

## Comparison of Osmotic Stress on Growth and Pectinase Production by *Aspergillus flavus* in Liquid and Solid-State Cultures



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**Abstract :** A comparative study on the effect of osmotic stress on the growth and pectinase production by *Aspergillus flavus* was undertaken. *A. flavus* when grown on different naturally available pectic substrates as the sole carbon source in liquid and solid-state cultures (SSC). Fungal growth on Dead Tree Leaves (DTL) and Rice Straw (RS) as substrates was uninhibited in presence of potassium chloride (KCl). The presence of sodium chloride (NaCl) however was growth inhibitory to *A. flavus* cultures. The protein content of the cultures from RS spent cultures was highest in presence of KCl rather than in media containing NaCl. While the protein obtained from DTL substrate was not. Total Pectin Lyase/Pectate Lyase (TPNL/PL) activity was maximum, when *A. flavus* was grown in SSC with DTL as substrate and KCl as stressor. The fungus was able to resist up to 2.5 M KCl but, in culture media with DTL the salt resistance was poor in liquid culture. The RS substrate also showed low tolerance to salt stress in both modes of culture. The production of Exo-polygalacturonase (Exo-PGL) from RS by *A. flavus* was unaffected by salt up to 2 M KCl in both cultures. On the other hand, Exo-PGL production from DTL in SSC was unaffected to salt stress of 2 M NaCl. For both substrates SSC mode of *A. flavus* was better than liquid culture for enzyme production; however the study could not establish the best substrate.

**Key words :** Osmotic stress, *Aspergillus flavus*, Pectinases production, Solid-state culture.

### Introduction

Microorganisms differ in their tolerance to osmotic stress. In general, yeasts and fungi are more tolerant than bacteria. Among the various yeast strains, *Debaryomyces hansenii* and *Saccharomyces rouxii* have been reported to be halotolerant (Norkrans, 1966; Onishi, 1963). Very few strains such as *Candida boidinii* (Nakagawa, *et al.*, 2000), *Paenibacillus* sp. and *Bacillus* sp. (Soriano, *et al.*, 2005) are known to produce both pectate lyase (PL) and polygalacturonase (PGL) (elaborate full form) in appreciable amounts. (Reframe sentence) However, the application of these enzymes is restricted because of their instability in harsh

environments such as extremes of temperature, pH and ionic strength. Therefore, much attention has been given to the microorganisms that are able to thrive in extreme environments (Gomes and Steiner, 2004). It is, therefore, obvious that the strains capable of growing and producing enzyme at high salt conditions will have high biotechnological significance. The price of commercially available enzymes which are produced mostly by submerged fermentation is usually too high for agrobiotechnological applications (Philippidis, 1994; Ruth *et al.*, 1999). An alternative technique of enzyme production is solid state cultures (SSC) (Fonseca *et al.*, 1991; Lonsane and Ohildyal,

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1993). Solid state cultures have more advantages than liquid cultures: that is higher product yield; better product quality; cheaper product recovery and cheaper technology (Doelle *et al.*, 1992). Solid state cultures (SSC) are becoming a viable alternative technique for large-scale industrial processes for enzyme production (Cen and Xin, 1999). Filamentous fungi are the most important group of microorganisms used in SSC processes owing to their physiological, enzymological and biochemical properties, the hyphal mode of fungal growth and their good tolerance to low water activity ( $A_w$ ) and high osmotic pressure conditions. All these characteristics make fungi efficient and competitive for bioconversion of solid substrates (Gervais *et al.*, 1988; Oriol *et al.*, 1988a, 1988b). Also the hydrolytic enzymes which are excreted in this case are more concentrated than with liquid substrate culture (LSC) (Moo-Young *et al.*, 1983). Pectic substances are naturally occurring heterogeneous macromolecular polyuronides widely distributed in plant tissues. Based on the degree of esterification, pectic substances are classified into three main types: protopectin, highly esterified parent pectic substance, which is insoluble in water; pectin where the degree of polymerization varies between 70-90% and polygalacturonic acid (unesterified) (Whitaker, 1991). The degradation of pectic substances involves the combined action of different pectinases, viz., esterases and depolymerases (hydrolases and lyases). Pectic transeliminases or pectic lyases are one among the pectinases, which degrade pectic substances by 3-elimination mechanism yielding 4:5 unsaturated oligogalacturonates. Pectin Lyase acting on pectin and polygalacturonate lyase or pectate lyase acting on polygalacturonic acid is two important transeliminases acting on pectic substances. Fungal strains are mainly found to produce pectate lyase (PL) and bacterial strains were used for the production of polygalacturonase (PGL) (Gummadi and

Kumar, 2005). PL and PGL can be differentiated by their substrate requirement and the absolute requirement of calcium for PGL activity (Henrissat *et al.*, 1995). Pectic lyases are extensively used in extraction and clarification of fruit juices, degumming of ramie and jute fibers, scouring of crude cotton fibers, pretreatment of wastewater from food processing industries (Bruhlmann *et al.*, 1994; Naidu and Panda, 1998; Tanabe *et al.*, 1998; Kashyap *et al.*, 2001; Hoondal *et al.*, 2002; Gummadi and Panda, 2003; Gummadi and Kumar, 2005).

## Materials and Methods

### Chemicals

Apple pectin (Sigma) was used as the substrate of pectinases assays, whereas all other chemicals and media components used were of highest purity grade available commercially. The naturally available pectic substrates were used i.e. Dead Tree Leaves (DTL) and rice straw (RS) were obtained and collected locally, also used as the sole carbon source for fungal growth in liquid and solid-state cultures (SSC).

### Media and Fungal Strain

*Aspergillus flavus* fungal strain under study was identified by Central Laboratories, Faculty of Agriculture, Alexandria University, Egypt. The screening medium had the following mineral salts composition ( $\text{gL}^{-1}$ ):  $\text{NaNO}_3$ , 2;  $\text{KH}_2\text{PO}_4$ , 1;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5;  $\text{KCl}$ , 0.5; were dissolved and completed up to one liter of tap water, pH was adjusted at 6.8. Erlenmeyer conical flasks 250 ml capacity were used in this study, there are two culture condition protocols of media used, which are liquid and SSC. Each flask containing 100 and 10 ml of mineral salts were added to 3 and 7 g of washed, dried and ground pectic substrate in both liquid and SSC conditions, respectively. The flasks were autoclaved, and inoculated with 2 ml of an evenly prepared spore suspension ( $\sim 10^6 \text{ ml}^{-1}$  spores) and incubated at  $30^\circ\text{C}$  for 7 days.

### **Statistical Analysis**

All the experiments were performed independently in triplicate and the results given here are the mean of three values. Reducing sugar was determined using 3,5-dinitrosalicylic acid (DNS) reagent (Ghose, 1987; Thygesen *et al.*, 2003). The protein content was determined as described by (Bradford, 1976), using bovine serum albumin as standard. The fungal growth in liquid culture was measured by the fungal dry weight (DW g/100ml). The fungal growth was filtered and dried in an oven at 60°C for 5 days.

### **Total Pectin lyase (PNL) and Pectate lyase (PL) assay**

Total Pectin lyase and Pectate lyase (TPNL/PL) activity was evaluated by the method of (Collmer, *et al.*, 1988). The supernatant (cell-free filtrate) (CFF) was used as the source of enzyme for enzyme assay. Reaction mixture consisted of 0.25ml of CFF; 0.25ml of distilled water; and 2.0ml of 0.24 % pectin from apple in 0.05M Tris-HCl buffer (pH 8.0) with 0.5ml of 1mM CaCl<sub>2</sub> were incubated at 37°C for 10 minutes. The increase in absorbance was measured at 235 nm using UV-VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme which forms 1µmol 4,5-unsaturated product per minute under the assay conditions.

### **Exo-polygalacturonase (Exo-PGL) assay**

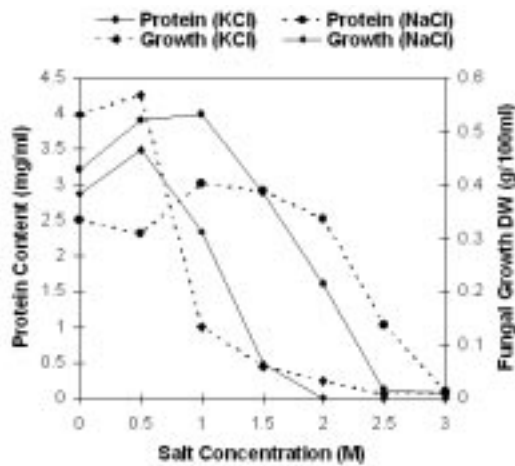
Exo-PGL was assayed by quantification of reducing sugars that were liberated by 0.1ml CFF mixed with 0.5ml 1.0 % pectin and 0.4ml acetate-buffer pH 5.0 incubated for 20 min at 45°C. The optical density (OD) was measured at 520 nm, and the boiled inactivated samples used as blanks. Results expressed as the amount of enzyme that catalyzes the formation of 1µmol galacturonic acid under the assay conditions (Carranco, *et al.*, 1997).

### **Effect of Different KCl and NaCl Concentrations**

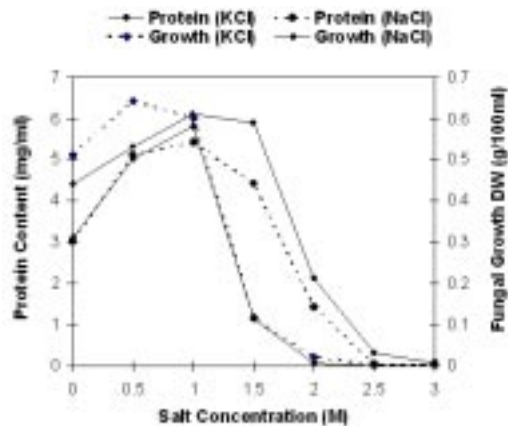
The effect of different KCl and NaCl concentrations on pectinase production was studied in liquid and SSC. The mineral medium was contained as mentioned above and supplied with the following different salt concentrations viz: 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 M, 100 and 10ml of each concentration was added to 3 and 7 g of each substrate, respectively, then autoclaved, inoculated with 2 ml *A. flavus* spores and incubated for 7 days at 30°C. At the end of incubation period, each flask contents were gathered and thoroughly mixed with cooled distilled water (10 ml in each solid-state flask only), rapidly filtered through Buchner's funnel, while the liquid cultures were filtered using 0.45 µm GHP cellulose acetate (Hydrophilic). The filtrate was assayed for protein content and pectinase activity.

### **Results and Discussion**

To study the effect of osmotic stress using different KCl and NaCl concentrations in comparative study on the growth and pectinase production by *A. flavus* under study, the isolate was grown on two naturally pectic substrates in liquid and SSC in the presence and absence of salts. It has been observed that the fungal growth of *A. flavus* was not affected up to 0.5 M (0.567 and 0.466 DW g/100ml) when grown on DTL of liquid culture in both KCl and NaCl, respectively, and thereafter the fungal growth gradually decreased (Fig. 1). The protein content was assayed under the same culture conditions and gave the maximal value up to (3.98 and 3.01 mg/ml) at 1 M in both KCl and NaCl salts, respectively for pectinase production (Fig. 1), in this case, thereafter the protein content was gradually declined. As shown in (Fig. 2), the fungal growth was grown on RS at different salt concentrations in liquid culture conditions and observed that the maximal growth detected up to 0.5 and 1 M



**Fig. 1 : Extracellular protein and fungal growth in different salts concentrations grown on DTL in liquid culture.**

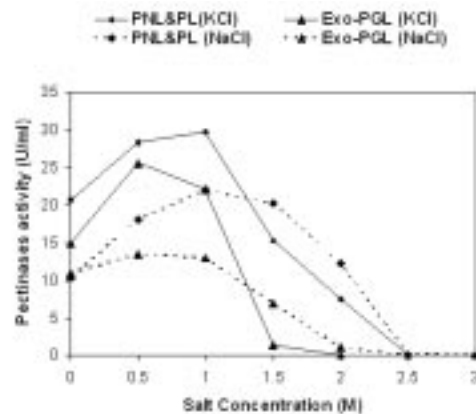


**Fig. 2 : Extracellular protein and fungal growth in different salts concentrations grown on RS in liquid culture.**

(0.643 and 0.581 DW g/100ml) in KCl and NaCl, respectively. While the protein content of pectinase production reached the maximal value up to (6.10 and 5.41 mg/ml) at 1 M of KCl and NaCl, respectively (Fig. 2).

Table (1) shows the protein and growth percentage determination of *A. flavus* in compared to highest value when grown on DTL and RS in different salts concentrations in liquid culture conditions. It has been observed that

the growth in KCl on DTL and RS was better and more resistant to osmotic stress than those grown in NaCl, while the protein determined from RS showed the highest values in KCl rather than that in NaCl, and the protein determined from DTL substrate was not. The effect of different salts concentrations on the production of pectinases from DTL in liquid culture conditions is as shown in (Fig. 3). TPNL/PL showed gradual increase in enzyme activity and exhibited maximal activity up to (29.66 and 21.94 U/ml) at 1 M KCl and NaCl, respectively, and thereafter, TPNL/PL production declined up to (7.44 and 0.13 U/ml) at 2 M and 2.5 M of KCl and NaCl, respectively, after these concentrations no enzyme activity was observed. While Exo-PGL gave optimal production up to (25.61 and 13.52 U/ml) at 0.5 M of KCl and NaCl, respectively when grown on DTL in liquid culture (Fig. 3).

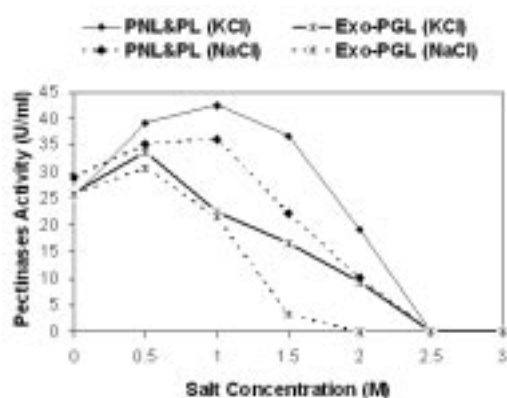


**Fig. 3 : Pectinases produced in different salts concentrations grown on DTL in liquid culture.**

The effect of osmotic stress using different salts concentrations on pectinase production from RS in liquid culture conditions is as shown in (Fig. 4). TPNL/PL exhibited gradual increase in enzyme activity and showed maximal production up to (42.69 and 36.19 U/ml) at 1 M KCl and NaCl, respectively, and thereafter, the activity declined up to (19.21 and 10.11 U/ml) at 2 M in both KCl and NaCl,

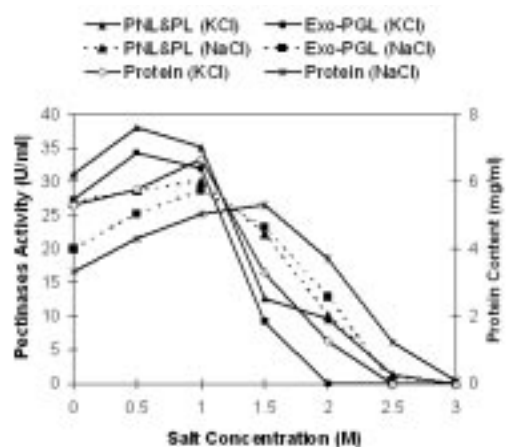
**Table 1 : *A. flavus* protein and growth determination (in % compared to highest value) grown on DTL and RS substrates in different salt concentrations in liquid culture conditions.**

Salt Conc. (M)	DTL				RS			
	KCl		NaCl		KCl		NaCl	
	Protein	Growth	Protein	Growth	Protein	Growth	Protein	Growth
0	80.65	93.65	83.39	81.97	72.29	79.47	55.64	53.87
0.5	98.24	100	77.08	100	87.05	100	94.64	86.57
1	100	23.81	100	66.95	100	93.62	100	100
1.5	72.36	10.76	96.68	13.52	96.56	18.04	81.7	19.28
2	40.2	5.64	83.72	0.43	34.59	3.26	26.06	1.03
2.5	3.27	1.41	34.22	-	5.08	0.47	0.39	-
3	2.01	1.41	2.99	-	0.98	0.47	0.39	-



**Fig. 4 : Pectinases produced in different salts concentrations grown on RS in liquid culture.**

respectively, after this concentration no enzyme activity was recorded. On the other hand, Exo-PGL at the same conditions gave optimal production up to (33.69 and 30.72 U/ml) at 0.5 M of KCl and NaCl, respectively (Fig.4). The effect of osmotic stress using different concentrations of KCl and NaCl on pectinase production from DTL in SSC conditions was studied as shown in (Fig. 5), and showed that, TPNL/PL enzyme exhibited the maximal activity up to (38.09 and 30.11 U/ml) at (0.5 M and 1 M) KCl and NaCl, respectively. With an increase salt concentration the activity declined up to (1.34 and 1.06 U/ml) at 2.5 M in both KCl and NaCl, respectively and thereafter no activity was observed. While at the same conditions Exo-PGL showed the maximal

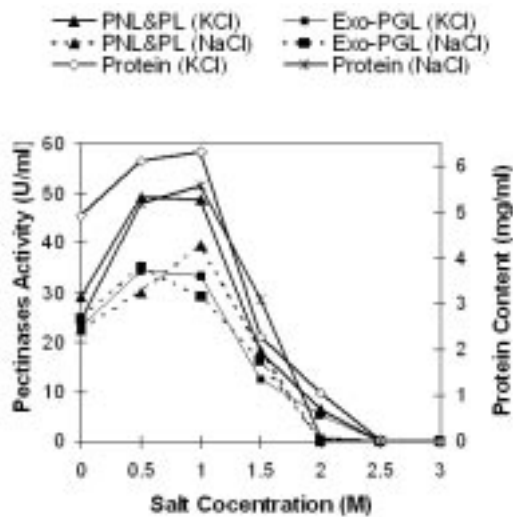


**Fig. 5 : Pectinases and protein content produced in different salts concentrations grown on DTL under SSC.**

activity up to (34.29 and 28.66 U/ml) at (0.5 M and 1 M) KCl and NaCl, respectively, thereafter the activity declined with increasing osmotic stress and reached up to (9.29 and 13.01 U/ml) at (1.5 M and 2 M) KCl and NaCl, respectively, above these concentrations no activity was recorded (Fig. 5). The protein content assayed for the fungal growth when grown on DTL in SSC conditions and gave the maximal value up to (6.66 and 5.31 mg/ml) at (1 M and 1.5 M) KCl and NaCl, respectively, thereafter the protein declined gradually with an increasing salt concentration (Fig. 5).

Also, the effect of osmotic stress on

pectinase production from RS substrate in SSC conditions was observed as shown in (Fig. 6) and revealed that TPNL/PL showed maximal activity up to (49.22 and 39.32 U/ml) at (0.5 M and 1 M) KCl and NaCl, respectively, thereafter, the activity decreased up to (6.31 and 18.11 U/ml) at (2 M and 1.5 M) KCl and NaCl, respectively, above these concentrations no activity was observed (Fig. 6), while Exo-PGL showed maximal activity up to (34.22 and 35.21 U/ml) at 0.5 M in both KCl and NaCl, respectively, and the activity declined up to (5.31 and 16.21 U/ml) at (2 M and 1.5 M) KCl and NaCl, respectively, thereafter, no growth obtained (Fig. 6). Also, the protein determined for *A. flavus* that grown on RS in SSC conditions and the maximal value obtained up to (6.32 and 5.56 mg/ml) at 1 M in both KCl and NaCl, respectively, thereafter, above this concentration the protein decreased (Fig. 6).



**Fig. 6 : Pectinases and protein content produced in different salts concentrations grown on RS under SSC.**

Gummadi *et al.*, (2007), found that a new strain *Debaryomyces nepalensis*, grows better in different saline stress conditions, and greater tolerance was observed for  $K^+$  when compared to  $Na^+$  and  $Li^+$ . Also, it was observed

that  $Na^+$  and  $Li^+$  were more toxic to *Saccharomyces cerevisiae* compared to *D. nepalensis*. In this study we found that the pectinase enzyme activity was decreased with an increase of salt concentration in both liquid and SSC conditions. Also, it was observed only in SSC that the TPNL/PL activity was determined up to 2.5 M KCl and NaCl when grown on DTL, also on the same substrate the activity obtained up to 2.5 M NaCl in liquid conditions. These results may be able to tolerate the highest osmotic stress and may report that the reason of decreasing the fungal growth and enzyme activity by increasing salt concentration that may be due to the toxicity effect of  $K^+$  and may be inhibit some steps in the metabolism of either the fungal growth or enzyme production.

The concentration of dextrose in culture medium was around 4-5 g/L at the time of PL and PGL determination. The synthesis of pectinases by microorganisms is usually a subject of a catabolic repression. In *Aureobasidium pullulans* ATHUM 2915, it has been observed that the production of polygalacturonase was subjected to catabolic repression by glucose (Galiotou-Panayotou, *et al.*, 1998). Similar findings were reported on pectinase production by *Aspergillus niger* where glucose has been shown to reduce the synthesis of polygalacturonase and polymethylgalacturonase to a great extent (Friedrich, *et al.*, 1990).

In this study, the growth and pectinases production were affected by osmotic stress in both liquid and SSC. It has been shown that the production of PL and PGL by *D. nepalensis* was not repressed by the presence of glucose (Gummadi and Kumar, 2006), unlike other pectin-degrading strain such as *Candida boidinii* and *Paenibacillus sp.* (Nakagawa, *et al.*, 2000; Soriano, *et al.*, 2005), where pectinases production was repressed by the presence of glucose in the medium. Recently,

several workers have reported about production of haloenzyme from halophilic bacteria, and no haloenzyme or halopectinase has been reported in yeast (Gomes and Steiner, 2004). There are reported about the production of halophilic enzymes such as amylase, xylanase, and protease from different bacterial species (Amoozegar, *et al.*, 2003; Sanchez-Porro, *et al.*, 2003; Tokuoka, 1993).

### Conclusions

These results clearly indicated that the production of TPNL/PL and Exo-PGL by *A. flavus* under osmotic stress in this study was subjected to some catabolic repression mechanism, at the same time; these enzymes were produced from naturally pectic substances especially enhanced from solid-state culture. This makes *A. flavus* an interesting strain for the study of molecular mechanisms of salt tolerance in fungi and also a potential candidate for development of new biotechnological applications of pectinases.

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