.6 and 541.44 U/g at the 5th and 7th day of incubation, respectively.

The high avicelase production by *A. flavus* L-2007/2012 at the range of 25-35ºC prove the mesophilic nature of the fungus. On the other hand, the decreases in avicelase production at 40 and 45ºC maybe due the the effect of high temperature in inhibiting or delaying spore germination of the fungus (Raimbault and Alazard, 1980), beside at high temperature the thermal denaturation of metabolic pathways needs high maintenance energy for cellular growth, resulting in low production of the metabolites including enzymes (Dutt and Kumar, 2014).

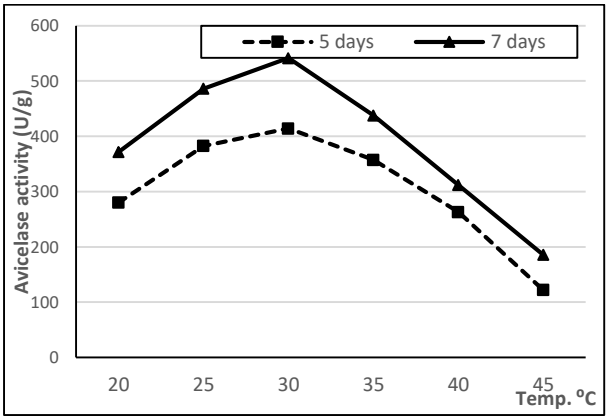

Fig. 7. Effect of incubation temperature on avicelase production by *A. flavus* L-2007/2012 under SSF process. Flasks were amended with 1% glucose, 1% $(NH_4)_2SO_4$, moisture 1.5:1.0 and pH 5. The enzymatic reaction was carried out at 40ºC and pH 5.5 for 30 min.

Effect of initial pH

Avicelase production by *A. flavus* L-2007/2012 appeared to be affected by adjusting the initial pH in the fermentation medium. Data in Fig. (8) show that avicelase production by *A. flavus* L-2007/2012 in acidic conditions is better than alkaline conditions. Data reveal that pH 6 is the most suitable for enzyme production by *A. flavus* L-2007/2012, which recorded the highest amounts of enzyme (526.56 and 572.56 U/g) after 5 and 7 days of incubation. On the other hand, avicelase production dropped hard with raising pH value over 7. Yoon *et al*., (2014) mentioned that most fungi prefer acidic pH for cellulases production, where initial pH around 5 is preferable.

Effect of inoculum size

Data in Fig. (9) reveal that adding 2 ml of the fungus spore suspension (10^6 spores / ml) gives the highest avicelase production by *A. flavus* L-2007/2012, which reached 799.23 U/g after 7 days of incubation. This amount of enzyme increased by 35.9% compared with control (1 ml), which

recorded 578.82 U/g at the 7th of incubation. Also data show that raising inoculum size over 2 ml resulted in a decrease in avicelase production at the 7th day of incubation.

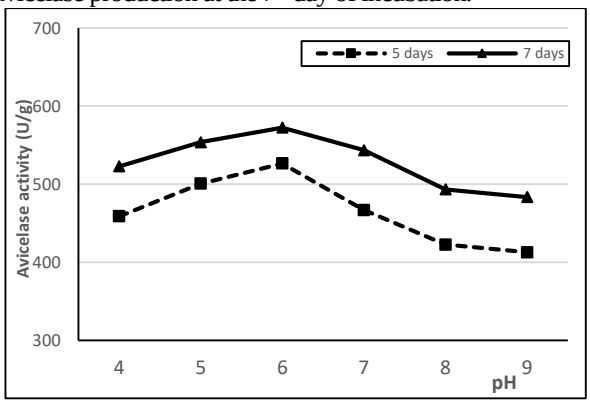

Fig. 8. Effect of pH on avicelase production by *A. flavus* L-2007/2012 under SSF process. Flasks were amended with 1% glucose, 1% $(NH_4)_2SO_4$, adjusted at moisture ratio 1.5: 1.0 and incubated at 30ºC. The enzymatic reaction was carried out at 40ºC and pH 5.5 for 30 min.

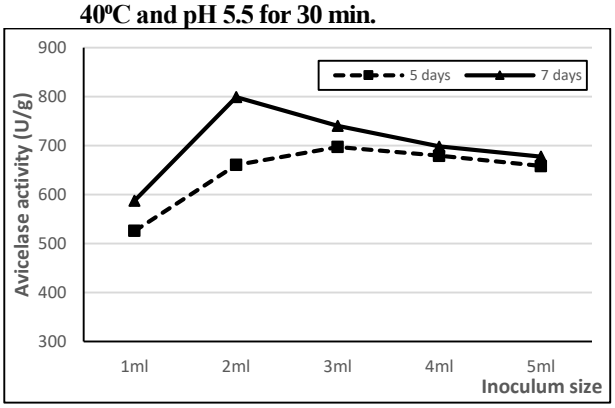

Fig. 9. Effect of inoculum size on avicelase production by *A. flavus* L-2002/2017 under SSF process. Flasks were amended with 1% glucose, 1% $(NH_4)_2SO_4$, adjusted at moisture ratio 1.5: 1.0, pH 6 and incubated at 30ºC. The enzymatic reaction was carried out at 40ºC and pH 5.5 for 30 min.

Factors affecting avicelase activity

Optimum pH of enzyme activity

Data in Fig. (10) show the avicelase activity under various pH conditions. Data show that enzyme activity raised with raising pH value over 4 to reach maximum activity at pH 6, then the activity of the enzyme decreased with pH higher than 6. Also, data reveal that avicelase produced from *A. flavus* L-2002/2017 maintains high activity in the range of pH from 5 to 6.5, while the activity decreased greatly at pH 7 and in the extremely acidic conditions at pH 4. These data reveal that the enzyme could be used in acidic conditions with optimum pH at 6, while it has a poor activity at neutral and alkaline conditions. The decreases in enzyme activity in some pH values possibly due to the instability of the enzyme protein due to the changes in ionic strength of the reaction mixture (Mahmood *et al*., 2013).

Optimum temperature of enzyme activity

The effect of incubation temperature on the avicelase enzyme activity is shown in Fig. (11). Data show that the optimum incubation temperature for avicelase activity is 50ºC. Also, data show that the activity of the enzyme reduced to 95% at 40ºC, 84.5% at 60ºC and 82% at 30ºC compared with enzyme activity at optimum temperature, while a severe decrease occurred at 70 and 80ºC. Data reveal that the produced enzyme can maintains its activity at high levels in reactions at temperatures between 30 and 60ºC. The severe decline in enzyme activity over 70ºC due to the denaturation of enzyme protein at high temperatures (Mahmood *et al*., 2013).

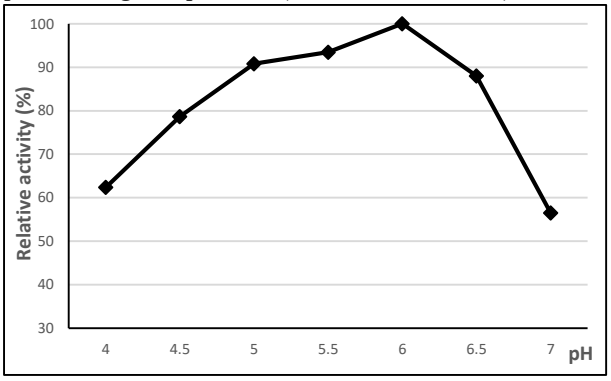

Fig. 10. Effect of the pH of reaction mixture on the avicelase activity. The enzymatic reaction was carried out at 40ºC for 30 min.

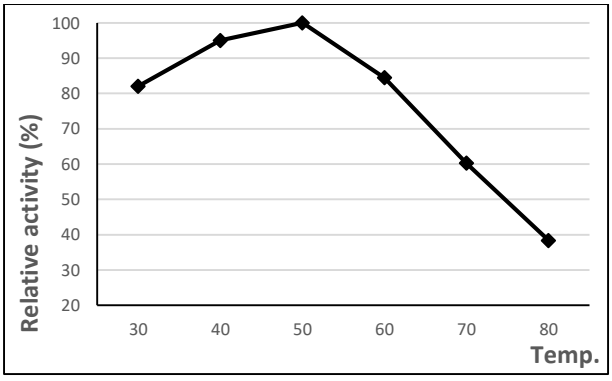

Fig. 11. Effect of incubation temperature on the avicelase activity. The enzymatic reaction was carried out at pH 6 for 30 min.

Avicelase stability at different pH values

Data in Fig. (12) show the stability of avicelase produced by *A. flavus* L-2002/2017 at different pH values. Data show that the enzyme is more stable in acidic conditions, the enzyme has high stability at pH 4 and 5, while the stability decreased with raising at pH 6 and 7. The enzyme kept approximately 70% of its activity at pH 4 and 5 after 4 hours, while at same period it kept approximately 45 and 36% of its activity at pH 6 and 7 respectively.

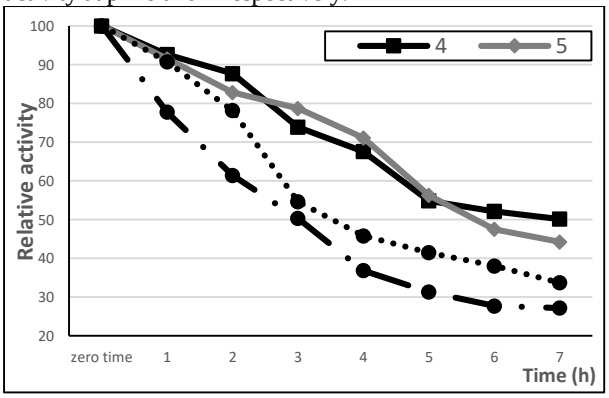

Fig. 12. Avicelase stability at different pH values. The enzymatic reaction was carried out at pH 6 and 50ºC for 30 min.

Thermal stability of avicelase

Data in Fig. 13 (a, b and c) show the temperature stability of avicelase activity at 50, 60 and 70ºC. Data show that the activity of the enzyme decreased with raising storage time at different temperatures, and with raising the temperature of storage. After one hour of storage, the enzyme lost approximately 3.5% at 50ºC, and 52.4% at 60ºC of its activity. Storage of the enzyme at 50ºC can keep 55% of activity for 5 hours. Also, data show that enzyme activity quickly collapsed with storage at 70ºC, the enzyme lost approximately 50% of its activity after 25 min. and 91% after 40 min. from these results, the avicelase produced from *A. flavus* L-2007/2012 shows a high temperature stability at 50ºC, moderate stability at 60ºC and poor stability at 70ºC.

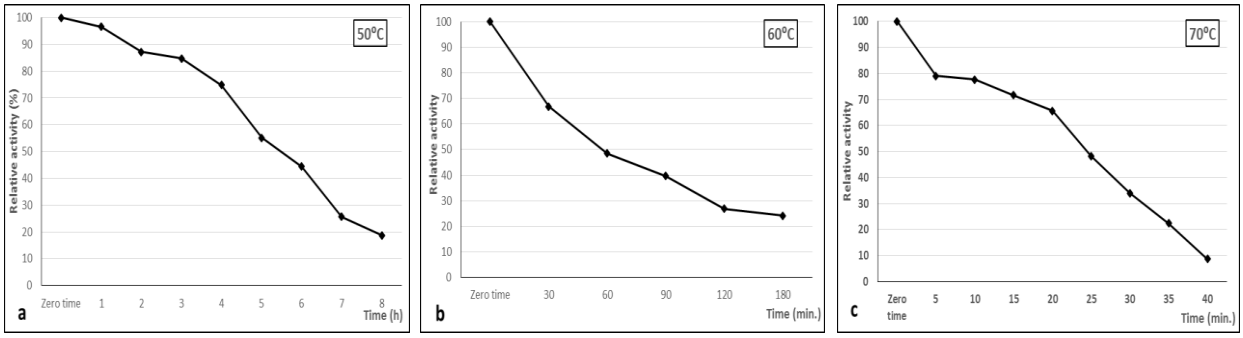

Fig. 13 a, b and c. Thermal stability of avicelase at 50, 60 and 70ºC. The enzymatic reaction was carried out at pH 6 and 50ºC for 30 min.

In conclusion, we recommend using clover husk as a raw material through SSF for producing a high active and good pH and thermal stable avicelase from *A. flavus*. To maximize the production, we recommend adjusting moisture ratio at 1.5:1 with supplementing clover husk with glucose and ammoinium sulphate and maintaining fermentation conditions at 30ºC and pH 6.

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استخدام تبن البرسيم لإنتاج إنزيم الأفيسيليز من الأسبرجيلس فلافس بطريقة التخمر الصلب

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يعتبر إنزيم الأفيسيليز أحد المكونات الهامة فى نظام إنزيمات تحليل السليلوز، حيث له أهمية كبيرة فى عملية تسكير المركبات السليلوزية للإستخدامات فى الصناعات البيوتكنولوجية. وهناك اتجاه نام مؤخرا لإنتاج الإنزيمات المحللة للمواد السليلوزية من خلال عمليات تخمر الحالة الصلبة بواسطة الكائنات الحية الدقيقة خاصة الفطريات. فى هذه الدراسة تم عزل سلالة فطرية عالية الإنتاج لإنزيم الأفيسيليز، حيث تم تعريفها على أنها أسبيرجيلس فلافس، وبعد اختبار عدة مخلفات نباتية تم اختيار تبن البرسيم كأفضل مادة يمكن استخدامها كبيئة لنمو السلالة الفطرية عليها لإنتاج الأفيسيليز. وقد أظهرت نتائج الدراسة أن أعلى إنتاج للإنزيم كان 799.23 وحدة/جرام تم تحقيقه بعد 7 أيام من التحضين على بيئة تبن البرسيم المدعمة بالجلوكوز كمصدر إضافى للكربون وكبريتات الأمونيوم كمصدر نيتروجين بنسبة 1% وظبط الرطوبة بنسبة 1.5 : 1 (حجم : وزن) والأس الهيدروجينى للبيئة عند 6 والتحضين على 30º م وكان حجم اللقاح المستخدم 2مل (يحتوى المليمتر الواحد على 10 [6] جرثومة فطرية). وبدراسة بعض العوامل المؤثرة على كفاءة إنزيم الأفيسيليز المنتج وجد أن أعلى نشاط للإنزيم كان عند درجة حرارة 50º م وأس هيدروجينى 6، بالإضافة إلى أن الإنزيم كان جيد الثبات فى الظروف الحامضية وذو ثبات عالى عند درجة حرارة 50º م.