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Potential Production of Tannin Acyle Hydrolase, Gallic acid and Ellagic acid by *Aspergillus aculeatus* Using Green Tea and Pomegranate as Solid Substrates

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Abstract Eleven fungal species were isolated from the tannery soil sample on tannic acid agar medium by dilution plate method. They were identified as *Aspergillus aculeatus, A. subolivaceus, A. fumigatus, Penicillium . janthinllum, P. purpurogenum, P. implicatum, P. oxalicum, P. simplicissium, P. citrinum, Trichoderma viride* and *Ulocladium alternariae.* Solid State Fermentation technique with 80% moisture content using GT and/or PO as tannin rich substrates were used as selective media in the survey. *Aspergillus. aculeatus* showed the most active strain in tannin acyl hydrolase and gallic acid production on green tea, while ellagic acid was the maximum on pomegranate. Higher yield of extracellular tannin acyl hydrolase (117.3 U/ml) and gallic acid (651.9 μ g/ml) were attained in culture medium containing green tea than that obtained on pomegranate culture under one factor-at-a-time optimization process; while, maximum yield of ellagic acid from green tea and pomegranate was 19.70 and 26.50 μ g/ml, respectively. Plackett–Burman design was applied for the screening of 11 variables on the production of tannin acyl hydrolase, gallic acidA, and ellagic acid using green tea as a substrate. The effect of the tested variables was screened at the probability (p) level of 0.0. The Significant variables effect of A, C, H, J, K and L were estimated for tannin acyl hydrolase production, while, A, B, C, E, F, G and L, were significant for gallic acid. In addition, A, C, H, J and L were the significant variables for ellagic acid production. The analysis of variance of models after the exclusion of insignificant coefficients showed that the coefficient of determination (R^2) was 0.9993, 0.9926 and 0.9898 for gallic acid, ellagic acid, and tannin acyl hydrolase respectively. Due to the value of R^2 predicted, the calculated values were 0.6315, 0.7321 and 0.9893 for tannin acyl hydrolase, ellagic acid, and gallic acid respectively, indicating that the gallic acid model is the greater predictive ability compared with the other models.

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Introduction

Tannins are high molecular mass plant polyphenols divided into two chemically and biologically distinct groups: condensed tannins (CT) or proanthocyanidins, and hydrolysable tannins: ellagitannins (ETs) and gallotannins (GTs) (Hashimoto *et al.*, 1992). Tannins gained original popularity in the tanning industry where animal hides were converted into leather by using plant extracts, but have attracted much attention recently due to their numerous biological activities and implications in potential benefits to human health (Landete 2011)

Pomegranate (*Punica granatum* L.) wastes, contain a significant amount of phenolic compounds, including anthocyanins (derived from delphinidin, cyanidin and pelargonidin), hydrolysable tannins (catechin, epicatechin, punicalin, pedunculagin, punicalagin, gallic and ellagic acid esters of glucose) (Cuccioloni *et al.*, 2009). These phenolic compounds confer antioxidant, anti-mutagenic, anti-inflammatory and anticancer activities to the pomegranate wastes (Naveena *et al.*, 2008). However, pomegranate is a rich source in the ET punicalagin (Landete *et al.*, 2011). In recent studies, pomegranate husks were successfully used as support and nutrient sources for Ellagic acid(EA) production by solid state fermentation (SSF) by *Aspergillus niger* GH1 (Hernández-Rivera 2008). This process is economically interesting since from each ton of waste, it is possible to produce 8 kg of ellagic acid by SSF (Robledo *et al.*, 2008). This process is also quite profitable from an industrial point of view, considering the commercial price of this acid and the low cost and abundance of the husks.

Green tea (*Camellia sinensis* L.) is one of the most widely consumed beverages in the world. Epidemiologic research has revealed that individuals who drink large quantities of green tea are less likely to develop cancer (Yu *et al.*, 1995). Green tea contains many compounds considered to promote health, such as polyphenolic flavonoids, of which epigallocatechin gallate (EGCG) is the major constituent. The cancer chemo-preventive function of green tea catechins has been well documented, and in particular, EGCG has been shown to have anti-carcinogenic activity in vitro, EGCG often described as the major biologically active component in green tea, is one of the most potent catechins capable of inhibiting cell proliferation and inducing apoptosis in cancer cells (Shimizu *et al.*, 2011). Recently, tea residue was selected as tannin-rich substrate for the production of TAH by filamentous fungi under SSF as reported by Sherief *et al.* (2011) and Sharma *et al.* (2014)

Tannin acyl-hydrolases (TAH), commonly referred to as tannases (E.C.3.1.1.20), are inducible enzymes produced by fungi, yeast and bacteria (Mehta *et al.*, 2013). It has mostly been characterized by their activity on complex polyphenolics and are able to hydrolyze the ester bond (galloyl ester of an alcohol moiety) and the depside bond (galloyl ester of gallic acid) of substrates such as tannic acid, epicatechin gallate, epigallocatechin gallate, and chlorogenic acid (Garcia-Conesa *et al.*, 2001). In this connection, Macedo *et al.* (2011), indicated that the antioxidant capacity of the tea increased after tannase treatment, The tannase hydrolyzed the substrates contained in the tea by the same way it did toward the pure epigallocatechin gallate (EGCG) standard, and the products of hydrolysis apparently contributed to the observed increase in the tea's antioxidant capacity. The antioxidant capacity of the GT sample was increased by 55% after enzymatic treatment. Similarly, biotransformation increased the antioxidant activity of the commercial EGCG by 46%. These results indicate that the fungal TAH from being able to hydrolyze the ester bonds of natural substrates. Epigallocatechin and gallic acid can be formed by the degalloylation of the gallate (epigallocatechin gallate) present in the tea extract.

A lot of literatures proved that fungi are the most prominent TAH producers, in which *Aspergillus* and *Penicillium* genera were found to be most promising genera. Tannin acyl hydrolase is an inducible enzyme, industrially important, finds wide applications in the food, beverages, pharmaceuticals, bioremediation and as a sensitive analytical probe for determining the structure of naturally occurring gallic acid esters (Haslam *et al.* 1970). It is mainly used in

the manufacture of ice tea, and production of gallic acid and ellagic acid (Chavez-Ganzalez *et al.*, 2011).

Gallic acid is an important precursor for tri-methoxy-benzaldehyde which is the precursor for tri-methoprim production. Trimethoprim is a broad spectrum antibiotic, which is largely imported by pharmaceutical companies engaged in trimethoprim synthesis (Lokeswari and Jaya Raja 2007). Gallic acid is regarded as a non-toxic for human; also, due to the poly-OH structure of gallic acid; it is characterized as a heavy metal chelating agent, anti-microbial, anti-oxidative, anti-carcinogenic hypotensive and serum lipid reducing. Furthermore, it found to show cytotoxic activity against cancer cells without any harming effects on normal cells. In this connection, the current global requirement of gallic acid is around 8,000 tons/year (Verhagen *et al.*, 2002, and Sariozlu and Merih 2009).

Ellagitannins (ETs) and EA are consumed constantly in fruit, seeds, and in the foods or beverages based on fruit juices and jam, etc. (Clifford and Scalbert, 2000). Ellagic acid, a dimeric derivative of gallic, is present in the plant vacuole, either in its free forms as EA or EA derivatives, or else bound as water-soluble ETs (Amakura *et al.* 2000). Numerous derivatives of EA exist in plants, formed through methylation, glycosylation and metoxylation of its hydroxyl groups (Maas *et al.*, 1991). Furthermore, during food processing ETs change to free EA and EA derivatives (Bakkalbasi *et al.*, 2009). Like other polyphenols, ETs, EA and their derived metabolites possess a wide range of biological activities, which suggest that they could have beneficial effects on human health. Ellagitannins, Ellagic acid and derived metabolites have antioxidant functions, estrogenic and/or antiestrogenic activities and anti-inflammatory and prebiotic effects (Landete 2011)

The use of a sequential experimental design strategy is a useful tool for optimization of production process. Response surface methodology (RSM) provides important information regarding the optimum level of each variable along with its interactions with other variables and their effects on yield production. It reduces the number of experiments without neglecting the interactions among the parameters. This multivariate approach also improves statistical interpretation possibilities and evaluates the relative significance of several contributing factors even in the presence of complex interactions (Dilipkumar *et al.* 2011). Response surface methodology is widely used for multivariable optimization studies in several biotechnological processes such as media optimization (Pan *et al.*, 2008; Abou-Bakr *et al.*, 2013)

In the present work, the local fungal strain, *Aspergillus aculeatus*, which is considered as an excellent tannin-degrading fungus and TAH producer, has been used to evaluate its ability to convert green tea (GT) and pomegranate (PO) as cheap tannins rich sources to gallic acid (GA) and ellagic acid (EA) in solid-state fermentation. The sequential strategy of the experimental design was applied to optimize the factors affecting the accumulation of antioxidants and the enzymes production.

Material and methods

All the experiments were run in triplicate, and the average values were adopted.

Isolation of tannin degrading fungi from tannery soil as a source of microorganisms

In spring of 2012, soil samples were collected from different localities such as Batra Village, Dakahlia province, Egypt. Through continuous 15 days, 1.0 kilogram of soil sample was mixed and witted daily by adding domestic residues of tea and coffee as additives tannins. After that time, 25 grams of tannery soil were used for the isolation of tannin decomposing fungi on tannic acid agar medium by the dilution plate method.

Tannic acid agar medium

According EL-Tanash, (1997), this medium has the following constitution; 10.0 g tannic acid, 0.5 g $KH2PO_4$, 0.5g $K2HPO_4$, 1.0g NH4Cl, and 0.5g $MgSO_4$, 0.01g CaCL2, and 0.5 g glucose, all these contents were dissolved

in 1000 ml 0.1M acetate buffer (pH 5.5). Media without tannic acid and agar is considered as basal medium.

Identification of tannin degrading fungi

The isolated tannin-degrading fungi were morphologically and microscopically identified by the Regional Center of Mycology and Biotechnology (RCMB), Al-Azhar University, Nasr City, Cairo, Egypt. Survey results indicated that *Aspergillus aculeatus* was found to be the most active tannin degraders compared with others and its maintenance code number is "SHTA 92"

Chemicals

All chemicals used were of analytical grade and obtained from Sigma, Aldrich, and Merck (Germany); BDH and LAB (England); and El-Nasr Pharmaceutical Chemicals Co. (Egypt).

Solid substrate

Leaves of green tea (GT) and the mixture of the husk and peels of pomegranate (PO) as a natural tannin rich substrate were bought from the local supermarket in Al-Mansoura, Egypt. The samples were cut into fine particles (0.1-0.5 mm) and dried at 60°C for 5.0 days.

Inoculum preparation

Fungal strain was sub-cultured on modified agar media containing 2.0% tannic acid as a sole carbon source, at 30°C for 5.0 days and maintained at 4°C. Induced slant was mixed with 10 ml sterile basal medium for preparing a spore suspension.

Solid state fermentation and extraction of metabolites

Solid state fermentation medium was prepared according to Trevino-Cueto *et al.*, (2007) with slight modification. 1.0 g of GT/or PO was transferred to 250 ml Erlenmeyer flask and mixed with 2.0 ml of freshly prepared basal medium (0.1 M acetate buffer; pH 5.5). The flasks were autoclaved at 121°C at 15 lbs for 30 min. The cooled substrates were inoculated under aseptic conditions and distributed carefully with 2.0 ml spore suspension; then, incubated for 2.0 days incubation at 30°C under static conditions. After the incubation period, the fermented substrates were mixed properly by adding 50 ml of 0.1 M acetate buffer pH 5.5 containing 1% NaCl to culture medium. The mixture was kept on the rotating shaker for 1.0 h at 10°C; then, centrifuged at 5000 rpm for 10 min to remove all fungal cells and substrate residue. The clarified extract represented was used for TAH, GA and EA estimation.

Tannin acyl hydrolase assay

Tannin acyl hydrolase activity was estimated by detection the liberated GA after precipitation un- hydrolyzing tannic acid as reported by Sherief *et al.*, (2011). One tannase unit is the amount of enzyme that liberates 1.0 *u* mol GA per ml per min under the assay conditions.

Estimation of Gallic acid

The amount of GA in 1.0 ml culture filtrate was estimated according the method explained by Sherief *et al.*, (2011), using the μg standard curve of Sigma gallic acid.

Estimation of Ellagic acid

The method proposed by Wilson and Hagerman (1990) was used for the determination of EA with some modification. The Standard curve of Sigma EA was conducted in μg/ml.

Optimization of process parameters (one factor-at-a-time)

The effect of different incubation periods (1.0-7.0 days), different incubation temperatures (20-45°C) and different initial pH (pH; 3.0-7.0) on TAH, GA, and EA production by *Aspergillus aculeatus* were examined at the constant substrate level of tannin substrates (1.0 g $flsk^{-1}$), and other nutrients of basal medium. The level of each factor was

estimated by the single factor experiment technique (one factor-at-a-time).

Statistical optimization

Response surface methodology (RSM) consists of a group of empirical techniques used for evaluation of the relationship between cluster of controlled experimental factors and measured response. A prior knowledge with an understanding of the related bioprocesses is necessary for a realistic modeling approach. In this study, statistical software package "Design Expert 7.0.0" is used to prepare such design and analyze the experimental data. Green tea was selected as a carbon source for the optimization process of TAH, GA, and EA production by *A. aculeatus* under SSF.

Plackett–Burman experimental design

The Plackett–Burman design (PB) is an efficient technique for the optimization of medium component. PB is very useful for picking the most important factors from a list of candidate factors. At this early problem-solving stage, the methodology assumes that important main effects will be much larger than two-factor interactions, so we were willing to confound the main effects. The technique was used to identify the most important independent variables, to verify if the investigated levels were inadequate range and to select them to realize another fractional design or a complete factorial design (Plackett and Burman 1946). The results of PB experimental design were fitted by the first-order model as follows:-

$$Y = \beta_0 + \Sigma \beta_i X_i \quad ,(i=1, 2, \ldots k)$$

Equation (1)

[Where; **Y** is the estimated target function; β_0 is the model intercept and β_i is the regression coefficient and X_i is the coded independent factor and k is the number of studied factor].

Each variable was defined at two levels, namely high and low level that was coded by (+1) and (-1). The difference between the average of at least three measurements obtained at (+1) and (-1) level of each factor was simply taken as the effect of such design (Reddy *et al.*, 2008). The studied 11 variables in our experimental design were as the following; incubation time (A), incubating temperature (B), initial pH (C), moisture level (D), inoculums size (E), substrate level (F), additive glucose (G), additive sucrose (H), NH_4Cl (J), yeast extract (K) and $CaCl_2.2H_2O$ (L). Selected variables with low level (−1) and high level (+1) are illustrated and screened in 12 experimental runs deign. The statistical software package, Design Expert 7.0.0 was used to analyze the experimental data.

Reproducibility

All the experiments were repeated at least three times and the results were reproducible. The data points represented the mean values of the individual values.

Results and Discussion

Isolation and screening of tannin degrading fungi

Tannin-degrading fungi from tannery soil amendment with residues of tea and coffee as additives tannins were isolated on tannic acid agar medium, identified and arranged according to their taxonomical positions (Table 1). Low fungal populations were obtained, these results may attribute to the antimicrobial properties of tannins (2% tannic acid in isolation medium) as previously reported by Lim *et al.*, (2006). Only eleven fungal species belonging to four genera were isolated. These fungi belong to two classes: Ascomycetes, and Hyphomycetes. A number of Ascomycetes were the most frequent (9 species belonging to 2 genera). The two genera, *Aspergillus* and *Penicillum* include 3 and 9 species, respectively. *Penicillum* was the most frequent genus in this class and was represented by six species namely; *Penicillium janthinllum, P. purpurogenum, P. implicatum, P. oxalicum, P. simplicissium* and *P. citrinum*; while, *Aspergillus* was represented by three species namaly; *Aspergillus aculeatus, A. subolivaceus* and *A. fumigatus*. Hyphomycetes include two genera and two species and identified as *Trichoderma viride* and *Ulocladium alternariae*. The dominance of Acomymcetes was mainly *Aspergillus* and *Penicillium* species which are in agreement with those

obtained by Batra and Saxena (2005). Moreover, higher frequency of *Aspergillus* and *Penicillium* species on different tannin rich substrates were reported by Sherief, *et al.*, (2011) who isolated 11 fungal species from tannin rich substrates of GT and RT (red tea), most of them are belonging to genus *Aspergillus* (6 species). Higher occurrence of *Aspergillus* and *Penicillium* species in different tannin rich sources may attribute to their ability for TAH production, that catalyze the hydrolysis of hydrolysable tannins (as tannic acid in dilution plats) producing GA and glucose (Belur and Mugeraya 2011).

Eleven fungal isolates cultivated on GT and/or PO as tannin rich residues were tested using SSF to determine their abilities for TAH, GA, and EA production. Triplicate flasks were autoclaved at 121°C at 15 lbs for 30 min. The cooled substrates were inoculated under aseptic conditions and distributed carefully with 2.0 ml spore suspension, then, incubated for 3.0 days at 30°C under static conditions. Results indicated that *Aspergillus aculeatus* belonging to *Aspergillus niger* group was the most active fungus able to degrade natural tannins of GT and PO producing 82.9 and 45.3 U/ml of TAH, respectively compared with the others fungal species. In addition, higher yield of GA at the end of incubation periods was shown by the same fungus; it produces 586 and 188 µ g/ml GA from GT and from PO, respectively. Higher production of TAH and GA from GT compared with PO may attribute to the richness of GT of *Camellia sinesis* L. with epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG) which is the most abundant, accounting for 50-80% of the total catechins in GT as reported by Feng (2006), Lu and Chen, (2007) and Akroum *et al.*, (2009). Recently, ***Aspergillus niger*** species are the most tannins decomposing fungi for TAH and GA production when grown on natural tannin rich substrates by SSF (Lekha and Lonsane, 1994; Pinto *et al.*, 2001). In this connection, *A. foetidus* and *A. aculeatus* (Mukherjee and Banerjee, 2006) and *A. ruber* (Kumar *et al.*, 2007) were also reported as higher tanninolytic fungi.

The present results also indicated that *A. aculeatus* was the most EA producer when cultivated on PO compared with the other tested fungi. It produces about 23.3 and 13.3 ug/ml of EA from PO and GT respectively. These results may attribute to the richness of PO with ellagitannins as reported by Cuccioloni *et al.*, (2009). For these reasons, PO husks were successfully used as support and nutrient sources for EA production by SSF (Aguilar *et al.*, 2008). Recently, elagitannin acyl hydrolase has been related with the bioconversion of elagitannin into EA during SSF of PO husks. Biodegradation process of PO residues is economically interesting since from each ton of waste, it is possible to produce 8 kg of EA by SSF (Robledo *et al.*, 2008). This process is also quite profitable from an industrial point of view, considering the commercial price of this acid and the low cost and abundance of the husks. Many tannin rich plant residues have been used in SSF processes. However, extracts of its fermentable debris using organic/ or in organic solvents have shown great potential regarding biological properties due to antioxidant and antifungal activities. Recently, it has been demonstrated that some tannin rich substrates have a potential for TAH, GA and EA production by SSF using *Aspergillus niger* Aa-20 (Treviño-Cueto *et al.*, 2007). High concentrations of GA and EA were also obtained by *Aspergillus niger* PSH during SSF of tannin-rich aqueous extracts (Ventura *et al.*, 2008). *Aspergillus niger* GH1 has also been reported as being a fungus with great ability to hydrolyze natural ellagitannins into EA during SSF (Aguilera-Carbo *et al.*, 2009).

Tannin acyl-hydrolase that hydrolyze tannins is the future direction of research. So far, TAH can be found everywhere in a variety of fungal strains, nevertheless the production level is far from being enough for commercialization. For that reason, isolating a promise local TAH, GA and EA producing fungi of *Aspergillus aculeatus* and optimization for high productivity using low cost substrates of GT and PO are of great importance to the industry.

Table (1): Screening of most active tannin degrading fungi and their ability for TAH, GA and EA production fungi on GT and PO using SSF.

Fungal species	GT				PO			
		TAH (U/ml)	GA (ug/ml)	EA (ug/ml)		TAH (U/ml)	GA (ug/ml)	EA (ug/ml)
Aspergillus aculeatus	++++	82.9	586.0	13.3	++++	45.3	188.0	23.3
Aspergillu subolivaceus	++	9.0	40.7	4.6	++	6.7	94.0	13.6
Aspergillus fumigatus	+++	22.6	156.6	11.6	+++	9.0	62.7	13.3
Penicillium janthinllum	++	9.8	62.6	7.0	++	6.0	62.7	13.0
Penicillium purpurogenum	++	9.8	125.3	6.3	+++	9.8	94.0	17.0
Pencillium implicatum	++	10.5	84.6	4.0	++	9.0	78.4	13.6
Pencillium oxalicum	++	8.29	31.3	2.0	+++	12.0	81.5	13.0
Pencillium simplicissium	+	6.7	94.0	5.6	++	7.5	72.0	5.3
Penicillium citrinum	+	4.5	28.2	0.3	++	10.5	34.4	7.0
Trichoderma viride	++	16.6	112.8	13.0	++	9.8	50.1	10.3
Ulocladium alternariae	+	11.3	94.0	7.3	+	12.0	43.8	19.0

Optimization of TAH, GA and EA production by *A. aculeatus* cultivated on GT and PO using SSF (one factor at-a-time)

Time course for TAH, GA and EA production

Time course for TAH, GA and EA production by culture of *A. aculeatus* were carried out through 7 days on both tannin rich residues of GT or/and PO. Results in (Fig. 1 A) show that TAH activity was gradually increased from the first day of incubation and reached maximum activity after 2.0 days in case of green tea (80.0 U/ml) and after 3.0 days in case of pomegranate (57.14 U/ml), then gradually decreased. The lower incubation period for enzyme production is an industrially advantage and the decrease of enzyme activity on prolonged incubation time could be attributed to inhibition and de-naturation of the TAH protein (Kumar *et al.*, 2007). These results are in agreement with that obtained by *A. foetidus* (Mukherjee and Banerjee, 2004) and another strain of *A. aculeatus* DBF9 (Banerjee *et al.*, 2007). However, optimal tannase of *Rhizopus oryzae* was detected after 5.0 days (Chatterjee *et al.*, 1996) and after 4.0 days incubation from *A. niger* (Lekha and Lonsane, 1997, and Kumar *et al.*, 2007) using other tannin rich residues.

Pattern of GA and EA released (Fig. 1 A and B) from the culture of GT and PO was clearly associated to evaluate their TAH, which were detected at its maximum level at the same time and decreased afterward. The maximum production of GA (651.87 and 316.53 ug/ml) and EA (21.00 and 28.67) were observed within 2.0 and 3.0 days of incubation on GT and PO, respectively. These results are in agreement with that obtained by Robledo et al., (2008), who reported that two *Aspergillus niger* strains GH1 and PSH are able to degrade PO ellagitannins during the first 72 h of culture into EA in a SSF. Higher yield of GA and EA in the primary phase of fungal growth may attribute to the higher yield of TAH activity. Tannins of solid GT or PO residues cannot penetrate the fungal cell membrane due to its high molecular weight, but the excretion of microbial TAH as extracellular form can breakdown its content into GA/ or EA and glucose. The glucose is a readily available carbon source; therefore, it is assimilated first, which results in rapid growth. However, as the glucose concentration falls, GA/or EA is utilized by as a substrate for energy production. This also explains the decrease of GA/or EA after the optimum incubation periods. For this reason, it is very important to stop the fermentation at this time. Furthermore, higher yield of EA in case of PO compared to GT may attribute to its richness with ellagitannins of punicalagin isomers as reported by Lansky (2006) and Landete (2011).

In this connection, Sharma *et al.* (2007), and Rodrigues *et al.* (2008) found that 2.0 days of incubation period were the optimum for the

production of TAH and GA. No report was found in EA production. Moreover, maximum of EA released was reached at 36 h from the culture of *Aspergillus niger* grown on Creosote Bush Ellagitannins by SSF. However, the EA was decreased, possibly because EA represents one of the substrates generated by the action of ellagitannin-hydrolyzing enzymes (EHA) (Aguilera-Carbo *et al.* 2008 and 2009). . In addition, 4.0 days incubation was the optimum for higher EA production from *Aspergillus oryzae* grown on tea waste by SSF (Paranthaman *et al.*, 2013).

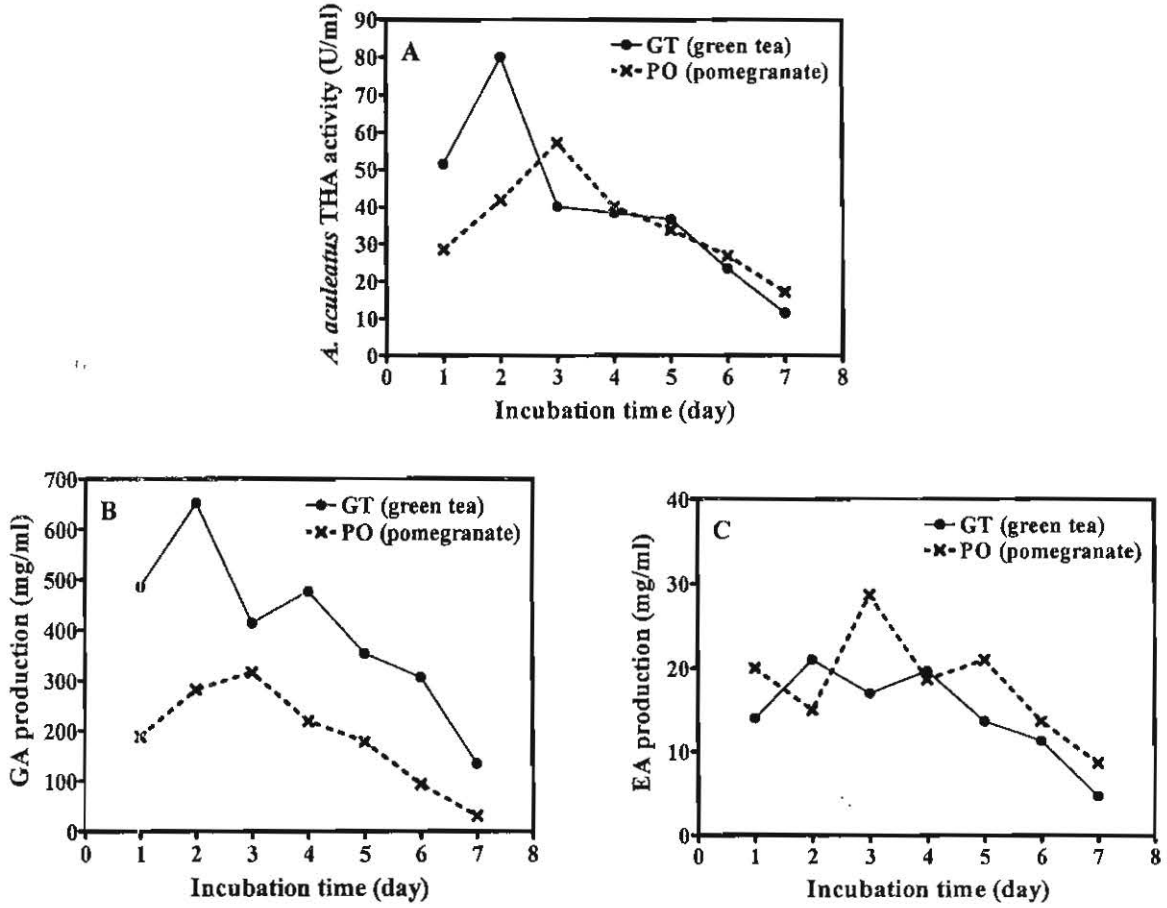


Fig.1 A, B and C: Time course for TAH, GA and EA production by *A. aculeatus* cultivated on GT and PO residues as solid substrates

Effect of different incubating temperatures on TAH, GA and EA production

Temperature is one of the most important cultural fermentation factors; strongly affect SSF process and the enzyme productivity (Pandey *et al.*, 1999). The results obtained in(Fig. 2 A) show the significant effects of temperatures on TAH, GA and EA production. Higher TAH activity was obtained on GT compared with PO at the range of temperatures used. The optimum temperature for maximum *A. aculeatus* TAH production appears to be at 30°C on both GT (103.0 U/ml) and PO (51.0 U/ml). The further increase in temperatures above 30°C was a sharp decrease in enzyme productivity; where, nearly no detectable fungal growth or TAH productions were detected at 60°C. These results are in agreement with that obtained by some investigators using various strains for TAH production including; *A. japonicus* (Bradoo *et al.*, 1997), *A. foetidus* (Mukherjee and Banerjee, 2004), *A. niger* ATCC 16620 (Sabu *et al.*, 2005), *A. ruber* (Kumar *et al.*, 2007) and another strain of *A. aculeatus* (Banerjee *et al.*, 2007).

The maximum release of GA and EA was also detected at 30°C (Fig. 2 B and C), the optimum temperature for *A. aculeatus* TAH

productivity for GT and PO. This temperature is also optimized for most fungal lignocellulosic enzymes production under SSF which enhancing the release of GA and EA from the tannin rich sources. In this connection, Huang *et al.*, (2008) recorded that the combination of *Aspergillus oryzae*, *Endomyces fibulige* and *Trichoderma reesei* enzymes (β-Glucosidase, Cellulase, Xylanase and Ellagitannase acyl hydrolase) appeared more effective for EA production than the single enzyme did. Furthermore,the yield of EA from non-heat-treated acorn fringe by the use of enzymes increased, compared with that from heat-treated material. In addition, 35°C was the optimum for higher EA production from *Aspergillus oryzae* grown on tea waste by SSF (Paranthaman *et al.*, 2013).

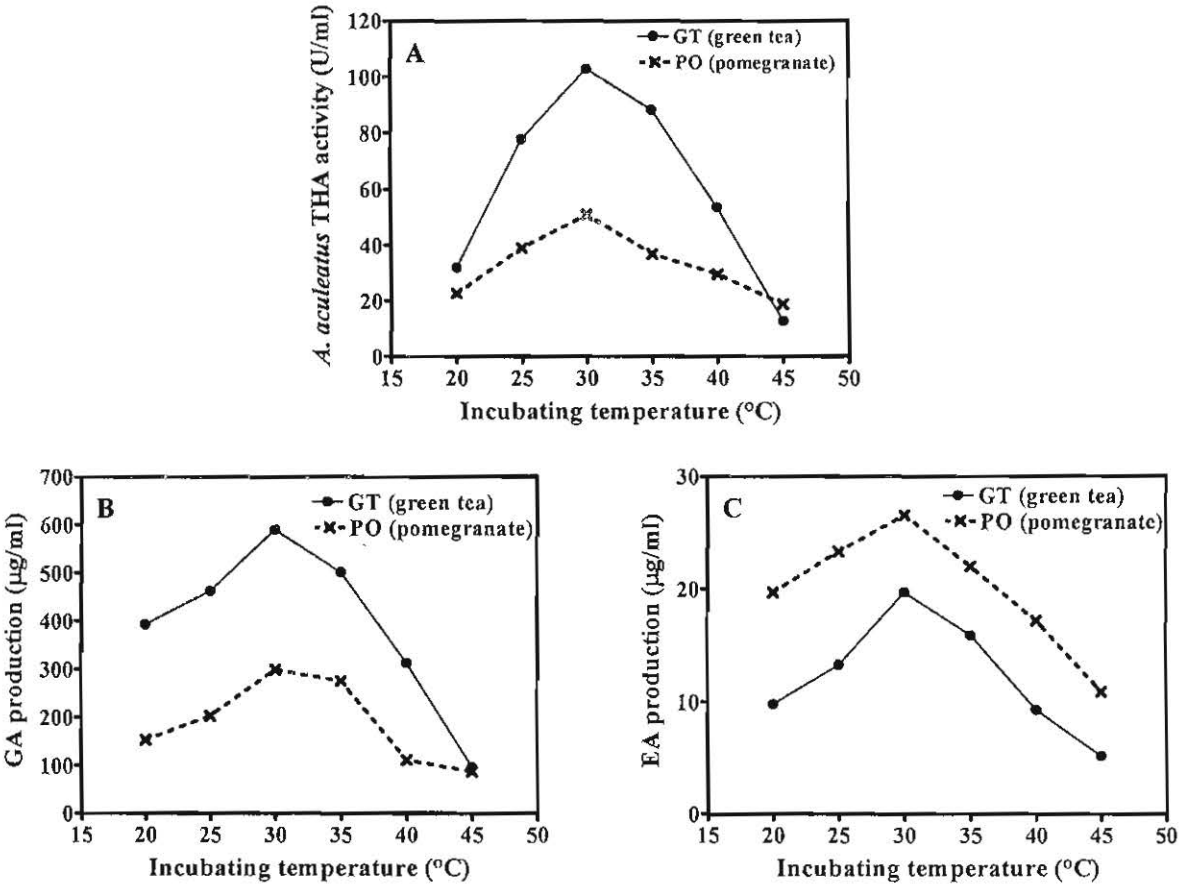


Fig. 2 A, B and C: Effect of different incubating temperatures on TAH, GA and EA production by *A. aculeatus* cultivated on GT and PO residues as solid substrates

Effect of different initial pH on TAH, GA and EA production

The role of initial pH on enzyme productivity during fermentation may be attributed to increase or decrease the permeability of microbial cells that effect on enzyme yield and stability (Mase *et al.*, 1996). The pH profile of *A. aculeatus* TAH production (Fig.2 A) indicated that pH 5.5 was the optimum for TAH from GT (117.3 U/ml) and PO (49.6 U/ml). Then, it gradually decreased with increasing the pH. Optimum acidic pH range from 4.5 to 6.5 has been reported for TAH production by other *Aspergillus* strains including; *A. foetidus* grown on powdered fruits of *Terminalia chebula* and *Caesalpinia digyna* pod cover powder (Mukherjee and Banerjee, 2004), *A. niger* ATCC 16620 grown on Tamarind seed powder and palm kernel cake (Sabu *et al.*, 2005) and *A. ruber* grown on jamun leaves (Kumar *et al.*, 2007).

The optimum pH for GA and EA was found to be 5.5 (Fig. 2 A and B.). Upon varying the pH of the medium from 3.0 to 7.0,

the yields were decreased as pH approached the neutrality. Our results are in accordance with the finding of microorganisms including fungi (Chhokar *et al.* 2009) and bacteria (Beniwal *et al.*, 2010).

From the previous, the final yield of TAH (117.3 U/ml) and GA (651.9 ug/ml) by *A. aculeatus* is significantly higher when cultivated on GT than that obtained on PO under one factor-at-a-time optimization process; while, maximum yield of EA from GT and PO was 19.70 and 26.50 ug/ml, respectively. Therefore, GT as a solid tannin rich source was selected in the following optimization process of ranking the effects of different culture conditions and evaluate TAH, GA and EA production.

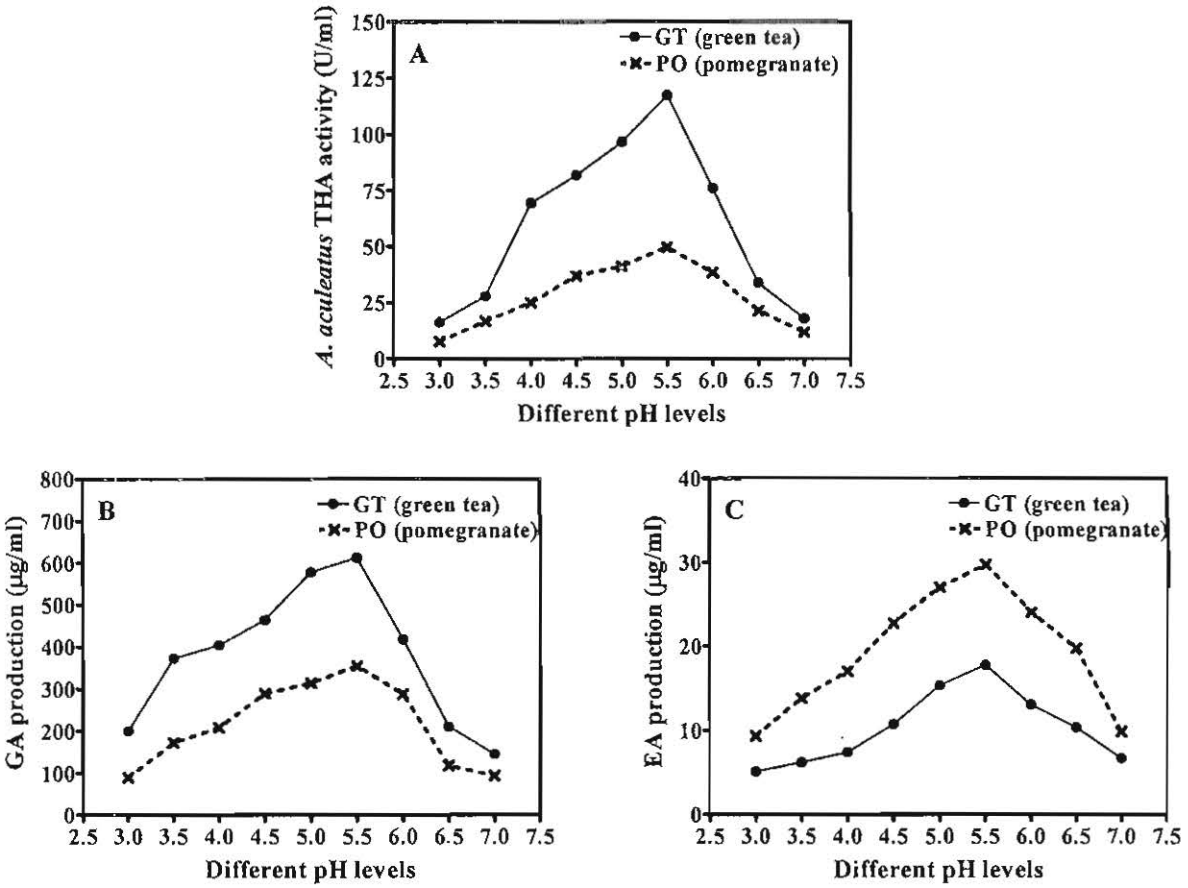


Fig. 3 A, B and C: Effect of different initial pH on TAH, GA and EA production by *A. aculeatus* cultivated on GT and PO residues as solid substrates

Statistical optimization

Statistical screening of GT culture fermentation using Plackett-Burman design to optimize TAH, GA and EA production by A. aculeatus

The **Plackett-Burman** (PB) designs experimental runs or trials is a multiple of four, i.e. N = 4, 8, 12, 16 ... and so on, where N is the number of trials/runs. PB designs are suitable for studying up to $k = (N-1)/(L-1)$ factors, where L is the number of levels and k is the number of factors. For instance, using a 12 run experiment, it is possible to study up to 11 process or design parameters at 2-levels. One of the interesting properties of PB designs is that all main effects are estimated with the same precision. This implies that one does not have to anticipate which factors are most likely to be important when setting up the study. For screening designs, experimenters are generally not interested to investigate the nature of interactions among the factors. The aim is to study as many factors as possible in a minimum number of trials and identifying those that need to be studied in further rounds of experimentation in which interactions can

be more thoroughly assessed. In the present study, PB design was used to analyze the effect of 11 variables on GT degradation to evaluate the yield of TAH, GA and EA by *A. aculeatus*. In the experimental design, each row represents an experiment and each column represents an independent variable. The sign +1 and −1 represent the two different levels (high and low) of the independent variable under investigation (Table 2). TAH, GA and EA were estimated for each experiment design as shown in Table (3). The results based on PB model showed a considerable rate of *A. aculeatus* growth in all experiments (12 trails), with variations in different estimated responses. Result (Table 3) indicated no significant co-relationship between responses and the rate of growth. TAH activity was ranged from 2.2 Uml^{-1} in run number 1 to a maximum of 216.40 Uml^{-1} in run no. 9. This Optimization recorded 1.85 fold increase of TAH compared with the activity before optimization. Comparing the highest run with the lowest one, it showed 98.40, 2.34 and 13.50 fold increases in TAH, GA and EA production, respectively; this reflecting the great influence of selected variables and their levels on responses (TAH, GA and EA). High GA (654.00 ug/ml) and EA (28.30 ug/ml) was detected in run 2 and 9 respectively. Statistical optimization of THA, GA, and EA production using SSF is rare, however, few studies on the production of TAH using PB design during SmF was recorded (de Melo 2014).

Table 2: the levels of the screened independent variables used for TAH, GA and EA production during solid-state fermentation of GT by *A. aculeatus*

Independent variables		Unit	Low level (-1)	High level (+1)
A	Incubation time	Day	2.0	6.0 days
B	Temperature	°C	25	40
C	pH	Degree	4.5	6.5
D	Moisture level	ml	1.0	5.0
E	Inoculums size	ml	0.5	2.5
F	Substrate level	%	0.25	1.25
G	Additive glucose	%	0.1	0.5
H	Additive sucrose	%	0.1	0.5
J	NH_4Cl	%	0.1	0.5
K	Yeast extract	%	0.0	0.2
L	$CaCl_2.2H_2O$	%	0.05	0.2

Table 3: The Plackett-Burman design with uncoded values and corresponding activities of TAH, GA, and EA by *A. aculeatus*

Std	Run	Independent variables											Responses					
													TAH (U/ml)		GA (μ g/ml)		EA (μ g/ml)	
		A	B	C	D	E	F	G	H	J	K	L	Actual	Predicted	Actual	Predicted	Actual	Predicted
4	1	2.0	40	4.5	5.0	2.5	0.25	0.5	0.5	0.5	0.0	0.05	2.20	0.00	297.70	292.83	5.00	4.13
6	2	2.0	25	4.5	5.0	0.5	1.25	0.5	0.1	0.5	0.2	0.2	60.30	58.60	654.00	655.31	20.00	20.27
5	3	2.0	25	6.5	1.0	2.5	1.25	0.1	0.5	0.5	0.2	0.05	30.10	37.63	538.00	535.98	14.66	14.39
1	4	6.0	40	4.5	5.0	2.5	1.25	0.1	0.1	0.1	0.2	0.05	16.20	17.90	422.00	424.03	10.00	9.13
9	5	6.0	40	6.5	1.0	0.5	0.25	0.5	0.1	0.5	0.2	0.05	15.00	7.47	279.00	283.16	2.10	2.97
11	6	6.0	25	6.5	5.0	2.5	0.25	0.1	0.1	0.5	0.0	0.2	75.40	82.93	329.00	330.31	19.33	18.46
8	7	6.0	40	4.5	1.0	0.5	1.25	0.1	0.5	0.5	0.0	0.2	37.70	39.40	570.00	567.98	6.66	7.53
3	8	6.0	25	6.5	5.0	0.5	1.25	0.5	0.5	0.1	0.0	0.05	33.90	32.20	548.40	544.24	11.00	11.27
2	9	2.0	40	6.5	1.0	2.5	1.25	0.5	0.1	0.1	0.0	0.2	216.40	208.87	634.00	638.88	28.30	28.03
7	10	6.0	25	4.5	1.0	2.5	0.25	0.5	0.5	0.1	0.2	0.2	45.30	43.60	307.00	305.69	6.30	6.03
12	11	2.0	25	4.5	1.0	0.5	0.25	0.1	0.1	0.1	0.0	0.05	82.90	90.43	329.00	333.88	20.33	21.20
10	12	2.0	40	6.5	5.0	0.5	0.25	0.1	0.5	0.1	0.2	0.2	75.40	77.10	423.00	418.84	16.66	16.93

Analysis of variance (ANOVA) of TAH, GA, and EA production for PB design

Analysis of PB designs showed that the corresponding fitted values (mathematically calculated) for TAH, GA and EA was close to those recorded from the response values (laboratory calculated). Fitted values are essential for determining whether the model fits the data, which means the accuracy of the variable selection. The analysis of variance (ANOVA) for TAH, GA and EA designs were calculated and summarized in Table (4).

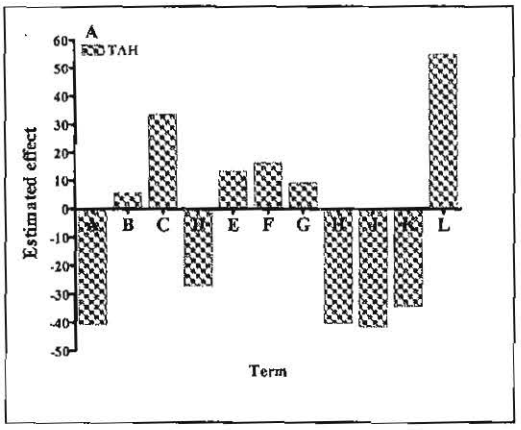
The effect of the tested variables was screened at the probability (p) level of 0.05. Also, (-) indicates that the tested variable is effective on the response, but the amount required is lower than the low (-1) level in Plackett- Burman design. If the effect is positive, a higher concentration than high level (+1) is required during further optimization studies. Results from (Fig. 4A) indicated that A, D, H, J, and K had negative effects; while, B, C, E, F, G and L had a positive effect on the production of TAH during degradation of GT by *A. aculeatus* under SSF. Incubating temperature (B), moisture level (D), inoculums size (E), substrate level (F) and additive glucose (G) were insignificant variables, had lower effects on TAH production and higher *p*-value. *P-value* greater than 0.05 indicate the model terms are not significant. The Model p-value 0.0452 implies the model is significant. *P*-value less than 0.050 indicate model terms are significant. In this case $CaCl_2.H_2O$ (L) is the most significant factor (*p*-value 0.0191) affecting TAH production followed by, NH4Cl (*p*-value 0.0328), incubation time (*p*-value 0.0343), additive sucrose (*p*-value 0.0349), yeast extract (*p*-value 0.0470) and pH (*p*-value 0.0490). Mohan *et al.*, (2014) applied the PB design technique for the screening of 12 medium nutrients for the production of tannase by *Aspergillus flavus* under submerged fermentation; the significant nutrients were identified as tannic acid, magnesium sulfate, ferrous sulfate and ammonium sulfate.

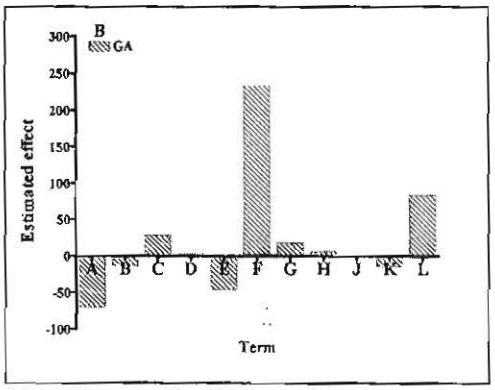
High significant model for GA (p-value = 0.0001) was obtained compared with TAH and EA models. Incubation time (-70.05), inoculums size (-45.95), yeast extract (-14.18) and incubating temperature (-13.83), had negative effect; while, the other variables had a positive effect on the yield of GA produced under study conditions (Fig.4B). Furthermore, incubation time, temperature, pH, inoculums size, substrate level, additive glucose, yeast extract and $CaCl_2.2H2O$ were significant GA model terms.

Results also showed that the EA model was significant due to the lower *p*-value 0.0331. However, incubation time (*p*-value 0.0120), pH (*p*-value 0.0493), Additive sucrose (*p*-value 0.0184), NH_4Cl (*p*-value 0.0454) and $CaCl_2.2H2O$ (*p*-value 0.0248) are significant GA model terms.

Table 4: Estimated effect, regression coefficient and corresponding p-values for TAH, GA, and EA recovered by *A. aculeatus* using Plackett-Burman design

Term	TAH			GA			EA		
	Effect	Coefficient	p-value	Effect	Coefficient	p-value	Effect	Coefficient	p-value
Model		57.57	0.0452		437.08	0.0001		12.50	0.0331
A-Incubation time	-40.63	-20.32	0.0343 *	-70.05	-29.88	0.0004	-8.26	-4.33	0.0120
B-Temperature	5.83	2.92	0.3000	-13.28	-13.83	0.0437	-3.82	-1.89	0.0528
C-PH	33.60	16.80	0.0490 *	28.62	9.16	0.0054	3.96	2.33	0.0493
D-Moisture level	-27.33	-13.67	0.0714	2.85	8.61	0.1568	0.61	1.50	0.2000
E-Inoculums size	13.40	6.70	0.2249	-45.95	-28.13	0.0014	1.14	0.22	0.3113
F-Substrate level	16.40	8.20	0.1677	233.62	109.63	< 0.0001	3.48	2.11	0.0624
G-Additive glucose	9.23	4.62	0.3587	18.18	3.94	0.0193	-2.49	-2.28	0.1123
H-Additive sucrose	-40.27	-20.13	0.0349 *	6.18	8.24	0.0968	-6.63	-2.50	0.0184
J-NH4Cl	-41.57	-20.78	0.0328 *	0.72	5.51	0.0500	-4.14	-1.22	0.0454
K-Yeast extract	-34.37	-17.18	0.0470 *	-14.18	0.09	0.0371	-3.48	-1.28	0.0624
L-$CaCl_2$.2H2O	55.03	27.52	0.0191 *	83.82	34.73	0.0002	5.69	2.50	0.0248
R^2		0.9898			0.9993			0.9926	
Adj R^2		0.9437			0.9976			0.9591	
Pred R^2		0.6315			0.9893			0.7321	





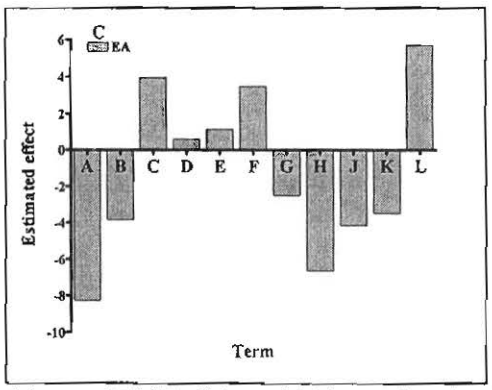


Fig. (4A, B and C): Effect of independent variables on TAH, GA and EA production by *A. aculeatus* based on PB experiments

The Pareto chart allows detecting the significant effects of variables which are most important to the process or design optimization study has to deal with. It displays the absolute values of the effects, and draws a reference line on the chart. Any effect that extends past this reference line is potentially important. Pareto chart from Design-Expert 7.0.0 reproduce the relation between t-value (effect) vs. rank. Results in (Fig 5A) showed that $CaCl_2.2H_2O$ (L) was the most significant variable at 95% confidence in TAH production followed by NH4Cl, incubation time, additive sucrose, pH. Other independent variables are insignificant (p-value < 0.05). Also, it is obvious to note that the significant variables of $CaCl_2$ $2H_2O$ (55.03) and pH (33.60) had positive effects on TAH production and; while, NH4Cl, incubation time, additive sucrose, and yeast extract has A negative effect (-41.57, -40.63, -40.27, and -34.37, respectively).

Whereas, (Fig. 5B) show that substrate level (F), $CaCl_2$ $2H_2O$ (L), incubation time (A), Inoculums size (E), and incubating temperature (B) were the highly significant variable in GA production. It is obvious to note that substrate level and $CaCl_2$ $2H_2O$ had positive effects on the GA response (233.62 and 83.82, respectively); while, incubation time, and inoculums size have a negative effect (**-70.05**, and **-45.95** respectively).

Pareto chart of EA response (Fig. 5C) was clear that incubation time (A), additive sucrose (H) $CaCl_2$ $2H_2O$ (L), and NH_4Cl (J) were significant variable on the yield of EA; while, the other factors are insignificant. It is obvious to note $CaCl_2.2H_2O$ had positive effects (5.69); while, incubation time (-8.26), NH_4Cl (-4.14) and additive sucrose (-6.63) has a negative effect.

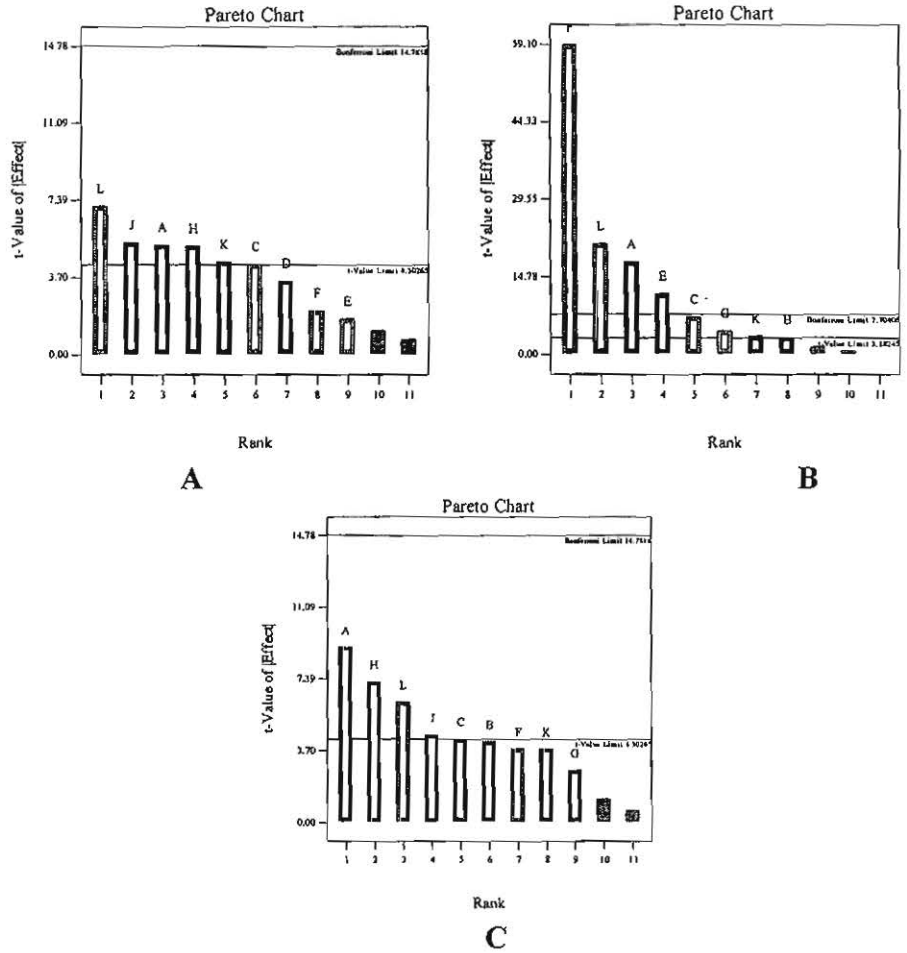


Fig. 5: Pareto chart of the standardized effects of the tested parameters on TAH (A), GA (B) and EA (C).

Coefficient of determination (R^2) and adjusted R_2 are measures of how well the fitness of the data (Table 4). These values can help selection the model with the best fit. R^2 describes the amount of variation in the observed response values that is explained by the factor (s). Because of the continuous increment of R^2 with additional predictors, the adjusted-R^2 is a modified R^2 that has been adjusted for the number of terms in the model. Unlike R^2, adjusted R^2 may get smaller when terms are added to the model; however, the higher the adjusted R^2 the more accuracy of the relationships between the variables and responses (TAH, GA and EA). Similar to adjusted R^2, predicted R^2 indicates how well the model predicts responses for new observations, whereas the adjusted R^2 indicates how well the model fits the data. Predicted R^2 can prevent over-fitting the model and is more useful than adjusted R^2 for comparing models. Larger values of predicted R^2 suggest models of greater predictive ability. However, all the kinds of R^2 range from 0 to 1.

On the bases of the previous explanation, values of TAH production could be predicted with 63.15 % (R^2 predicted) accuracy, and this explains 94.37 % (R^2 adjusted) of the fitness. The value of R^2 predicted is not as close to the R^2 adjusted which indicate large block effect. Whereas for GA, it could be predicted with 98.93% (R^2 predicted) accuracy, and this explains 99.76 % (R^2 adjusted) of the fitness. On the other side, the value of predicted R^2 for EA production is equal to 73.21% lower than the R^2 adjusted of 95.91%. Due to the value of R^2 predicted, the calculated values were 0.6315, 0.7321 and 0.9893 for TAH, EA, and GA respectively, it means that GA model is the greater predictive ability followed by EA, and finally TAH model.

The best-fitting mathematical model was determined by elimination of the non-significant regression coefficients; further, the best reduced equations are shown below (equation 3, 4 and 5). These equations are a fitted first-order model included the coefficients of significant terms for each model to approach the neighborhood of the optimum responses (TAH, GA and EA) from the PB designs:

$$Y_{1\,(TAH)} = +57.57 - 20.32A + 16.80C - 20.13H - 20.78J - 17.18K + 27.52L \ldots\ldots \quad \textbf{Eq (3)}$$

$$Y_{2(GA)} = = +444.26 - 35.02A - 6.64B + 14.31C - 22.97E + 116.81F + 9.09G - 7.09K + 41.91L\ldots\ldots \quad \textbf{Eq (4)}$$

$$Y_{3\,(EA)} = +13.36 - 4.13A + 1.98C - 3.32H - 2.07J + 2.85L \ldots\ldots \quad \textbf{Eq (5)}$$

The analysis of variance of models after exclusion of insignificant coefficients showed that the coefficient of determination (R^2) were 0.9993, 0.9926 and 0.9898 for GA, EA, and TAH respectively.

Conclusion

Eleven tannin degrading fungi were isolated from tannery soil, promising fungus isolated was identified as *Aspergillus aculeatus* SHTA 92 and capable to produce TAH as an inducible extracellular enzyme. TAH hydrolyze tannins from GT and PO is the future direction of research to increase the yield of antioxidants (GA and EA). TAH can be found everywhere in a variety of fungal strains, nevertheless the production level is far from being enough for commercialization. Therefore, isolating of local TAH producing fungus and optimization for high productivity are of great importance to the industry. Traditional fermentation process requires a complete series of experiments for important factors of interest, which is laborious and time consuming; such methods could not provide information for the interactions of the factors. Statistical methodology could locate the most important factor levels with minimum effort and time; moreover, it could reveal the interaction among the factor. In this study, a sequential statistical methodology comprising of Plackett-Burman was applied to enhance the production of TAH, GA and EA. The effect of the tested variables was screened at the probability *(p)* level of 0.0. The analysis of variance of models after exclusion the insignificant coefficients showed that the coefficient of determination (R^2) were 0.9993, 0.9926 and 0.9898 for GA, EA, and TAH respectively. Due to the value of R^2 predicted, the calculated values were 0.6315, 0.7321 and 0.9893 for TAH, EA, and GA respectively, it

means that the GA model is the greater predictive ability compared with the other models. Finally, to search the proper direction, four factors in 5 coded levels (-2, -1, 0, 1 and 2) were selected for further optimization analysis by a central composite design (CCD).

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إنتاج إنزيم التانيزوحمضى الجاليك والالاجيك بواسطة فطر أسبراجيلس اكيولاتس مستخدما الرمان والشاى الاخضر

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**العنوان الحالي-كلية العلوم-جامعة تبوك-المملكة العربية السعودية.*

يهدف هذا البحث الى انتاج انزيم التانيز المحلل للتانينات وانتاج حمضى الجاليك والالاجيك مستخدما الشاى الاخضر والرمان.

١- تم عزل وتعريف بعض السلالات الفطريه المحلله للتانينات الموجودة فى التربه المحلية وتشمل:

Aspergillus aculeatus,Ulocladium alternariae,Penicillium janthinllum, Aspergillus subolivaceus, Penicillium purpurogenum, , Asperagillus fumigatus , Trichoderma viride , Pencillium implicatum, Pencillium oxalicum, Pencillium simplicissium , and Penicillium citrinum.

٢- تبين أن النشاط الانزيمى لفطر Aspergillus aculeatus النامى على بيئة الشاى الاخضر والرمان هو اكثر الفطريات المعزوله انتاجا لانزيم التانيز وحمض الجاليك والاكثر انتاجا لحمض الالاجيك فى حالة استخدام الرمان كوسط .

٣-تم استخدام بلاكت برمان كبرنامج لعمل افضل انتاجيه لانزيم التانييز وحمضى الجاليك والالاجيك حيث تم عمل تجربه لاحد عشرة متغيرا من العوامل اللتى يمكن ان تاثر فى الانتاج مستخدما الشاى الاخضر كوسط.

٤- وجد ان العوامل A,C,H,J,K,Lهى العوامل الاكثر تاثيرا فى انتاج انزيم التانيز.

٥-ايضا العوامل A,B,C,E,F,G,L هى العوامل الاكثر تاثيرا فى انتاج حمض الجاليك.

٦-العوامل A,C,H,J,Lهى العوامل الاكثر تاثيرا فى انتاج حمض الالا جيك.

٧- تم عمل تحليل احصائى بعد استبعاد العوامل الغير مؤثره فى انتاج التانييز وحمض الجاليك والالاجيك فوجد ان حمض الجاليك هو الاكثر توافق مع النتائج المتوقعة.